Fibrillar Organization of Fibronectin Is Expressed Coordinately with Cell Surface Gangliosides in a Variant Murine Fibroblast

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Abstract. NCTC 2071A cells, a line of transformed murine fibroblasts, grow in serum-free medium, are deficient in gangliosides, synthesize fibronectin, but do not retain and organize it on the cell surface. When the cells are exposed to exogenous gangliosides, fibrillar strands of fibronectin become attached to the cell surface. A morphologically distinct variant of NCTC 2071A cells was observed to both retain cell surface fibronectin and organize it into a fibrillar network when the cells were stained with anti-fibronectin antibodies and a fluorescent second antibody. A revertant cell type appeared to resemble the parental NCTC 2071A cells in terms of morphology and fibronectin organization. All three cell types were subjected to mild $NaIO_4$ oxidation and reduction with KB^3H_4 of very high specific radioactivity in order to label the sialic acid residues of surface gangliosides. The variant had much more surface gangliosides than the parental, particularly more complex gangliosides corresponding to G_{M1} and G_{D1a}. The surface gangliosides of the revertant were intermediate between the parental and the variant. By using sialidase, which hydrolyzes G_{D1a} to G_{M1}, and ^{125}I-labeled cholera toxin, which binds specifically to G_{M1}, the identity and levels of these gangliosides were confirmed in the three cell types. When variant cells were exposed to sialidase for 2 d, there appeared to be little change in fibronectin organization. Concomitant treatment of the cells with the B subunit of cholera toxin, which bound to all the surface G_{M1} including that generated by the sialidase, however, eliminated the fibrillar network of fibronectin. In addition, exposure of the variant cells to a 70,000-mol-wt fragment of fibronectin, which lacks the cell attachment domain but contains a matrix assembly domain, inhibited the formation of fibers. Finally, all three cell types were assayed for their ability to attach to and spread on fibronectin-coated surfaces; no significant differences were found. Our results further establish that the ability of a cell to organize fibronectin into an extracellular matrix is dependent on certain gangliosides, but they also indicate that cell adhesion to fibronectin is independent of these gangliosides. We suggest that matrix organization and cell attachment and spreading are based on separate mechanisms and that these functions are associated with different cell surface "receptors."

FIBRONECTINS are large, well-characterized, extracellular structural glycoproteins that have been implicated in a wide variety of cellular events. These include cell adhesion, morphology, cytoskeletal organization, migration, differentiation, oncogenic transformation, phagocytosis, and hemostasis (17). Fibronectins exist both as a soluble plasma component and as an insoluble connective tissue protein that is found on cell surfaces and in the extracellular matrix, where it appears to function as an adhesive protein for cell attachment and tissue organization. Fibronectins have a complex molecular structure consisting of multiple functional domains, many of which contain specific binding sites for extracellular proteins such as collagen, fibrin, and proteoglycans, as well as cell surface components (27). The complex biological phenomena in which fibronectins participate can be considered in terms of this complex structure (17). The cell binding and attachment function of fibronectin has been localized to smaller regions within the molecule (13, 32) and recently to a tetrapeptide sequence (31, 39). Based on a variety of studies, this domain of fibronectin interacts with a 140-kD glycoprotein found on the surface of a variety of cells (1, 3, 14, 16, 19, 33). Thus, attachment of cells to substrates via fibronectin appears to involve a specific cell surface receptor and a specific domain on the fibronectin molecule.

An important, but still unanswered question concerns the mechanism of cell interactions with fibronectin to form fibrils,

as well as the relationship of fibril formation to the better-characterized process of cell attachment to fibronectin during adhesive events. Based on previous studies by our laboratories (18, 35, 36, 40, 41), gangliosides have been implicated as cell surface components that mediate the binding and organization of fibronectin. We have shown that ganglioside-deficient NCTC 2071A cells are unable to retain or organize their fibronectin into a fibrillar network unless exogenous gangliosides are added to the culture medium (36, 40). In addition, exogenous gangliosides promote the organization of exogenous fibronectin into fibrils attached to the surface of the cells (36). To pursue the possibility that gangliosides may play a role in fibronectin organization, we have used a variant of NCTC 2071A cells. It was found that changes in growth conditions of the parental NCTC 2071A cells produced variant cells which differed significantly in their morphology and their responsiveness to cholera toxin and Sendai virus, two ganglioside-specific probes (21). We now demonstrate that the observed differences in morphology of these cells correlated with changes in fibronectin retention and organization into fibrillar strands and in expression of complex gangliosides on the cell surface. Furthermore, the ability of these cells to adhere to and spread on fibronectin-coated substrates appears to be independent of ganglioside expression.

Materials and Methods

Materials

Vibrio cholerae sialidase (EC 3.2.1.18), cholera toxin, and its B subunit were obtained from Calbiochem-Behring Corp. (La Jolla, CA). $NaIO_4$ and KBH_4 were obtained from Sigma Chemical Co. (St. Louis, MO). KB^3H_4 (68 Ci/mmol) was purchased from Research Products International Corp. (Mt. Prospect, IL). Fluorescein- and rhodamine-labeled rabbit anti-goat IgG were obtained through Miles Laboratories, Inc. (Naperville, IL). Cholera toxin was iodinated as described previously (8). Affinity-purified goat anti-chicken cellular fibronectin antibodies were prepared as described by Yamada (37). The 70,000-mol-wt, amino-terminal fragment of fibronectin was prepared as described by McKeown-Longo and Mosher (24).

Cell Culture

NCTC 2071A cells, which were derived from clone 929 of mouse strain L cells by adapting the clonal cells to grow in chemically defined medium (7), were cultured in serum-free NCTC 135 medium prepared by the NIH Media Unit as described previously (36, 40) and are designated the parental cells. The variant cells were obtained by growing the NCTC 2071A cells in NCTC 135 medium obtained from GIBCO (Grand Island, NY). The revertant cells were produced when the variant cells were returned to the original culture medium. Strain L cells originally were derived from mouse connective tissue and have a fibroblast-like appearance as do the clone 929 and NCTC 2071A cells. The parental, variant, and revertant cells were karyotyped by Biotech Research Labs Inc. (Rockville, MD) for chromosome frequency distribution and for C-banding of the chromosomes.

