Fluorescent Gangliosides As Probes for the Retention and Organization of Fibronectin by Ganglioside-deficient Mouse Cells

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ABSTRACT Ganglioside-deficient transformed mouse fibroblasts (NCTC 2071A cells), which grow in serum-free medium, synthesize fibronectin but do not retain it on the cell surface. When fluorescent derivatives of gangliosides, containing either rhodamine or Lucifer yellow CH attached to the sialic acid residues, were added to the culture medium, the cells incorporated the derivatives and their surfaces became highly fluorescent. When the cells were stained with anti-fibronectin antibodies and a fluorescent second antibody, fibrillar strands of fibronectin were observed to be attached to the cell surface, with partial coincidence of the patterns of direct ganglioside fluorescence and indirect fibronectin immunofluorescence at the cell surface. When the cells were exposed to bacterial neuraminidase during the time of ganglioside insertion, similar patterns of fluorescence were observed. Because the fluorescent gangliosides are resistant to the enzyme, these results suggest that neuraminidase-sensitive endogenous glycoconjugates were not involved in the ganglioside-mediated retention and organization of endogenous fibronectin. After cells were exposed to exogenous chicken fibronectin, most of the fibronectin was attached to the substratum and only a few fibrils were attached to the cells. When exogenous gangliosides were included in the incubation, there was a striking increase in cell-associated exogenous fibronectin, which was highly organized into a fibrillar network. Conversely, cells incubated for 18 h with exogenous unmodified gangliosides exhibited a highly organized network of endogenously derived fibronectin. Upon further incubation of the cells for 2 h with fluorescent gangliosides, there was considerable co-distribution of the fluorescent gangliosides with the fibronectin network as revealed by immunofluorescence. Our results support the concept that gangliosides can mediate the attachment of fibronectin to the cell surface and its organization into a fibrillar network.

Fibronectin is a high molecular weight glycoprotein associated with the surface and extracellular matrix of a variety of cells and tissues. It is believed to be involved in various cellular processes, including attachment to and spreading on various substrates (1–3). In addition to the cellular form of fibronectin, there is also a soluble plasma form which, although chemically different, can bind to cells and induce attachment and spreading (4, 5). Recently, the cell attachment domain of fibronectin was isolated and sequenced (6, 7). The molecular basis for the interaction of fibronectin with cells, however, remains unclear, and the nature of the cell surface receptor for fibronectin has not yet been elucidated.

Different reports are consistent with the proposal that the cellular receptor(s) for fibronectin may be glycoproteins (8–14), glycosaminoglycans (15), gangliosides (16–21), or phospholipids (22). In a recent study (20), it was demonstrated

that exogenous gangliosides could mediate the retention and reorganization of endogenous fibronectin by NCTC 2071A cells, a line of transformed mouse L cells adapted to grow in serum-free medium. These cells are deficient in gangliosides (23, 24) and cannot retain endogenously synthesized fibronectin at the cell surface (20). Cells treated with exogenous gangliosides restored a more normal fibrillar arrangement of fibronectin attached to the cell surface (20). It was not established whether the gangliosides might interact directly with fibronectin or mediate their effects indirectly through some other cell surface component. It also was not determined whether they could modify the organization of exogenously supplied fibronectin rather than simply alter the patterns of secretion and retention of endogenous fibronectin. With the recent availability of fluorescent derivatives of gangliosides (25, 26) and species-specific anti-fibronectin antibodies, we have addressed these questions.

MATERIALS AND METHODS

Materials: *Vibrio cholerae* neuraminidase (EC 3.2.1.18) was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Fluorescein-labeled rabbit anti-goat IgG was from Miles Laboratories (Elkhart, IN) and rhodamine-labeled rabbit anti-goat IgG was from Cappel Laboratories (Cochranville, PA). Affinity-purified goat anti-chicken cellular fibronectin antibodies were prepared as described previously (27). Antibodies crossreacting with mouse fibronectin were removed by passage of the affinity-purified antibodies through a second affinity column containing mouse plasma fibronectin covalently linked to agarose. Removal of the crossreactive antibodies was verified by immunofluorescence staining of NCTC 2071A cells secreting endogenous mouse fibronectin. Cellular fibronectin was purified from chick embryo fibroblasts as described by Yamada (28). Rhodamine- and Lucifer yellow CH-labeled gangliosides were prepared as described by Spiegel et al. (25, 26). Bovine brain gangliosides were obtained from ICN Nutritional Biochemicals (Cleveland, OH).

