Fibronectin Glycosylation Modulates Fibroblast Adhesion and Spreading

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Abstract. The role of the carbohydrate residues of fibronectin concerning the specificities of that glycoprotein to interact with fibroblastic cell surfaces, gelatin, and heparin was examined. Tunicamycin was used to produce carbohydrate-depleted fibronectin; it was synthesized by cultured fibroblasts. Unglycosylated and glycosylated fibronectins were analyzed for their ability to bind gelatin and heparin, using affinity columns. Fibronectin-coated surfaces were used to quantitatively measure cell adhesion and spreading. The results showed that the lack of carbohydrates significantly increased the interaction of the protein with gelatin and markedly enhanced its ability to promote adhesion and spreading of fibroblasts. In contrast, the binding of fibronectin to heparin was not influenced by glycosylation. The composite data indicate that the Asn-linked oligosaccharides of fibronectin act as modulators of biological functions of the glycoprotein.

FIBRONECTIN is one of the best characterized cell adhesion-promoting factors, known to be involved in mediating the binding of fibroblastic cells to collagen (16) and proteoglycans (10), as well as possessing domains along its primary structure which bind heparin, DNA, and actin (7). The cell-binding domain of fibronectin has been the subject of many studies (for review see reference 18) and recently the interaction of this domain with a surface receptor complex has been well documented (4, 9).

The role of carbohydrate residues in these binding functions is not well understood. A study using tunicamycin to inhibit N-glycosylation of fibronectin (17) suggested that the carbohydrate moiety was not required for the proper synthesis, secretion, or fibroblast cell spreading and agglutination activity, but protected the externalized protein against proteolysis. However, recent evidence from work on the highly glycosylated forms of intact human fetal fibronectin (26) demonstrated that, quite separate from its capacity to protect against proteolysis, the carbohydrate serves to weaken the binding affinity of fibronectin to gelatin. This finding, taken together with the observation that isolated domains of fibronectin often appear to have greater biological activity than those domains present in the intact molecule (14, 24), led us to re-examine the effect of deglycosylation on the adhesion and spreading of fibroblasts. In this communication, we present evidence that carbohydrates weaken the binding affinity of human cellular fibronectin to gelatin and to human fibroblast surface receptor.

Materials and Methods

Cell Culture and Fibronectin Collection

Normal human skin fibroblasts (American Type Culture Collection, CRL 1493) were maintained in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (Gibco, Grand Island, NY). For isolation of radiolabeled fibronectins, fully confluent cell layers were split 1:2 and used 3–4 d later when dishes were post-confluent and contained $\sim 2 \times 10^6$ cells per 100-mm dish. Cultures were extensively washed in DME, then preincubated in 5 ml of DME containing 2% fibronectin-depleted fetal calf serum with or without tunicamycin (catalog No. T-7765, lot No. 15F/4050, isomer composition: 9% A, 32% B, 46% C, 12% D; Sigma Chemical Co., St. Louis, MO) at 0.5 μg per ml. After 4 h, this medium was exchanged for 5 ml of DME plus 2% fibronectin-depleted serum, with or without tunicamycin as required and including radiolabel ([^{35}S]methionine, 50 μCi/ml, 1,084 Ci/mmol; New England Nuclear, Boston, MA). After 24 h of culture, medium was collected, chilled, and brought to 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM *N*-ethylmaleimide, and 10 mM EDTA (protease inhibitors).

The collection of nonradiolabeled fibronectins followed the protocol given above, omitting the addition of radiolabeled methionine. Medium was collected at 24 and 48 h incubation. After centrifugation of the medium (10,000 *g* for 10 min), fibronectin was isolated by gelatin affinity chromatography as described elsewhere (13). Aliquots of fibronectins were checked for purity and molecular weight analysis by SDS PAGE (11). Affinity-purified fibronectin, collected in 1 M NaBr, 20 mM NaAc (pH 5), was checked for protein content using both Lowry determinations and OD_{280}, taking the extinction coefficient of fibronectin to be 1.28 (15). Samples were diluted into equal volumes of pure glycerol and stored in aliquots at −20°C.

Chromatography of Fibronectins on Gelatin- and Heparin-Agarose Columns

After centrifugation, radiolabeled culture medium was applied to a gelatin-agarose column (0.5 × 2.5 cm) containing 5 mg of gelatin per ml of packed agarose (Sigma Chemical Co.). The column was extensively washed with phosphate-buffered saline (PBS) and the bound fibronectin was eluted with a linear gradient formed by 30 ml each of PBS and 6 M urea in PBS. 0.8-ml fractions were collected. Recoveries from the column were from 80–90%. The purity of the bound fibronectins was checked by SDS PAGE followed by fluorography.