Immunofluorescence

Immunofluorescence was performed as described previously (36, 40). Briefly, the three cell types were subcultured on glass coverslips for 3 d in the appropriate medium. The cells then were rinsed with Dulbecco's phosphate-buffered saline (PBS), fixed for 1 h in PBS containing 3.7% formaldehyde and 5% sucrose, and rinsed three times with PBS over a 15-min period. The cells then were incubated with anti-fibronectin antibodies (20 μg/ml for 1 h). Indirect immunofluorescence was performed exactly as described before using a fluorescent second antibody (diluted 1:10). Routine controls included incubating the cells with an equal concentration of preimmune IgG from the same goat or omitting the first antibody and were always negative.

Labeling of Cell Surface Gangliosides

The three cell types were cultured in 75-cm^2 flasks and each flask was incubated with 7.5 ml of ice-cold PBS containing 2 mM $NaIO_4$ (25). After 30 min at 0°C, the cells were washed with ice-cold PBS, scraped into the same, and collected by centrifugation (1,200 rpm for 5 min). The cells were suspended in 1 ml of PBS to which 10 mCi of KB^3H_4 in 10 μl of 0.05 M NaOH was added. After 30 min at 25°C, 1 mM KBH_4 was added for an additional 30 min. Then 10 ml of ice-cold PBS was added and the cells were collected by centrifugation. The cells were washed three more times by this procedure. Gangliosides were isolated from the cells by established procedures (9, 25, 26). Briefly, the cells were sonicated in 1 ml of H_2O to make a homogenous suspension and a portion was removed for protein determination. Lipids were extracted with chloroform/methanol (1:2 [vol/vol] (25) and gangliosides were isolated from the crude lipid fraction by Sephadex G-25 and DEAE-Sephadex column chromatography, alkaline hydrolysis, and Sephadex G-25 and Unisil column chromatography (9). The radiolabeled gangliosides were separated by thin-layer chromatography and detected either by radioscanning using a Berthold automatic TLC-linear analyzer (Model LB 2842) or by fluorography (26).

Analysis of Gangliosides by Overlay Techniques

Modifications of methods developed by Magnani et al. (20) and Saito et al. (34) were used. Gangliosides purified as described above were separated by thin-layer chromatography on aluminum-backed Silica Gel sheets using chloroform/methanol/0.2% $CaCl_2$ (5:4:1 [vol/vol]). The air-dried chromatogram was quickly dipped twice in a solution containing 0.4% polyisobutylmethylacrylate (34). After drying in air, the chromatogram was sprayed with 100 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl and 9 mM $CaCl_2$. The chromatogram was soaked in the same buffer, drained, and overlaid with sialidase (0.1 U/ml) dissolved in the same buffer. After 2 h at 25°C, the chromatogram was drained and dipped in four successive changes of the same buffer (ice cold). After drying in air, the chromatogram was sprayed and then soaked with 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 1% bovine serum albumin. The chromatogram was then overlaid with the same solution containing ^{125}I-cholera toxin (10^6 cpm/ml). After 30 min at 4°C, the chromatogram was extensively washed in the same solution (ice cold and lacking bovine serum albumin). The chromatogram was air dried and the bound toxin was detected by autoradiography using Kodak X-Omat AR-2 film.

Assay of Cholera Toxin Receptors

Binding of cholera toxin to intact cells was performed by means of established procedures (9, 11). Briefly, the three cell types, cultured in multicluster trays (6 × 35 mm), were incubated with 1 ml of Hanks' balanced salt solution containing 0.1 nM ^{125}I-cholera toxin (1,050–1,660 cpm/fmol) and 0.01% bovine serum albumin for 3 h at 4°C. Then the cells were rapidly washed three times with 1 ml of ice-cold buffer, dissolved in 2 ml of 1 M NaOH, and analyzed for radioactivity and protein. Binding of cholera toxin to cell homogenates was determined by a modification of an established procedure (8, 26). Briefly, the cells, cultured in 75-cm^2 flasks, were washed, detached by scraping, and collected by centrifugation as described above. The cells were suspended in a Tris-buffered saline solution containing 3 mM NaN_3 and 1 mM EDTA (26) and disrupted by freezing and thawing. Portions were incubated with 0.5 nM ^{125}I-cholera toxin in the same solution containing 0.1% bovine serum albumin for 1 h at 34°C. The samples then were filtered on Millipore GVWP filters and counted as described previously (8). In both assays, nonspecific binding was determined by exposing the cells to 0.6 μM unlabeled toxin before adding the labeled toxin.

Assay of Cell Attachment and Spreading

The capacity of the different cell types to adhere to fibronectin-coated substrates was quantified using the cell spreading assay described in detail previously (12, 38). Although the results were qualitatively similar using the standard adhesion buffer or serum-free NCTC 135 medium, a higher percentage of each cell type tended to adhere in the serum-free medium; consequently, most of the assays were performed in the latter. Briefly, the wells of cluster dishes (6 × 35-mm from Costar, Cambridge, MA) were pre-coated with the indicated concentrations of purified human plasma fibronectin for 1 h; then nonspecific attachment sites were blocked by incubation for 30 min with 10 mg/ml of heat-denatured bovine serum albumin. Cells were detached mechanically from the substrate of normal cultures as for normal passage, centrifuged, and transferred to fresh serum-free medium, and then replated into the fibronectin-coated wells. After 2 h at 37°C, the cultures were fixed with glutaraldehyde and scored for percentage of spread cells as previously described (38), counting a total of 600 cells in random fields per point and calculating the mean and standard error.