Cell Culture: NCTC 2071A cells were cultured in serum-free NCTC 135 medium on glass coverslips as described previously (20). Cells were cultured 2–3 d before an experiment and were not fed. The cell density was ~50,000 cells/cm^2. The growth medium was then changed and unmodified, and fluorescent gangliosides, dissolved in sterile water, were added to the coverslip cultures at a final concentration of 20–50 μg in 1 ml of NCTC 135 medium. The cells were incubated without further medium change for 2 or 18 h in a humidified incubator (20). Cells were treated with exogenous chicken cellular fibronectin (20 or 50 μg/ml) after being washed with NCTC 135 medium.

Immunofluorescence: After the various treatments, the cells were rinsed three times with Dulbecco's phosphate-buffered saline (PBS) and fixed for 1 h with a solution containing 3.7% formaldehyde and 5% sucrose in PBS. After extensive rinsing, the cells were incubated with 20 μg/ml of affinity-purified goat anti-fibronectin for 1 h at room temperature. Indirect immunofluorescence was performed using rabbit anti-goat IgG labeled with fluorescein (diluted 1:20) or with rhodamine (50 μg/ml) as described previously (20). After extensive washing, the cultures were examined with Zeiss microscopes equipped for epifluorescence microscopy. Photographs were taken with either a Pentax camera or Photomicroscope III optics using interference filters (models 487710 and 48771; Carl Zeiss, Inc., Thornwood, NY) specific for rhodamine or fluorescein fluorescence. With this filter combination, no crossover was observed; cells labeled with Lucifer yellow CH-gangliosides were not detected in the rhodamine channel, and cells labeled with rhodaminyl gangliosides were not detected in the fluorescein channel. The filters were changed without altering the focus to compare direct ganglioside fluorescence with fibronectin immunofluorescence on the same cells. Routine controls included incubating the cells with equal amounts of preimmune IgG, omitting the first antibody, or omitting the ganglioside incorporation step.

Other Methods: Lucifer yellow CH-labeled gangliosides (50 μg) were incubated in 0.1 ml of neuraminidase solution (0.1 U) at 25°C for 18 h; then 0.4 ml of PBS was added, and the sample was dialyzed against distilled water and lyophilized. The gangliosides were separated by thin-layer chromatography on silica gel 60-coated glass plates using the solvent system chloroform/methanol/0.25% aqueous $CaCl_2$ (60:35:8, vol/vol).