For heparin-agarose chromatography, the nonspecific binding sites of heparin-agarose (780 μg heparin per ml packed gel; Sigma Chemical Co.) were initially blocked with bovine serum albumin, followed by addition of plasma fibronectin with the subsequent removal of plasma fibronectin by 0.5 M NaCl/6 M urea/0.05 M Tris, pH 7.4. Gelatin affinity-purified fibronectins in 6 M urea/0.05 M Tris, pH 7.4, were loaded onto the heparin-

agarose column and eluted with a linear gradient to 0.5 M NaCl in 6 M urea/0.05 M Tris, pH 7.4. By initially blocking the nonspecific sites of heparin-agarose as described, the recoveries from the column ranged from 85 to 90%.

Preparation of Substrata

Fibronectin solutions purified from culture medium of tunicamycin-treated cells (T-FN)[1] and control cultures (C-FN) were added to the wells of clean glass multiwell slides (Flow Laboratories, McLean, VA; U.K. cat. No. 60-418-05; 50.24 mm^2 well area), 50 μl per well, at concentrations of 1, 2.5, 5, 10, and 20 μg/ml. Adsorption of fibronectin to the glass proceeded for 18 h at 4°C, followed by one rinse in 1 M NaBr buffer and two rinses in distilled water. Slides were countercoated with 2% bovine serum albumin (Sigma Chemical Co.) for 2 h at room temperature in a humid atmosphere, rinsed in distilled water and washed three times in DME. Slides were kept under DME until used in cell adhesion assays, but were discarded if the interval was >2 h. Every slide prepared in this manner had at least one well which was coated with 2% BSA only.

Quantitation of the amounts of C-FN and T-FN which became adsorbed to the glass wells, at initial concentrations of 2.5, 5, 10, and 20 μg/ml, was carried out. The multiwell slides were cut into sections using a diamond tip pencil, with one well per section. Each well received radiolabeled ([^{35}S]methionine; see above) fibronectin solutions at the concentrations indicated; the protocol for adsorption and for countercoating with 2% bovine serum albumin was followed as described above. The rinse solutions and albumin solutions were combined to measure the content of radiolabeled fibronectin which did not adsorb to the glass. In addition, the amount of labeled protein which adhered to the glass was also measured by scintillation spectrometry. The amount of adsorbed fibronectin was calculated using the measured specific activities of C-FN and T-FN.

Preparation of Fibroblasts

Confluent monolayers were rinsed with DME and the medium replaced with medium containing 50 μg/ml cycloheximide (Sigma Chemical Co.), a concentration known to give 90% inhibition of protein synthesis (Jones, G. E., and J. A. Pizzey, manuscript in preparation). After 2 h, the medium was replaced with either 0.25% trypsin (Gibco) in DME or 0.25% trypsin plus 5 mM EDTA in calcium- and magnesium-free DME. In either case, cells were incubated at room temperature for 30 min, with gentle agitation on a Lab-Quake (Labindustries, Berkeley, CA), before dissociation. Three washes in DME or calcium- and magnesium-free salt solution as appropriate was followed by resuspension of cell pellets in DME with 50 μg/ml cycloheximide to a concentration of 2×10^5 cells/ml.

Cell Seeding and Spreading

Fibroblasts were seeded onto fibronectin lawns prepared on multiwell slides, 50 μl per well. Slides were incubated at 37°C in a tissue culture cabinet for 30 min, removed to room temperature and agitated for 5 min on a Lab-Quake under empirically determined shaking conditions (which were maintained for the duration of this study). Slides were rinsed in buffer and cells fixed using 2.5% glutaraldehyde as previously described (19). Cell viability was assessed on residual aliquots of material after each experiment and was found to be >85% on the basis of trypan blue exclusion. Seeding efficiency for each fibroblast population on the various substrata was calculated from random area counts which were compared with the number of cells known to have been in the initial seeding suspension.

The degree of cell spreading for each population was measured and calculated using differential interference contrast optics and a semi-automatic digitizer (Kontron Analytical, Everett, MA) interfaced to a microcomputer as previously described (8, 20). From each preparation, 50 cells were identified that fell on orthogonal diameters drawn randomly across the visual field of the microscope, and their areas were calculated using the digitizer and appropriate software. Cells that were observed to be in contact with a neighbor were excluded from the analysis.

Statistical Evaluation

In a population of spreading cells, the measured cell areas fit a negative binomial and not a Gaussian distribution (20), thus cell area frequency distributions were compared by using the Kolmogorov-Smirnov two-sample test (21). Cell areas were also logarithmically transformed to allow a parametric test of significance (Student's t test) to be performed. As a measure of uniformity of radial spreading in the seeded fibroblasts, a form factor was calculated for all cells measured. This is a measure of circularity of an object and was adapted in this study to identify cells that were irregularly spread or had become polarized after a phase of radial spreading. All cells measured in this study were found to have form factors >0.55 and can be regarded as radially spread (20).