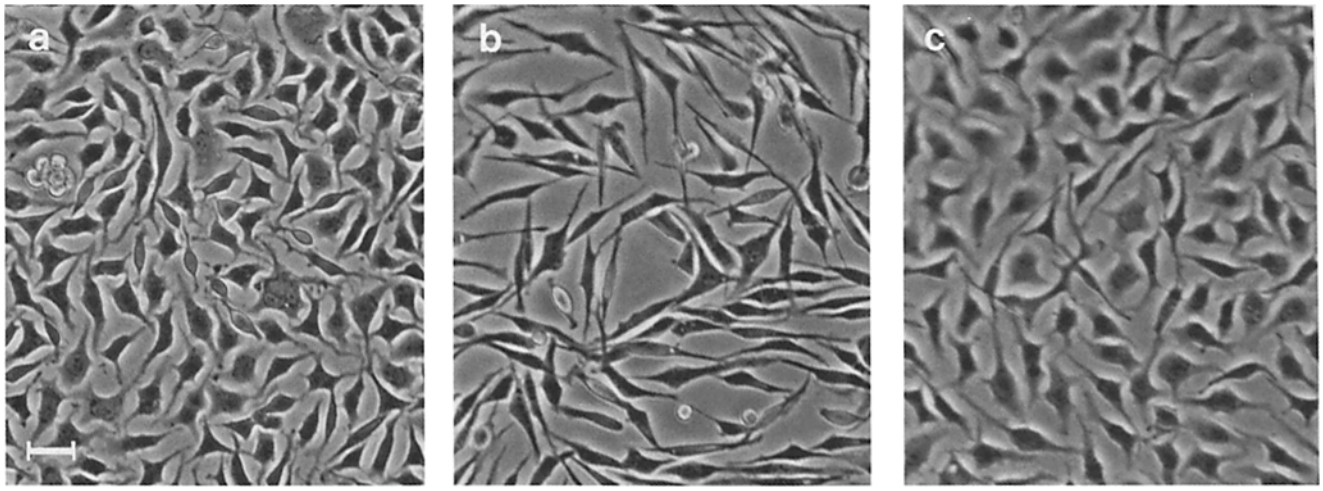

Figure 1. Morphological appearance of the three sublines of NCTC 2071A cells. The parental (*a*), variant (*b*), and revertant (*c*) cells were cultured on glass cover slips for 3 d in the appropriate media as described in Materials and Methods and examined under phase-contrast microscopy. Bar, 25 μm.

Results

Morphology and Endogenous Fibronectin Organization in the Different Sublines of NCTC 2071A Cells

The sublines of NCTC 2071A cells were found to be morphologically distinct as was observed by Markwell et al. (21). The variant cells were more spindle-shaped and flatter than the parental cells (Fig. 1 *b*). The latter had multiple processes (Fig. 1 *a*) as did the cells designated as revertants (Fig. 1 *c*). The cells were examined for endogenous fibronectin distribution by indirect immunofluorescence in order to determine whether the observed morphological differences correlated with changes in fibronectin retention and organization. Consistent with previous studies (36, 40), we observed that all of the fibronectin synthesized by the parental cells was confined to the substratum and almost no fibrils could be detected (Fig. 2, *a* and *b*). Examination of the morphologically distinct variant revealed that the fibronectin was organized into fibrillar strands attached to the cell surface and into an extensive pericellular fibrillar network (Fig. 2, *c* and *d*). The revertant cells exhibited a fibronectin distribution more similar to the parental cells (Fig. 2, *e* and *f*); but, in addition some fibronectin was detected in close apposition to the cell surface.

Analysis of Cell Surface Gangliosides Isolated from the Different Sublines

We had shown previously that a similar alteration in fibronectin retention and organization occurred in the parental cells upon exposure to exogenous gangliosides, particularly G_{M1}, G_{D1a}, and G_{T1b} (36, 40). We therefore examined the different sublines for endogenous cell surface gangliosides. The cells were exposed in situ to mild periodate oxidation and then reduction with KB^3H_4 of very high specific radioactivity in order to label surface sialic acid residues (9, 26, 29)[1]. The gangliosides then were isolated, separated by thin-layer chromatography, and detected by fluorography. Substantial amounts of ^3H were incorporated into the ganglioside fractions isolated from the oxidized cells; in the absence of periodate, there was very little incorporation (Fig. 3). The gangliosides were tentatively identified by their migration on the chromatogram relative to reference gangliosides.[2]

In the parental cells (Fig. 3, lane *6*), the majority of the radioactivity was located in the G_{M2} zone[1] but radioactivity also was detected in the G_{M3} zone. After a longer exposure, there was a trace of ^3H in the G_{M1} zone but none was detectable in the more complex gangliosides such as G_{D1a} and G_{T1b} (data not shown). In the case of the variant cells (Fig. 3, lane *4*), G_{M2} was the predominate radioactive zone but it was much more extensively labeled than the corresponding zone from the parental cells. Incorporation of ^3H into G_{M3} and G_{M1} also was increased compared to the parental gangliosides. The most striking differences were the appearance of labeled gangliosides corresponding to G_{D1a} and G_{T1b}. The ganglioside fraction from the revertant cells also exhibited increased labeling of the G_{M3}, G_{M2}, G_{M1}, and G_{D1a} zones compared to the parental gangliosides but the incorporation of ^3H was less prominant than that observed with the variant cells and there was very little labeling of the G_{T1b} zone (Fig. 3, lane *5*).

The qualitative results presented by fluorography were confirmed by quantitative radioscanning of the chromatogram. The variant gangliosides were the most highly labeled followed by the revertant and then the parental. In all three cell types, the distribution of label in the various zones was in the order $G_{M2} > G_{M3} \gg G_{M1} > G_{D1a} \gg G_{T1b}$. The most striking difference was the substantial labeling of the latter two gangliosides in the variant cells. G_{D1a} and G_{T1b} had been found to be the most effective gangliosides in the promotion of fibronectin organization by the parental cells (40).

Further Analysis of Gangliosides

To confirm the identity of G_{D1a} as well as its quantitative increase in the variant cells, two additional methods were

1. Previous attempts to label cell surface gangliosides of NCTC 2071A cells were unsuccessful due to the use of NaB^3H_4 of much lower specific radioactivity (29). Consistent with our present results, small amounts of G_{M2} were detected in these cells (10, 21, 28).

2. The multiple bands observed in Fig. 3 are presumably due to multiple molecular species of the various gangliosides. Variations in the lipid moiety of a ganglioside will affect its mobility (9) as will oxidation of the sialic acid by $NaIO_4$ (25, 26).