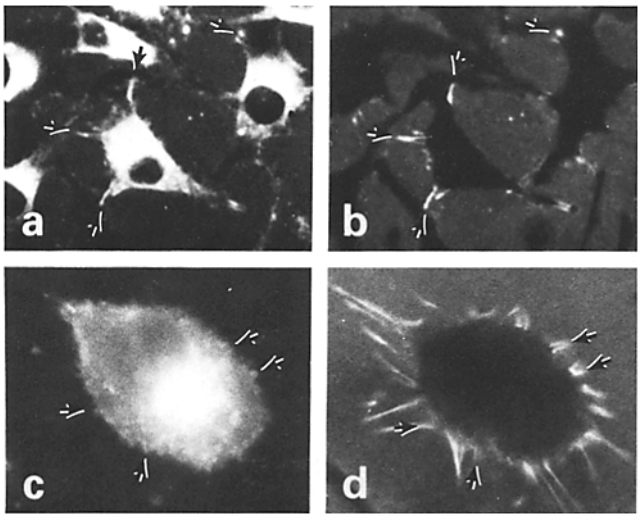

FIGURE 1 Effects of fluorescent gangliosides on the distribution of endogenous fibronectin on NCTC 2071A cells. Cells were cultured in serum-free medium supplemented with Lucifer yellow CH-labeled (*a* and *b*) or rhodamine-labeled (*c* and *d*) gangliosides for 18 h. The cells then were washed, fixed, and stained for fibronectin with goat anti-chicken fibronectin and either rhodamine- (*a* and *b*) or fluorescein- (*c* and *d*) conjugated rabbit anti-goat as described in Materials and Methods. The cells were then examined for the distribution of fluorescent gangliosides (*a*, Lucifer yellow CH; *c*, rhodamine) or fibronectin by immunofluorescence (*b*, rhodamine; *d*, fluorescein). Arrows indicate sites of correlation of ganglioside and fibronectin fluorescence patterns, either as coincidences or as a cell protrusion (ganglioside) associated with a fibril (fibronectin).

RESULTS

Effect of Fluorescent Gangliosides on Endogenous Fibronectin of NCTC 2071 Cells

NCTC 2071A cells were incubated 18 h in medium supplemented with either Lucifer yellow CH- (Fig. 1, *a* and *b*) or rhodamine-labeled gangliosides (Fig. 1, *c* and *d*), washed extensively, fixed, and then stained indirectly for cellular fibronectin using anti-fibronectin antibody and either rhodamine- (Fig. 1, *a* and *b*) or fluorescein- (Fig. 1, *c* and *d*) labeled second antibody. When we examined the cells for direct fluorescence, we observed that both of the ganglioside derivatives had become associated with the surfaces of the cells; in addition, there was some internal fluorescence, indicating that the gangliosides became internalized with time (Fig. 1, *a* and *c*). As had been observed with unmodified gangliosides (20), the fluorescent derivatives also promoted the reorganization of fibronectin into fibrillar strands bound to the cell surface (Fig. 1, *b* and *d*).

The specificity of the fibronectin staining was confirmed by appropriate controls. When cells enriched with fluorescent gangliosides were stained with preimmune IgG from the same goat followed by the appropriate fluorescent second antibody, no fluorescence was detected on the surfaces of the cells. The effect of the fluorescent gangliosides was also specific because control cells indirectly stained for fibronectin exhibited no surface fibronectin as reported previously (20).

A comparison of direct ganglioside fluorescence with indirect fibronectin immunofluorescence indicated considerable coincidence of the two patterns at the cell surface and sometimes on the fibers (Fig. 1, *a* vs. *b*, *c* vs. *d*; see also Figs. 3, 5, and 7). The effect of ganglioside treatment on fibronectin reorganization was not confined to only a few cells (Fig. 2*A*). Both unmodified and fluorescent gangliosides caused a similar

increase in the proportion of cells containing fibrillar strands of fibronectin.

Fluorescent Gangliosides Affect the Organization of Exogenous Fibronectin

To explore further the phenomenon of ganglioside-stimulated attachment of fibronectin to the cell surface, we added exogenous fibronectin from another species to the culture medium. The addition of purified chicken cellular fibronectin to NCTC 2071A cells for 18 h had minimal effects on the localization of fibronectin (Fig. 2*B*). Most of the fibronectin was confined to the substratum and only a few fibrils extending from the cell surface were observed (Fig. 3*a*; Fig. 4, *a* and *f*). When the cells were incubated at the same time with either fluorescent (Fig. 3, *b* and *d*) or unmodified (Fig. 4, *b*, *c*, and *h*) gangliosides, there was a striking increase in the intensity of fibrillar staining for fibronectin. Most of it was localized in fibrillar structures with increasing numbers of fibrils extending from the cell surface (Fig. 2*B*; Fig. 3, *b* and *d*; Fig. 4, *b*, *c*, and *h*). In addition, some of the fluorescent gangliosides were found localized on the strands of fibronectin (Fig. 3, *c* and *e*). Results with crossreacting (Fig. 3) and species-specific (Fig. 4) antibody yielded similar results.