1. *Abbreviations used in this paper*: C-FN, fibronectin solutions purified from control cultures; T-FN, fibronectin solutions purified from culture medium of tunicamycin-treated cells.

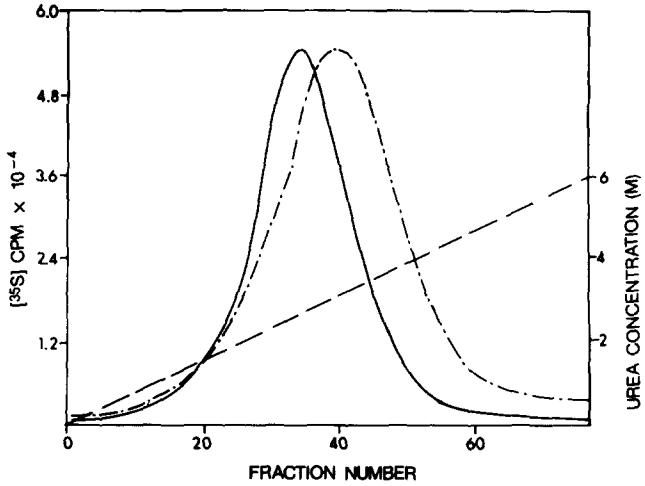

Figure 1. Gradient elution of fibronectins from gelatin-agarose. [^{35}S]Methionine-labeled culture media from control (———) and tunicamycin-treated (– · – · –) fibroblasts were applied to a gelatin-agarose column and the bound fibronectin was eluted with a 0–6-M (– – –) urea gradient.

Results

Chromatography of Fibronectins

The secreted fibronectins, from both control and tunicamycin-treated fibroblasts, quantitatively bound to the gelatin-agarose column and could be eluted with a linear gradient of 0–6 M urea in PBS. Such a linear gradient profile of C-FN and T-FN is shown in Fig. 1. The apex of the elution peak for C-FN appeared at 2.73 M urea while that of T-FN occurred at 3.16 M urea. Upon NaCl gradient elution of affinity-bound fibronectins from heparin-agarose columns, the apex of the elution peak for C-FN occurred at 0.2 M NaCl and there was no significant difference for T-FN (not shown). As anticipated, the T-FN polypeptides migrated more rapidly than the C-FN subunits when examined by SDS PAGE (Fig. 2). Furthermore, this figure indicates the fibronectins used in the present study were free of detectable contaminants or degradation products.

Fibronectin Adsorption

The amount of fibronectin adsorbed to the glass surface as a function of initial protein concentration is shown in Table I. The amount of fibronectin bound to the surface is dependent upon the initial concentration, for both C-FN and T-FN, and there is no apparent difference in the binding behavior of the two forms of fibronectin. The efficiency of binding ranged from 92–97% of the total fibronectin applied at the two lowest initial concentrations and decreased at the higher concentrations, perhaps reflecting increasing saturation of available binding sites. Note that there was a high de-

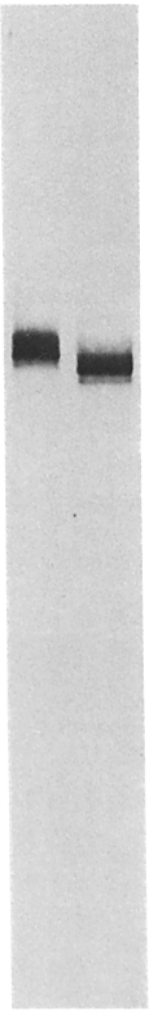

Figure 2. Fluorography of affinity purified C-FN (*left lane*) and T-FN (*right lane*) after SDS PAGE on a 4–7.5% gradient gel.

gree of reproducibility at each data point and that there was no significant difference in the corresponding values for C-FN and T-FN.

Fibroblast Seeding Efficiency

The promotion of cell-to-substratum adhesiveness induced by increasing concentrations of C-FN and T-FN is shown in Fig. 3. Fibroblasts did not appear to adhere to either form of fibronectin at a concentration below 2.5 μg/ml. Between

Table I. Quantitation of Fibronectin Adsorbed to Glass Surfaces

Concentration of FN	Amount of FN applied	Amount bound to the glass surface	
		C-FN*	T-FN*
μg/ml	*ng*	*ng*	*ng*
2.5	125	115.3 ± 5.9	115 ± 8.2
5	250	242 ± 6.4	243 ± 1.7
10	500	409 ± 22.4	394 ± 16.1
20	1,000	564 ± 71.3	544 ± 17.7

* Values are expressed as mean ± SEM of four separate experiments. 50 μl of fibronectin at the denoted concentrations was added to each well and the amount of fibronectin bound to the glass surface was calculated as described in Materials and Methods.