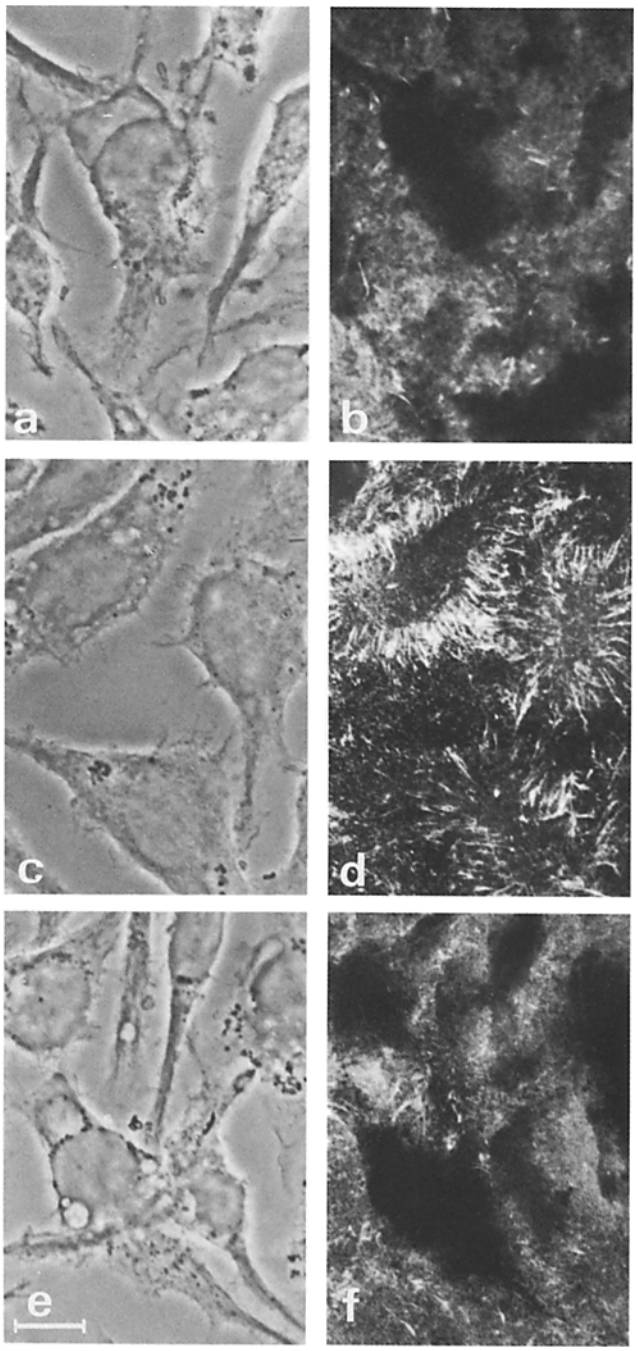

Figure 2. Fibronectin distribution in the three sublines of NCTC 2071A cells. The parental (*a* and *b*), variant (*c* and *d*), and revertant (*e* and *f*) cells were cultured in the appropriate medium for 2 d, fixed, and then stained with anti-fibronectin antibodies and rhodamine-conjugated second antibody. The cells were examined by phase-contrast (*a*, *c*, and *e*) and fluorescence (*b*, *d*, and *f*) microscopy. Bar, 10 μm.

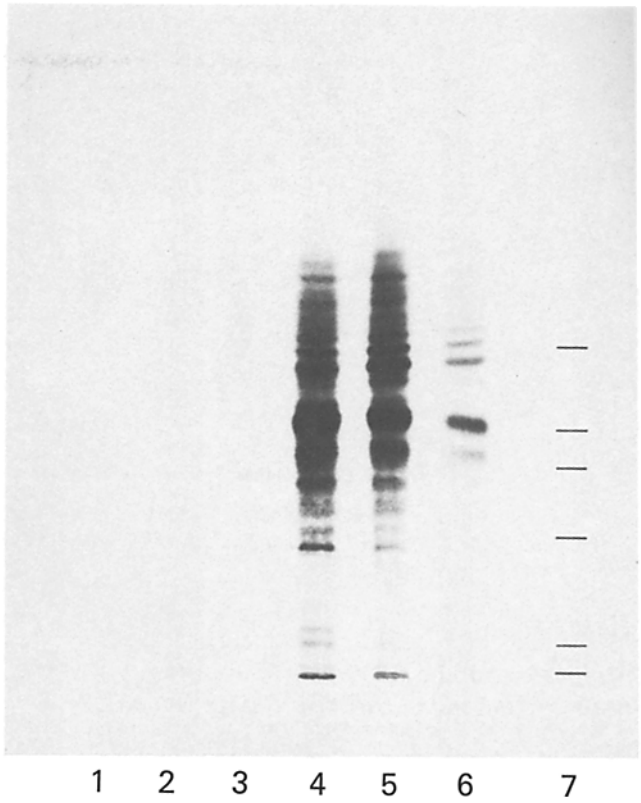

Figure 3. Comparison of surface gangliosides isolated from the three sublines of NCTC 2071A cells. The cells were incubated with and without 2 mM $NaIO_4$ for 30 min, washed, and treated with 10 mCi of KB^3H_4 for 30 min as described in Materials and Methods. Labeled gangliosides were isolated from the cells and portions equivalent to 3 mg of cell protein were separated by thin-layer chromatography and detected by fluorography. (Lanes *1–3*) Minus $NaIO_4$; (lanes *4–6*) plus $NaIO_4$. Gangliosides from parental (lanes *1* and *6*), revertant (lanes *2* and *5*), and variant (lanes *3* and *4*) cells. Mobilities of standard gangliosides are indicated in lane *7* from top to bottom: G_{M3}, G_{M2}, G_{M1}, G_{D1a}, and G_{T1b}; bottom bar indicates the origin.

used. In the first, gangliosides isolated from the cells were separated by thin-layer chromatography and the chromatogram was overlaid with *V. cholerae* sialidase, which converts more complex gangliosides to G_{M1}. The latter was then detected by overlaying the chromatogram with ^{125}I-cholera toxin, which was then visualized by autoradiography (Fig. 4). As shown in lane *1*, which contained standard gangliosides, the toxin bound to the regions of the chromatogram corresponding to G_{M1} and G_{D1a}. There was no binding to the G_{M3} and G_{M2} regions of the chromatogram. The purified ganglioside fraction from the parental cells contained only one toxin-binding component, which corresponded to G_{M1} (lane *2*) and could be detected without pre-treatment of the chromatogram with sialidase (not shown). In contrast, the gangliosides from the variant cells contained much more of this component as well as a component corresponding to G_{D1a} (lane *3*). Using this technique, the revertant cells contained an amount of G_{M1} similar to the parental cells and only a trace of G_{D1a} (lane *4*).