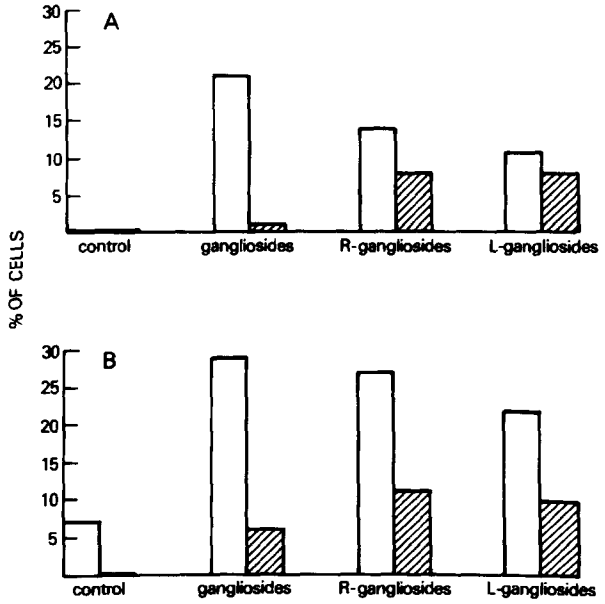

FIGURE 2 Gangliosides affect the organization of endogenous and exogenous fibronectin on NCTC 2071A cells. Cells were cultured in serum-free medium without (*A*) or with (*B*) exogenous chick fibronectin (20 μg/ml) for 18 h. Where indicated, no gangliosides (control), 20 μM bovine brain gangliosides, or 20 μg/ml of rhodamine-labeled (*R*-) or Lucifer yellow CH-labeled (*L*-) gangliosides were added to the medium during the incubation. The cells were then washed, fixed, and stained for fibronectin as described in Materials and Methods and examined for fibrillar strands of fibronectin. Values are based on counting at least 100 cells. □, percent of cells containing less than five fibrillar strands of fibronectin; ▨, percent of cells containing more than five fibrillar strands of fibronectin.

Fluorescent Gangliosides Co-distribute with the Fibrillar Network of Fibronectin

The above studies indicated some association of fluorescent gangliosides with the fibrillar network of fibronectin. Because a similar, tight association had been observed recently with dense cultures of human fibroblasts (21), it appears that gangliosides may have a higher affinity for fibrillar fibronectin than for soluble fibronectin because of some type of cooperative interaction. To test this possibility, NCTC 2071A cells were cultured for 18 h with unlabeled gangliosides. Then the cells were washed and incubated for 2 h more with Lucifer yellow CH-labeled gangliosides (Fig. 5*a*). The fluorescent gangliosides were not only distributed diffusely on the plasma membrane but also corresponded identically to the fibrillar network of fibronectin as revealed by rhodamine-labeled immunofluorescence (Fig. 5*b*). The more intense co-distribution of fluorescent gangliosides under these conditions, compared with those used in Fig. 1, appears to be the result of two effects. First, the fluorescent gangliosides became internalized by the cells with time (Fig. 1) and thus were not available to interact with the fibronectin as it was slowly organized at the cell surface. Second, during the long incubation period required to optimize the fibrillar network of fibronectin, most of the exogenous fluorescent gangliosides became adsorbed to the plastic dishes in which the glass coverslips were maintained. The ability of gangliosides to be adsorbed to plastic is well established and has been used to develop solid phase assays (19, 29).