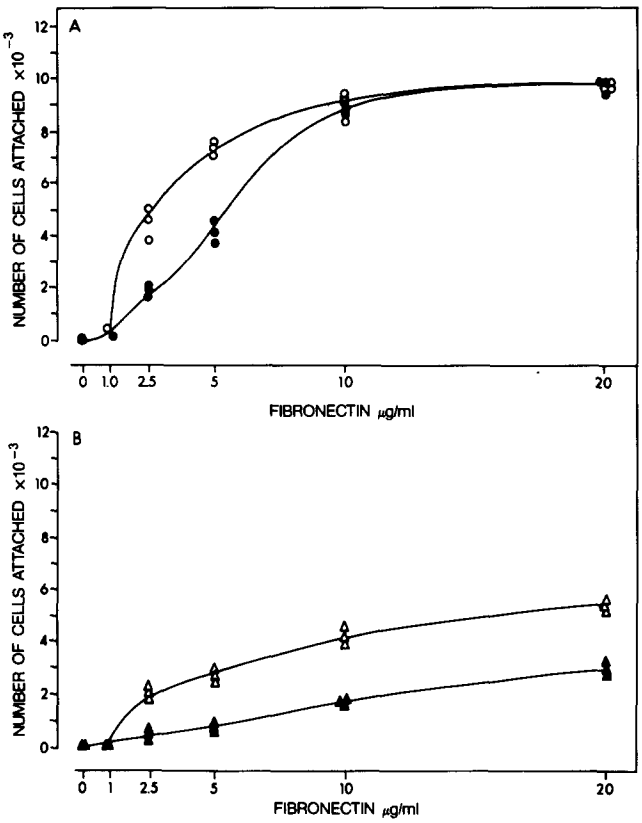

Figure 3. Adhesion of fibroblasts to C-FN (●, ▲) and T-FN (○, △) substrata. Cycloheximide-treated fibroblasts were released with trypsin (*A*) or trypsin/EDTA (*B*) for 30 min at room temperature. The cells in DME were then seeded onto the wells coated with increasing concentrations of fibronectin. After a 30-min incubation at 37°C, the substratum-bound cells were fixed and counted as described in Materials and Methods. Values are shown as the number of substratum-bound cells in each well. Each point represents a separate experiment. Values are significantly different between C-FN and T-FN substrata at 2.5 and 5 μg/ml concentrations in *A* and at 2.5, 5, 10, and 20 μg/ml concentrations in *B*.

2.5 and 5 μg/ml, trypsinized cells adhered with a significantly greater efficiency on T-FN than on C-FN, though both fibronectins were equally adhesive at and above 10 μg per ml (Fig. 3 *A*). Cells dissociated with trypsin/EDTA displayed a reduced capacity to bind to fibronectin, with only a small, but significant, improvement with increasing fibronectin concentration (Fig. 3 *B*). Even under these conditions, T-FN was seen to improve fibroblast seeding efficiency above that for C-FN, up to 20 μg/ml.

Fibroblast Spreading

The degree of cell spreading seen in adherent cells is shown in Figs. 4 and 5 for trypsinized cells and Figs. 6 and 7 for fibroblasts dissociated with trypsin/EDTA. Examination of multiwell slides revealed that cycloheximide-incubated cells retain the ability to spread on fibronectin lawns, though to a lesser degree than cells not incubated in this drug (data not shown). The mode of spreading appears normal as judged by cell morphology (Figs. 4 and 6), with the degree of spreading increasing with increasing concentrations of fibronectin. Trypsin-dissociated fibroblasts spread to a greater extent on T-FN compared with equivalent concentrations of C-FN up