We also used a cholera toxin binding assay to quantify the toxin receptors in the three cell types (Table I). The intact parental cells bound only trace amounts of ^{125}I-cholera toxin whereas the variant cells bound 4.5-fold more toxin. The revertant cells appeared to have only 1.7-fold more toxin receptors on their cell surface compared to the parental cells. After sialidase treatment, the number of toxin receptors on the variant cells increased by 24.2 fmol/mg protein compared to increases of only 2.5 and 5.1 fmol/mg protein for the parental and revertant cells, respectively. Thus, the variant

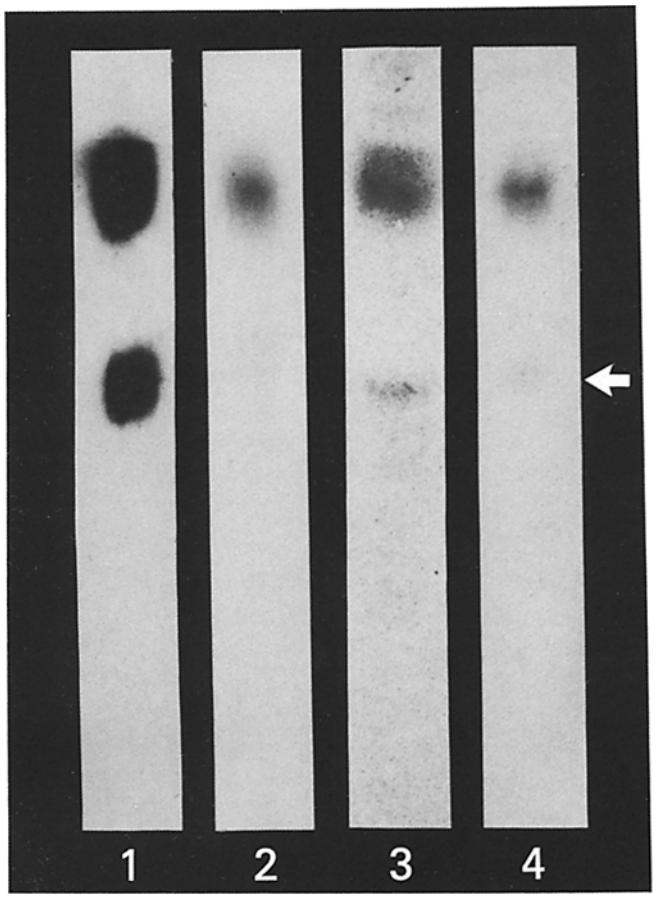

Figure 4. Detection of complex gangliosides from the three sublines by a sensitive overlay technique. The ganglioside fractions were isolated from the cells and portions equivalent to 1 mg of cell protein were separated by thin-layer chromatography on an aluminum-backed Silica Gel sheet as described in Materials and Methods. The chromatogram was overlaid first with *V. cholerae* sialidase and then with ^{125}I-cholera toxin. The bound toxin was detected by autoradiography (16-h exposure). (Lane *1*) G_{M1} and G_{D1a}; (lane *2*) gangliosides from parental cells; (lane *3*) gangliosides from variant cells; (lane *4*) gangliosides from revertant cells. Arrow indicates location of G_{D1a}.

Table I. Binding of ^{125}I-Cholera Toxin to Control and Sialidase-treated Cells

	^{125}I-cholera toxin bound			
	Intact cells		Cell homogenates	
Cell type	−sialidase	+sialidase	−sialidase	+sialidase
	fmol/mg cell protein			
Parental	1.6	4.1	5.7	5.3
Variant	7.2	31.4	22.2	52.7
Revertant	2.7	7.8	3.95	12.0

Cells were incubated without and with 0.01 U/ml of *V. cholerae* sialidase for 2 h and then assayed for binding of ^{125}I-cholera toxin to the intact cells or to homogenates prepared from the cells as described in Materials and Methods. Each value represents the mean of triplicate determinations, which varied <10% and have been corrected for nonspecific binding as measured in the presence of 0.6 μM unlabeled toxin. Similar results were obtained in several additional experiments.

cells have more complex gangliosides as well as G_{M1} on their surfaces compared to the latter cells. We also measured toxin binding to cell homogenates in order to detect any receptors not accessible on the surface of the intact cells (Table I). As binding was measured in a smaller volume with a higher concentration of toxin, the absolute values were higher than those obtained with the intact cells; but qualitatively, the results were similar. The only substantial difference was presence of more toxin receptors in the parental cell homogenate compared to that from the revertant and the lack of an increase in the former after treatment of the cells with sialidase. Thus, the parental cells appear to have as much G_{M1} as the revertant cells but less of it is accessible on the cell surface. This result is consistent with the data obtained both from labeling of the cell surface gangliosides (Fig. 3) and from the toxin overlay experiment (Fig. 4), which detects the total amount of G_{M1} extracted from the cells.

Effects of Sialidase and Cholera Toxin B-Subunit on Fibronectin Organization in the Variant Cells

From the above experiment it was clear that the complex gangliosides on the surface of the variant cells were sensitive to *V. cholerae* sialidase. To further explore the relationship between the cell surface gangliosides and the organization of fibronectin into fibrillar strands extending from the cell surface, the variant cells were incubated with sialidase for 2 d. This treatment had minimal effects on their morphology and the localization of fibrils of fibronectin. Most of the fibronectin was still localized in fibrillar structures attached to the cell surface (not shown). When the cells were incubated at the same time with the B subunit of cholera toxin, which binds specifically to G_{M1} on the cell surface including that generated by the action of the sialidase (9, 26), there was a striking decrease in the intensity of fibrillar staining of fibronectin (Fig. 5). Most of the fibronectin was now confined to the substratum and only a few fibrils extended from the cell surface.