Effect of Neuraminidase on Reorganization of Endogenous Fibronectin

We observed that Lucifer yellow CH-labeled gangliosides

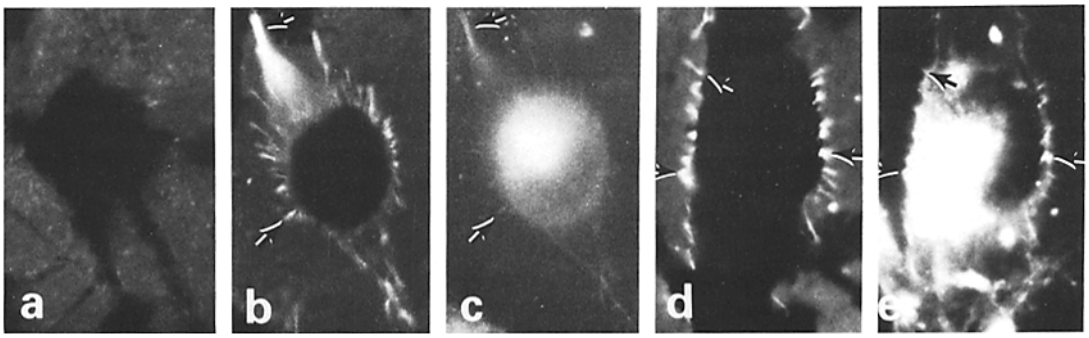

FIGURE 3 Effects of fluorescent gangliosides on the distribution of exogenous fibronectin on NCTC 2071A cells. Cells were cultured for 18 h in serum-free medium supplemented with 20 μg/ml of chick fibronectin without (a) and with (*b*–e) Lucifer yellow CH-labeled gangliosides. The cells then were washed, stained with anti-chicken fibronectin and rhodamine-conjugated rabbit anti-goat IgG, and examined for ganglioside (*c* and *e*) and fibronectin (*a*, *b* and *d*) fluorescence. Arrows indicate points of partial coincidence of ganglioside and fibronectin fluorescence.