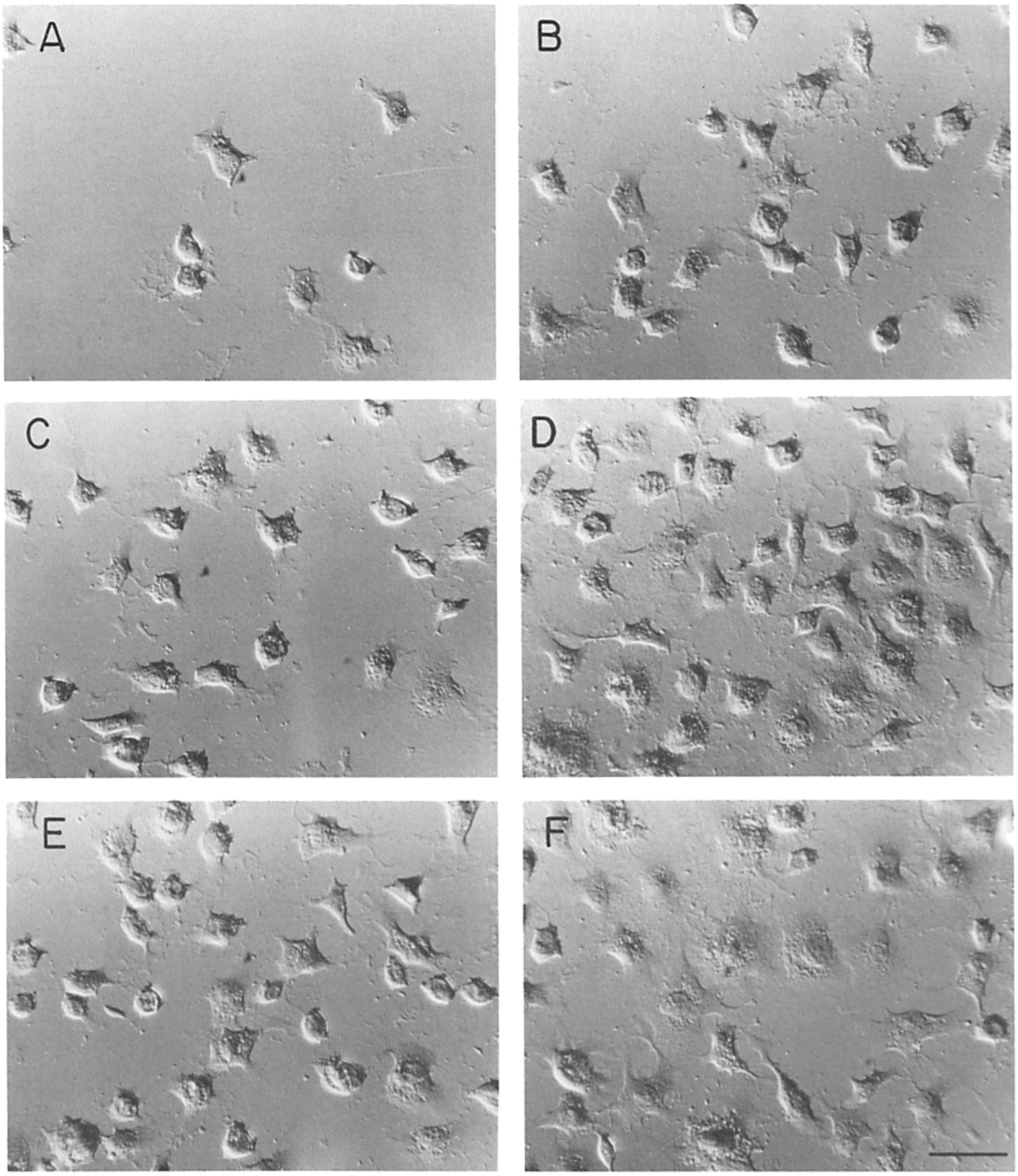

Figure 4. Cycloheximide-treated human fibroblasts were seeded for 30 min at 37°C in DME after a 30-min trypsinization. Substrata consist of either C-FN (*A*, *C*, and *E*) or T-FN (*B*, *D*, and *F*), which have been prepared as described in Materials and Methods. Fibronectin concentrations used in the preparation of the adhesive lawns were 2.5 (*A* and *B*), 5 (*C* and *D*), and 10 μg/ml (*E* and *F*). The micrographs demonstrate the degree of adhesion and cell spreading seen under the various conditions of the assay and are for illustration only. Quantitative data were obtained from samples selected according to the criteria described in Materials and Methods and from images examined under much greater magnification. Bar, 100 μm.

to 10 μg/ml, where both fibronectins appear equally suitable (Fig. 5). Fibroblasts treated with trypsin/EDTA spread less well than trypsinized samples (Fig. 7), and the differential effects of the two forms of fibronectin was shifted to 5–20 μg/ml (the highest concentration used). These data were obtained from cells which were found in areas of low density, where no cell-to-cell adhesive contacts were present. Cell area measurements obtained from zones of high cell density failed to give similar results to those just described; in particular, trypsinized fibroblasts spread to a degree which was not influenced by the nature of the fibronectin substratum at concentrations >5 μg/ml (data not shown).

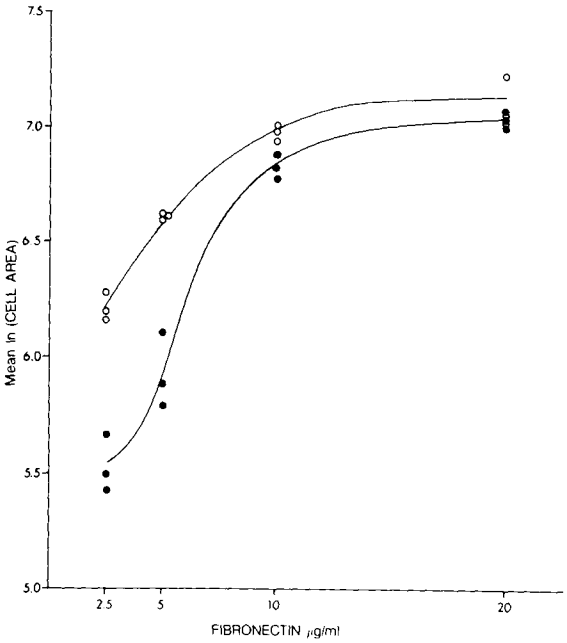

Figure 5. Spreading of cycloheximide-treated, trypsinized cells on fibronectin surfaces. Values are represented as the mean cell areas of 50 cells after natural logarithmic transformations. Trypsinized cells were seeded and allowed to spread for 30 min at 37°C on C-FN- (●) and T-FN- (○) coated surfaces. Results are significantly different at 2.5 and 5 μg/ml concentrations.