Effect of 70,000-mol-wt Fragment on Fibronectin Organization

Variant cells were subcultured, allowed to attach for 4 h, and then exposed to the 70,000-mol-wt amino-terminal fragment of fibronectin overnight (Fig. 6). As shown in Fig. 6, *b–d*, the fragment caused an inhibition of fibrillar organization in a concentration-dependent manner. As little as 10 μg/ml was effective (not shown). This effect was not observed when the cells were exposed to bovine serum albumin (Fig. 6 *e*) or the buffer against which the fragment was dialyzed (Fig. 6 *f*).

Attachment and Spreading of the Cells on Fibronectin

With the availability of these cell types with varying cell surface gangliosides, we decided to directly test the requirement of the latter for cell adhesion to fibronectin. The adhesion of each type to fibronectin was quantified in a standard cell attachment assay in which cells suspended in serum-free medium were allowed to attach and spread on substrates coated with increasing concentrations of fibronectin. As shown in Fig. 7, the parental, variant, and revertant cells exhibited identical sensitivities for fibronectin as an attachment protein. There were no differences in the initial slope or the concentration of fibronectin required for half-maximal spreading. The final extent of spreading at high concentrations of fibronectin also was similar. Thus, the differences in cell surface gangliosides appeared to have no effect on cell behavior in a standard adhesion assay for cell attachment to fibronectin.

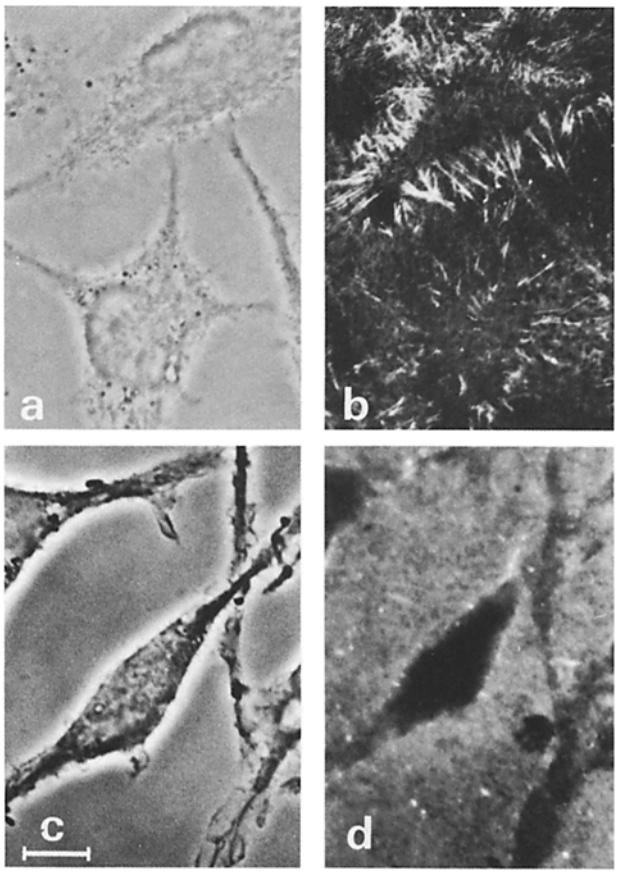

Figure 5. Effect of sialidase and B subunit of cholera toxin on the distribution of fibronectin on the variant subline of NCTC 2071A cells. The variant cells were incubated for 2 d in medium containing no addition (*a* and *b*) or 0.01 U/ml *V. cholerae* sialidase and 0.6 μM cholera toxin B subunit (*c* and *d*). The cells then were fixed, stained with anti-fibronectin antibodies and rhodamine-conjugated second antibody, and examined under phase-contrast (*a* and *c*) and fluorescence (*b* and *d*) microscopy. The sialidase and B subunit were added 4 h after the cells were subcultured; after 24 h more, the medium was replaced with fresh medium with the additions. The medium was changed on the control cells at the same times. Bar, 10 μm.

Chromosomal Analysis of the Sublines

As described in Materials and Methods, the variant cells were obtained by culturing the parental NCTC 2071A cells in NCTC 135 medium obtained from GIBCO instead of medium prepared by the NIH Media Unit. Morphological changes were observed within a week and the cells appeared to be fully converted after 2 wk. This conversion has been reproduced at least three times from frozen stocks of the parental cells. Although the chemical composition on the two media appears to be identical, it is also clear that the change in cell morphology is due to the change in the media, especially as the revertant cells were obtained by culturing the variant cells in the medium prepared at NIH. The fact that the revertant cells were biochemically different from the parental cells in terms of ganglioside composition led us to examine the karyotypes of the three cell types. A majority of the cells in each type contained a chromosome number between 55 and 57 and between 9 and 13 submetacentric and metacentric chromosomes and one subtelocentric chromosome. C-banding karyotype showed that the parental subline likely has two populations of cells: one population (25–35%) has a large acrocentric chromosome with three heterochromatin bands; the other population (65–75%) has a large submetacentric chromosome with two heterochromatin bands and a medium-sized acrocentric chromosome with three heterochromatin bands. The karyotype of the parental cells resembles that of the NCTC 2071 cell (CCL 1.1) described in the American Type Culture Collection catalogue except that it has fewer chromosomes per cell. The C-banding karyotypes of both the variant and revertant sublines showed that the cells have a large acrocentric chromosome with three heterochromatin bands which resembles the characteristic chromosome present in one of the populations of the parental subline.

Discussion

In previous studies, we demonstrated that complex gangliosides mediated the retention of fibronectin by the parental NCTC 2071A cells and its organization into a fibrillar network (36, 40). By using fluorescent gangliosides, we were able to observe a direct association between fibronectin and gangliosides at the cell surface (36). It also appeared that the organization of fibronectin required a ganglioside-dependent cell-associated function rather than some nonspecific self-polymerization of fibronectin that was induced by the gangliosides. Finally, gangliosides promoted the retention and organization of exogenous fibronectin from a different species by the parental cells (36).