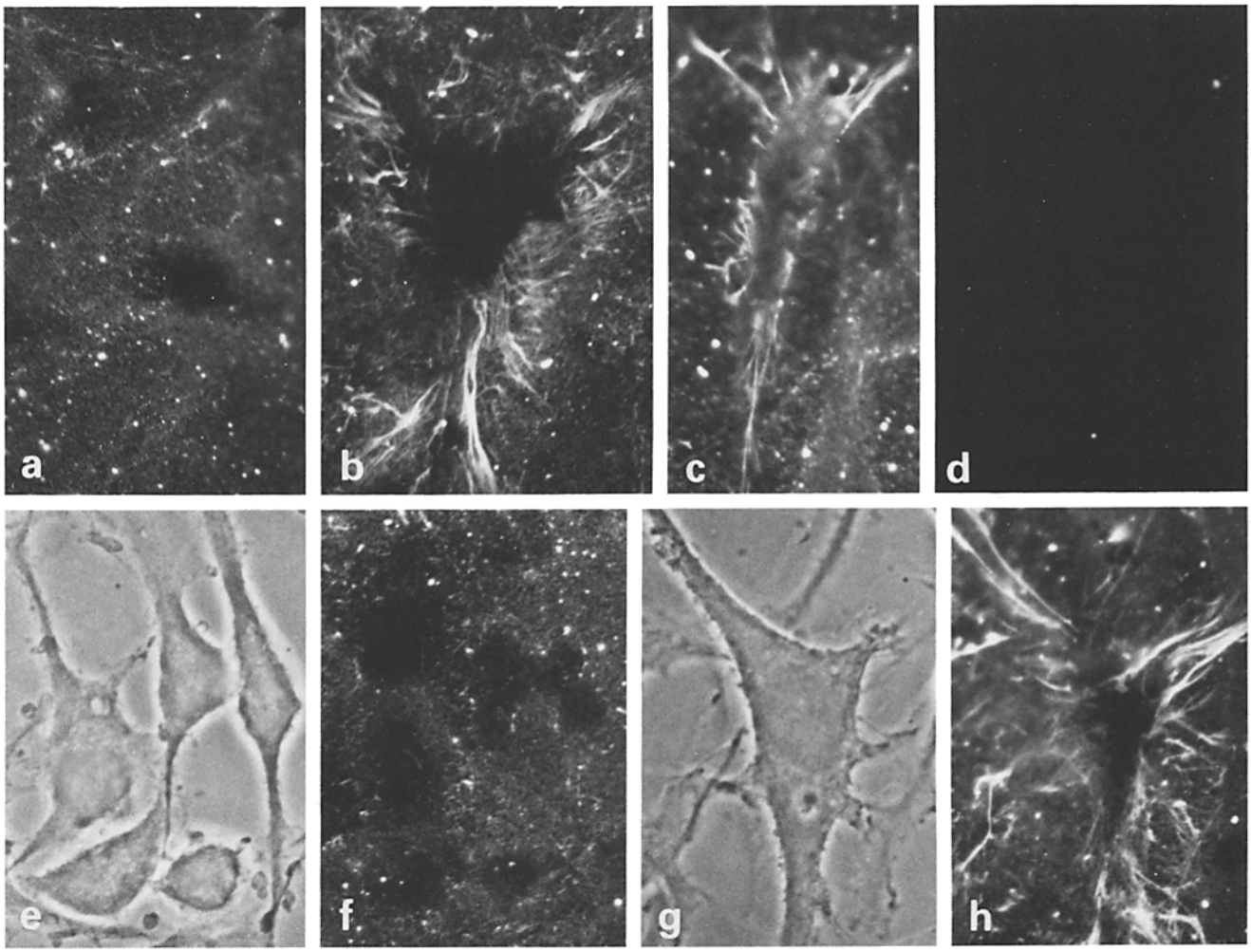

FIGURE 4 Interspecies reorganization of exogenous fibronectin by ganglioside-treated NCTC 2071A cells. Cells were incubated for 18 h with high concentrations (50 μg/ml) of purified chicken cellular fibronectin in the presence or absence of unmodified gangliosides (20 μg/ml). Control and treated cells were fixed and stained specifically for chicken fibronectin using affinity-purified anti-chicken fibronectin antibodies from which all antibodies recognizing endogenous mouse fibronectin had been removed by affinity chromatography. Fluorescein-labeled rabbit anti-goat was used as the second antibody. (*a*) Substrate-adsorbed fibronectin in control culture receiving exogenous fibronectin but no exogenous gangliosides; (*b* and *c*) fibrils of reorganized exogenous fibronectin around individual cells treated with gangliosides; (*d*) absence of immunofluorescence in a culture receiving exogenous gangliosides but no exogenous fibronectin, showing lack of staining of endogenous mouse fibronectin; (e and *f*) control culture receiving exogenous fibronectin but no exogenous gangliosides; and (*g* and *h*) cultures treated with both exogenous fibronectin and gangliosides. (e and *g*) Phase contrast micrographs and (*f* and *h*) the corresponding immunofluorescence images.

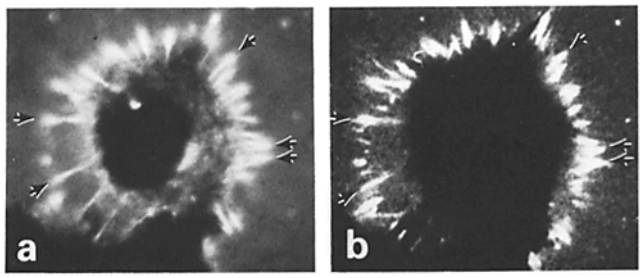

FIGURE 5 Interaction of fluorescent gangliosides with fibrillar network of fibronectin on NCTC 2071A cells. Cells were cultured for 18 h in serum-free medium supplemented with 20 μg/ml unmodified gangliosides. The cells then were washed and incubated in fresh medium for 2 h with Lucifer yellow CH-labeled gangliosides. The cells were washed, fixed, stained with anti-fibronectin and rhodamine-conjugated second antibody, and examined for ganglioside (*a*) and fibronectin (*b*) fluorescence. Arrows indicate coincidence between ganglioside and fibronectin fluorescence.

were resistant to neuraminidase (Fig. 6). The NCTC 2071A cells were incubated with both the enzyme and the Lucifer yellow CH-tagged gangliosides. Neuraminidase treatment did not have any noticeable effect on ganglioside insertion and distribution (Fig. 7*a*) or on the reorganization of fibronectin on the cell surface (Fig. 7*b*). When NCTC 2071A cells were incubated with several different proteolytic enzymes, they became severely damaged, and no useful information was obtained.