Discussion

Most of the fibronectin carbohydrates are located at the gelatin-binding domain, but a significant proportion are also present in the 75-kD fragment which contains the cell-binding region (5). However, the heparin-binding domains do not appear to be glycosylated. We have demonstrated that inhibition of fibronectin glycosylation causes an increase in the gelatin-binding, cell-binding, and cell-spreading activity of fibronectin, but not that of heparin-binding affinity. It is noteworthy that Zhu et al. (25) have presented evidence that polylactosamine glycosylation of human fetal placental fibronectin weakens the gelatin-binding affinity of fibronectin. In their studies, N-linked polylactosamine-containing fragments interact with gelatin with lesser affinity than equivalent fragments containing smaller, complex N-linked saccharides. Our results show that the absence of "complex" N-linked oligosaccharides of cellular fibroblast fibronectin results in increased affinity to gelatin (C-FN elution peaks at 2.73 M urea and T-FN elution peaks at 3.16 M urea). Hence, it is evident that the binding affinity of fibronectins to collagenous structures may vary greatly, depending upon the extent and chemical nature of their glycosylation.

The data on cell attachment and spreading to normal and nonglycosylated fibronectin also provide evidence of increased binding affinity to the unglycosylated protein. Where fibroblast populations are selected for measurement on the basis of negligible cell–cell interaction, T-FN promotes both higher cell-seeding levels and a greater degree of spreading than C-FN. This effect is most evident for cells dissociated with trypsin which have been seeded onto lawns of low (non-saturation) fibronectin concentration. The degree of initial seeding and subsequent spreading is much reduced in trypsin/EDTA–dissociated cells. These data may reflect the fact that the surface receptor for fibronectin is inactivated by trypsin action under reduced divalent cation conditions (22). Preliminary data (not shown) suggests that receptor inactivation is a function of trypsin exposure time (as has been suggested by Akiyama and Yamada [1]). After a 15-min incubation in trypsin/EDTA, fibroblasts spread to a much greater extent than reported here (30-min incubation). Attempts to remove all binding activity by longer incubation periods were discontinued as it was discovered that cell viability fell below 60% after 45 min incubation in trypsin/EDTA.

Superficially, the data presented here appears to contradict the findings of Olden et al. (17), who reported that non-glycosylated fibronectin was as effective as normal fibronectin in assays of cell agglutination, mediating cell attachment to collagen and cell spreading on plastic. However, several differences exist between the two studies which can account for the disparate findings. We have chosen to use a normal human fibroblast strain rather than the baby hamster kidney (BHK) cell line favored by Olden et al. (17). We have examined the spreading of cycloheximide-treated cells onto lawns of fibronectin, while Olden et al. (17) chose to seed cells onto plastic in medium containing various concentrations of fibronectin. Thus, the conditions under which the assay for cell spreading was performed are very different in the two studies. The assay for cell spreading is also very different; Olden et al. scored this parameter by counting 250 cells and calculating the percentage of cells that were fully spread, where fully spread is defined as no longer refractile by phase-contrast microscopy (17). In our study, cell spreading is measured directly as cell areas of fibroblasts, chosen merely on the basis that each measured cell did not contact a neighboring cell. Thus our data accurately quantifies the degree of spreading seen in a population of fibroblasts, rather than merely describing whether a cell is spread or not spread. Using the latter criterion, we cannot demonstrate any significant difference between the proportion of spread cells on lawns of C-FN and T-FN: above a fibronectin concentration of 2.5 μg/ml, >75% of cells can be counted as spread. This is in general agreement with the findings of Olden et al. (17), but fails to show that some cell populations spread more than others. This is what we demonstrate here. Furthermore, in areas of high cell density, the establishment of numerous cell–cell contacts diminishes the relative contribution that cell–substratum adhesions make to cell spreading (data not shown). Meaningful data on cell–substratum interactions can only be obtained from isolated cells.

Examination of the data on trypsin-dissociated fibroblasts indicates that at saturation levels of fibronectin (6), cell attachment and degree of cell spreading appear to be maximal and independent of whether fibronectin is glycosylated or not. When the density of the fibronectin lawn is reduced below 0.01 μg/mm^2, C-FN is not able to sustain the degree of cell attachment and spreading noted for T-FN. Only at very low levels of fibronectin (0.001 μg/mm^2) do attachment values for T-FN and C-FN again become similarly poor; we presume this to be a consequence of the provision of a subthreshold density of fibronectin molecules required for adequate specific interaction with the cell surface.