Our present studies confirm and extend the potential importance of gangliosides as mediators of fibronectin organization at the cell surface. The variant cells coordinately expressed a distinct morphology, a well-organized fibronectin matrix, and complex cell surface gangliosides in contrast to the parental cells. The presence of complex gangliosides on the surface of the variant cells and their absence on the parental cells was determined by several different techniques, all of which gave qualitatively similar results. These included cell surface labeling, sensitivity to sialidase, and recognition by cholera toxin. The revertant cells, although similar to the parental cells in terms of morphology and fibronectin organization, were intermediate between the latter and the variant in terms of labeling of cell surface gangliosides. These gangliosides, however, appeared to be more accessible to periodate oxidation than to sialidase and cholera toxin.[3] Based on the latter, the revertant cells more resembled the parental cells.

Further support for a role of gangliosides in fibronectin organization was provided by the dramatic effect that a combination of sialidase and cholera toxin B subunit had on the variant cells. Sialidase hydrolyzes complex gangliosides such as G_{D1a} to G_{M1}; thus under these conditions, all of the complex gangliosides on the variant cell surface are either eliminated or complexed with cholera toxin B subunit. After 2 d of treatment, the fibrils of fibronectin were absent from the cell surface and the fibronectin was localized on the substratum. Of particular interest was the observation that sialidase alone had no effect of the fibrillar network of fibronectin. Thus, cell

3. Using different approaches, Markwell et al. concluded that the gangliosides of variant cells were more accessible than those of the parental or revertant cells and that the gangliosides of the latter may be cryptic or masked (Markwell, M. A. K., J. Moss, B. E. Hom, L. Svennerholm, and P. H. Fishman, manuscript in preparation).

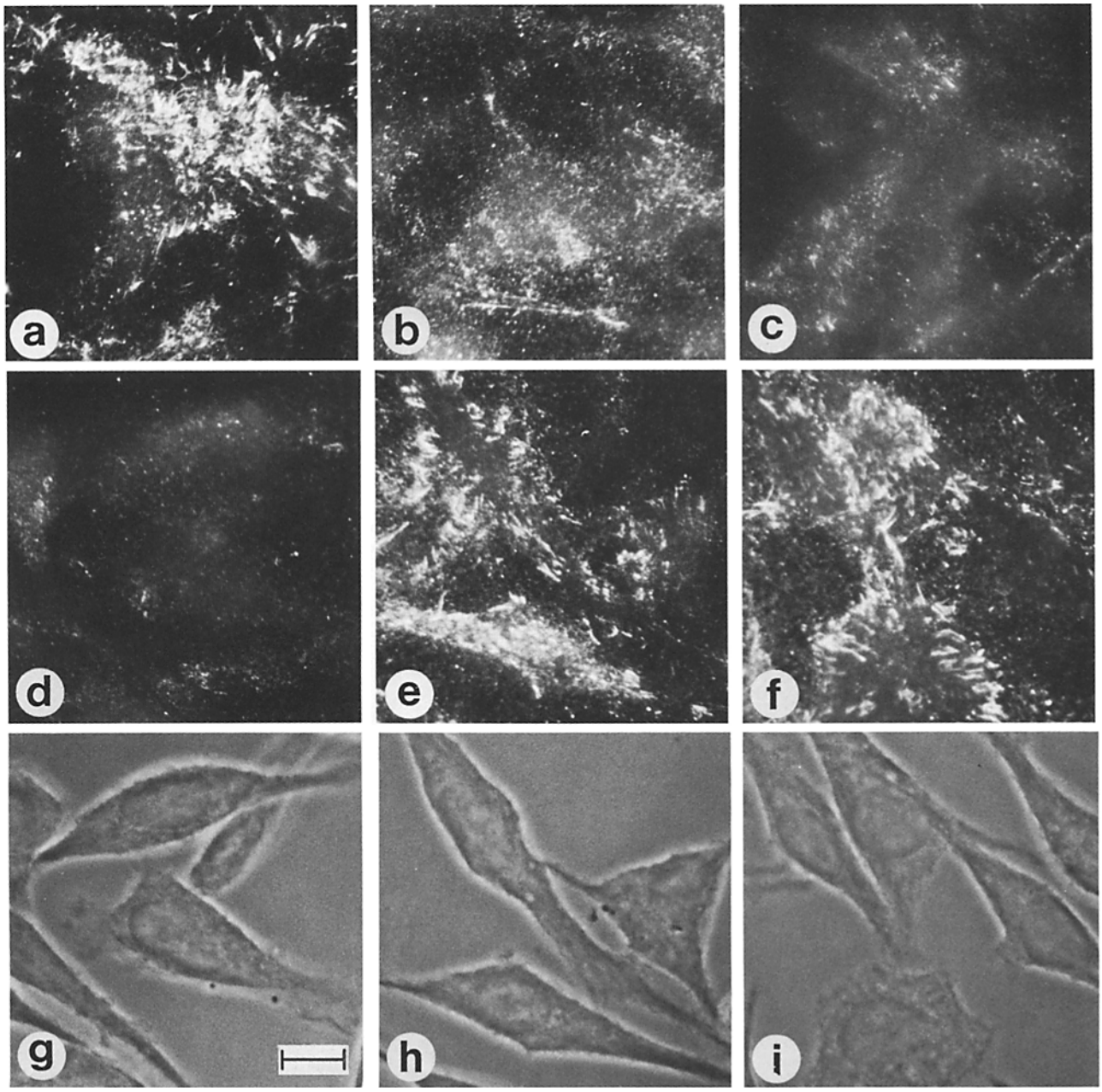

Figure 6. Effect of 70,000-mol-wt amino-terminal fragment of fibronectin on fibrillar organization. The variant cells were incubated in 2 ml of medium containing (*a*) no addition, (*b*) 25, (*c*) 50, and (*d* and *g*) 100 μl of 70,000-mol-wt amino-terminal fragment of fibronectin (2.4 mg/ml) or (*e* and *h*) 100 μl of bovine serum albumin (2.4 mg/ml) or (*f* and *i*) 100 μl of the solution against which the fragment was dialyzed. After 16 h, the cells were fixed, stained with anti-fibronectin antibodies and rhodamine-conjugated second antibody, and examined under fluorescence (*a–f*) and phase-contrast (*g–i*) microscopy. All of the additions were made 4 h after the cells were subcultured. Bar, 10 μm.

surface G_{M1} appears to be able to promote fibronectin organization. We observed in an earlier study that exogenous G_{M1}, though less effective than G_{D1a}, was able to mediate retention and organization of fibronectin by the parental cells (40).