DISCUSSION

Our present results indicate that fluorescent derivatives of gangliosides are taken up by ganglioside-deficient NCTC 2071A cells and initially are associated with the plasma membrane. There, the fluorescent gangliosides promoted the retention of endogenous fibronectin and its organization into fibrils in a manner analogous to that reported previously for un-

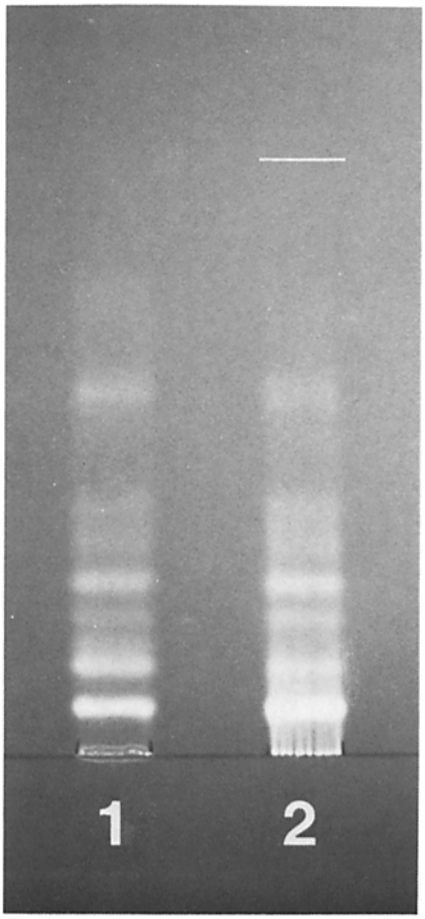

FIGURE 6 Effect of neuraminidase on fluorescent gangliosides. 5-μg portions of control (lane *1*) and neuraminidase-treated (lane *2*) Lucifer yellow CH-labeled gangliosides were separated by thin-layer chromatography as described in Materials and Methods and detected by ultraviolet light. The bar in lane *2* indicates the location of unmodified G_{M1} on the same chromatogram.

modified gangliosides (20). The use of fluorescent gangliosides also allowed us to determine whether there was any association between fibronectin and gangliosides at the cell surface. Under appropriate conditions, we observed a coincident distribution of the gangliosides by direct fluorescence and the fibrillar network of fibronectin as revealed by indirect immunofluorescence. In many cases with cells at low density, the fibrillar patterns of fibronectin could have represented binding to cell processes or extensions without matrix formation. In other cases, e.g., in Fig. 4 of this paper and in a previous publication (20), the fibronectin appeared to become organized into an extensive pericellular fibrillar network. It also appears that the organization of fibronectin requires a ganglioside-dependent function of the cells rather than some nonspecific self-polymerization event.

In addition, we were able to demonstrate that gangliosides promoted the retention and organization of exogenously added chick fibronectin. Finally, we observed that bacterial neuraminidase did not prevent the retention and organization of fibronectin in cells exposed to the fluorescent gangliosides. As the latter were resistant to the enzyme presumably because of their modified sialyl groups, only endogenous sialoglycoconjugates would be susceptible to the enzyme.

Our observations confirm and extend previous studies that implicate gangliosides as cell surface receptors for fibronectin.

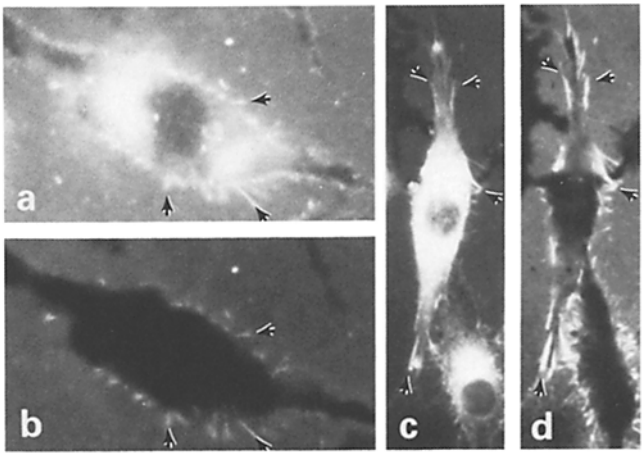

FIGURE 7 Effect of neuraminidase on the distribution of fluorescent gangliosides and fibronectin on NCTC 2071A cells. Cells were incubated in NCTC 135 medium containing Lucifer yellow CH-labeled gangliosides and neuraminidase (0.01 U/ml) for 18 h, washed, fixed, and stained with anti-fibronectin and rhodamine-conjugated second antibody. (*a* and *c*) Lucifer yellow CH-ganglioside fluorescence; (*b* and *d*) fibronectin as revealed by rhodamine immunofluorescence. Arrows mark examples of partial coincidence of ganglioside and fibronectin fluorescence.