The data obtained on cell attachment and degree of spreading for fibroblasts dissociated with trypsin/EDTA is consistent with the concept of surface receptor degradation. Within

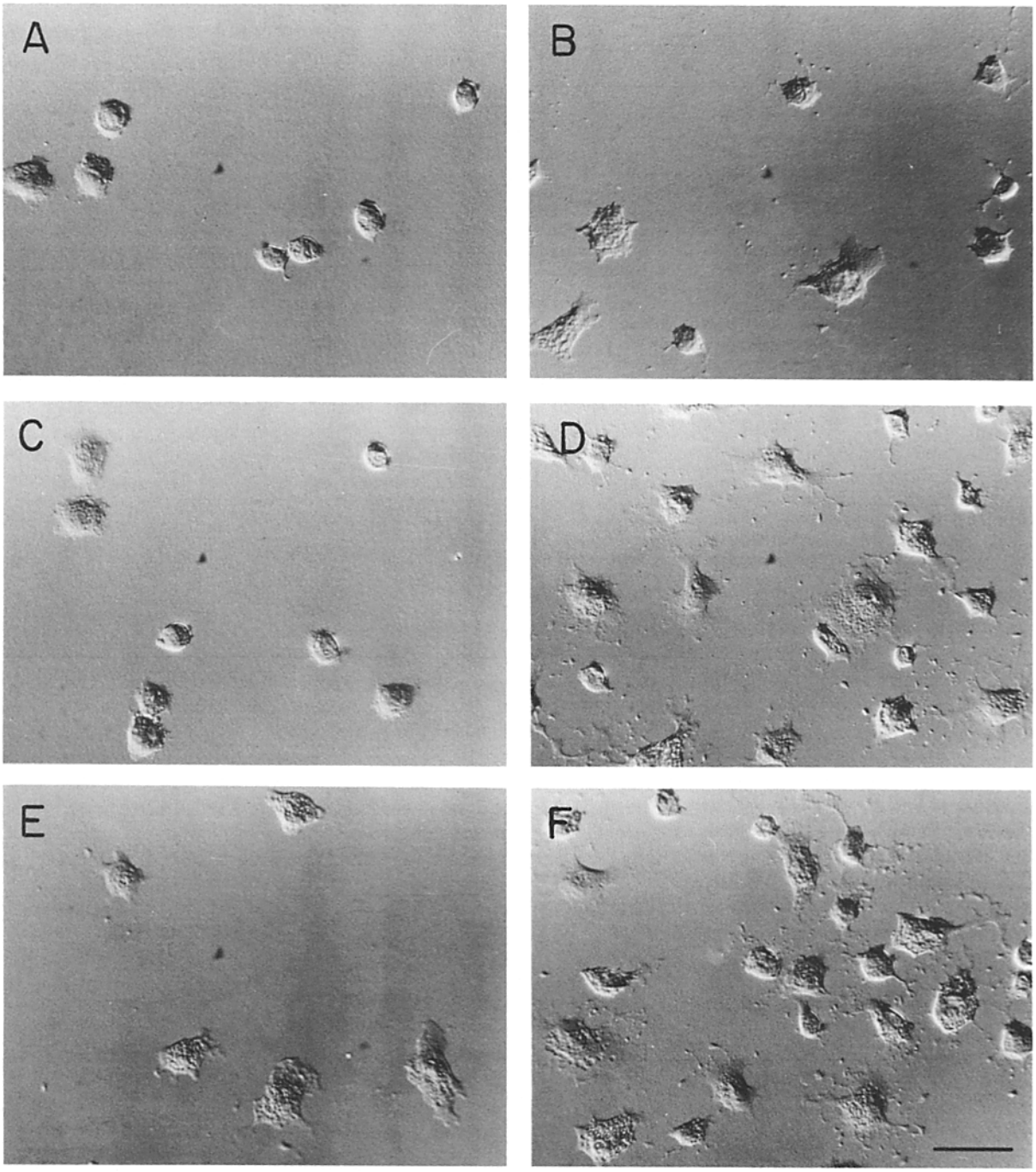

Figure 6. Cycloheximide-treated human fibroblasts seeded for 30 min at 37°C in DME after a 30-min trypsin/EDTA dissociation. Fibronectin lawn type and concentration follows the classification given in Fig. 4. Comparison with Fig. 4 demonstrates the lower seeding efficiency and reduced spreading of trypsin/EDTA–dissociated cells compared with only trypsin-dissociated cells. Bar, 100 μm.