In addition, we found that gangliosides were not required in the process of cell attachment and spreading on a fibronectin-coated substrate, as all three sublines attached and spread equally well. In this regard, Perkins et al. (30) reported that although substrates coated with the B subunit of cholera toxin supported limited spreading of BALB/c 3T3 cells, the morphology of the spread cells was clearly different from that of cells spread on fibronectin as was the organization of F-actin within the cells (30). These authors suggested that binding of fibronectin to cell surface gangliosides cannot lead to the cytoskeletal reorganization that is characteristic of cells spread on fibronectin (30).

The cell-binding domain of fibronectin has been proposed to be localized to a specific tetrapeptide sequence (31) which interacts with a 140-kD cell surface glycoprotein complex isolated from human and avian cells (1, 16, 33). Using monoclonal antibodies, such 140-kD glycoproteins have been identified as putative receptors for fibronectin in several different cell lines (3, 14, 19). In one case, the monoclonal antibodies were found to inhibit cell attachment to fibronectin but not

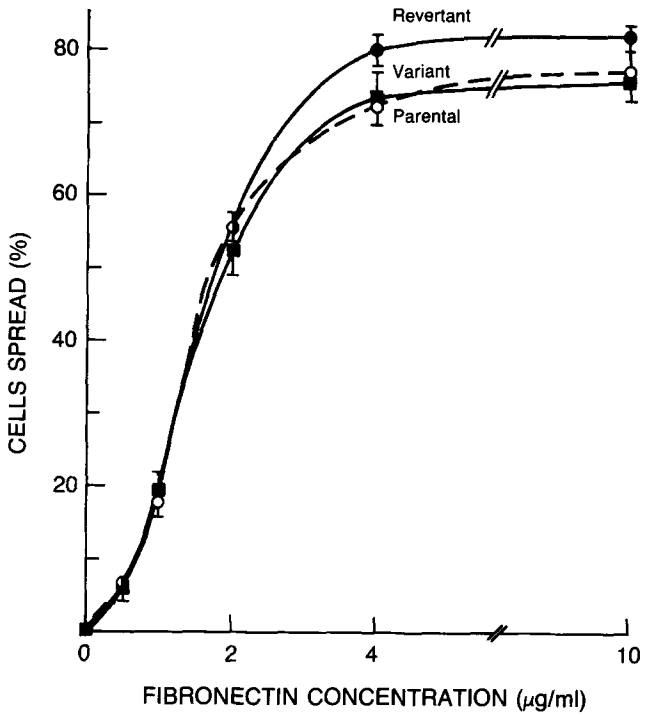

Figure 7. Adhesion of the three sublines of NCTC 2071A cells to fibronectin. The cells were allowed to adhere and spread in serum-free medium in wells coated with the indicated concentrations of fibronectin for 2 h. Each value represents the mean and standard error based on scoring 600 cells in random fields: (○) parental, (■) variant, and (●) revertant cells.

to other substrates (3). Thus, it appears that cell attachment to fibronectin involves the binding of a specific domain of fibronectin to a specific cell surface glycoprotein.

To place the above glycoprotein and complex ganglioside data in perspective, we postulate that there are two separate cell surface sites for interactions with fibronectin: one that is involved in cell attachment to fibronectin (the glycoprotein) and the other that mediates the organization of fibronectin into a matrix (the gangliosides). In support of this model, others have suggested that the cell surface receptor involved in the initial attachment and spreading on fibronectin is not required for matrix assembly (22, 23). Recently, McKeown-Longo and Mosher demonstrated that there is a domain on the amino-terminal 70-kD fragment of fibronectin distinct from the well-characterized cell attachment domain, and that this domain is involved in the assembly of fibronectin into an extracellular matrix (24). We observed that exposure of the variant cells to this fragment inhibited the fibrillar organization of fibronectin (Fig. 6). Thus, it is possible that the 140-kD glycoprotein is binding to the cell attachment domain of fibronectin and the gangliosides are interacting with the matrix assembly domain.

Previously, it was difficult to imagine how the interaction of fibronectin with gangliosides, which are too short to span the lipid bilayer of the plasma membrane, could lead to the reorganization of the cytoskeleton that usually accompanies cell spreading. Based on our above model, it is relatively easy to conceive how the interaction of fibronectin with an integral membrane glycoprotein could exert such an effect. In this regard, Chen et al. (4) and Damsky et al. (6) have described a membrane-associated 140-kD glycoprotein complex that co-localizes with fibronectin and microfilament bundles, and they suggest the complex may be a part of a cell surface linkage between fibronectin and the cytoskeleton.

From our present results, it was clear that the parental NCTC 2071A cells successfully attached to and spread on absorbed fibronectin, but failed to organize fibronectin into an extensive fibrillar network. The most likely explanation is that the cell attachment receptor is functional in these cells but the matrix assembly receptor is missing and its function can be restored either by adding exogenous gangliosides (36, 40) or altering the expression of complex cell surface gangliosides by a yet unknown mechanism.

Finally, it is important to point out that only minute amounts of complex gangliosides on the cell surface are required to mediate the organization of fibronectin into a fibrillar matrix. Based on the toxin-binding experiments, we estimate that each variant cell has ~60,000 molecules of G_{M1} or more complex gangliosides on its surface. Such trace amounts of gangliosides would not be readily detected by conventional techniques, which may explain their reported absence from cells such as Chinese hamster ovary (5, 15)[4] and baby hamster kidney (2) that have an extensive extracellular fibronectin matrix.

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