the range of fibronectin concentrations used, no amount can provide for maximal attachment or spreading of cells as seen with trypsinized samples. The shallow and linear increase in cell attachment seen with increasing fibronectin concentration may be explained by the recruitment of undamaged fibronectin receptors to available binding domains. The receptor is thought to be a complex of glycoproteins with lateral mobility in the plasma membrane (4, 9), and the 30-min incubation period would easily allow viable receptors at any point on the surface to be relocated to the substratum interface (10). Even under these constraints, T-FN promotes greater cell attachment than C-FN, providing further evidence for the increased affinity for the nonglycosylated form of fibronectin. Similarly, cell spreading is impaired in trypsin/EDTA–dissociated fibroblasts. The degree of spreading measured is equivalent on C-FN and T-FN at a concentration of 2.5 μg/ml, but T-FN is able to promote a higher level of spreading with increasing concentration.

The possibilities for the modulation of gelatin-binding, cell-binding, and cell-spreading activities of fibronectin by

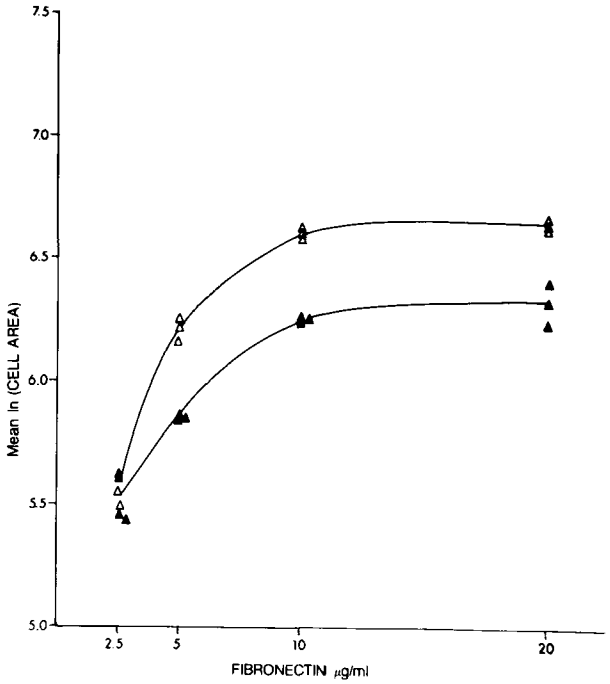

Figure 7. Spreading of cycloheximide-treated fibroblasts, previously released with trypsin/EDTA, on C-FN (▲) and T-FN (△) substrata. Results are significantly different at 5, 10, and 20 μg/ml concentrations. Other details are as described in Fig. 5.

its carbohydrates would include steric effects, the chemical structure, and the location of saccharides on the peptide backbone. Another consideration would be the manner in which fibronectin aggregates are organized in the extracellular matrix, the topography of cell surfaces in relation to such aggregates, and the influence of carbohydrates upon both aggregate organization and the cell–fibronectin interaction. Since the heparin-binding domains of fibronectin do not contain any carbohydrate units and their affinities do not appear to be affected, it may be that both location and steric hinderance of carbohydrates on the protein core may be important in modulating the functional properties of fibronectin. It would be interesting to know whether the polylactosamine structure of fetal fibronectins (25) affect their heparin-binding affinity.

Recently, Akiyama et al. (2) proposed two models for the mechanism of interaction between fibronectin and its cell surface receptor. One model involves two processes: "near interaction" recognition sites, containing Arg-Gly-Asp-Ser- recognition domains with weak affinity, plus "far interaction" regions, distinct from immediate cell-recognition regions, which increase the affinity of the fibronectin–cell binding. In another model, a 75-kD domain containing cell-binding region alters the affinity of the minimal recognition peptide, Arg-Gly-Asp-Ser. In either one of these models, the increased cell adhesion and cell spreading by unglycosylated fibronectin may be due to modulation of such recognition sites, although increased affinity of the gelatin-binding domain for cell surface collagenous structures may also be a factor. Furthermore, the 75-kD domain from human plasma fibronectin has been found to contain 40% of the total carbohydrate (5). In contrast, the extent of glycosylation of the cell-binding region of human cellular fibronectin has not been determined. More detailed analysis of the interactions of the purified cell-binding domains from C-FN and T-FN with fibroblasts may provide additional insight into participation of oligosaccharides in the binding process.

Altered glycosylation of fibronectin under certain physiological or pathological conditions may influence its biological behavior. For example, Cossu and Warren (3) have reported that lactosaminoglycans and heparan sulfate are constituents of fibronectins synthesized by F9 teratocarcinoma cells. Moreover, they have suggested that the presence of these carbohydrates is sufficient to interfere with the adhesive properties of fibronectin. Additional branching of oligosaccharides and an increase in the amount of carbohydrates of fibronectin also accompany neoplastic transformation (23) and fetal development (27). Such changes in the glycosylation of fibronectin may well alter its association with components of the extracellular matrix and with cell surfaces.

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