It was orginally shown by Kleinman et al. that gangliosides and their oligosaccharides inhibit the attachment of Chinese hamster ovary (CHO)[1] cells to fibronectin-collagen substrate (16) and the binding of fibronectin to human fibroblasts (18). Gangliosides and their oligosaccharides also blocked fibronectin-mediated hemagglutination of sheep erythrocytes, spreading of baby hamster kidney (BHK) cells on plastic, and restoration of a normal morphology to transformed mouse cells (17). More recently, Perkins et al. confirmed the ability of gangliosides to inhibit the spreading of CHO and BHK cells on fibronectin-coated plastic substrate (19). Direct binding of fibronectin to immobilized gangliosides was also reported (19). In all of these studies, the more complex, more sialylated gangliosides were the most effective.

Other observations, however, suggest that gangliosides are not receptors for fibronectin. The major ganglioside in CHO and BHK cells is *N*-acetylneuraminyl(α2→3)galactosyl-(β1→4)glucosyl(β1→1′)ceramide (G_{M3}) with only trace amounts of complex gangliosides (30, 31). Neuronal cells, which have large amounts of complex gangliosides, do not normally bind fibronectin (32). In addition to inhibiting the attachment of cells to fibronectin surfaces, gangliosides also block attachment to glycosidase and lectin surfaces, and it was suggested that the ganglioside effect was nonspecific (12). Cholera toxin, which binds specifically to galactosyl(β1→3)*N*-acetylgalactosaminyl-(β1→4)[*N*-acetylneuraminyl(α2→3)]galactosyl(β1→4)glucosyl(1→1′)ceramide (G_{M1}) supports cell attachment but not cell spreading in contrast to fibronectin (19). Protease treatment of cells inhibits certain fibronectin-mediated cell adhesive and binding events (11, 14). In addition, fibronectin binds to cell surface glycosaminoglycans (15) and to phospholipid vesicles (22). Finally, numerous reports have implicated plasma membrane glycoproteins as receptors for fibronectin (8–14).

The identification of these putative glycoprotein receptors

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; BHK, baby hamster kidney.

for fibronectin is still controversial. Aplin et al. showed that a 48-kD cell-surface glycoprotein is cross-linked to fibronectin in adherent cells (9). The same glycoprotein, however, was detected when cells were allowed to spread on photoactive derivatives of concanavalin A and ricin. Tarone et al. reported an unusual feature of the glycoproteins involved in the adhesion of cells to substrate-bound fibronectin (14). They found two glycoproteins (GP 120 and GP 80) that are resistant to trypsin. Another glycoprotein (GP 45) was absent, however, from trypsinized cells that retained their adhesiveness to fibronectin. A different conclusion was reached by Grinnell, who found that BHK cells treated with low concentrations of trypsin in the absence of calcium lose their ability to bind fibronectin-coated beads (11, 33). Oppenheimer-Marks and Grinnell reported that antibodies raised against wheat germ agglutinin receptors on BHK cells inhibit cell spreading on fibronectin-coated substrates, cause rounding up and detachment of the cells, and inhibit binding of fibronectin-coated beads to the cells (10). The antibodies react with glycoproteins of 48, 61, 83, 120, 165, 210, and 230 kD. Their results were inconclusive in terms of specificity for a fibronectin "receptor" since the antibodies also inhibited cell spreading on concanavalin A- and polycationic ferritin-coated surfaces. Wylie et al. also have described adhesive glycoproteins (GP 120 and GP 140) by using antisera to induce cell rounding and detachment from the substratum (34).

Nevertheless, our present studies with NCTC 2071A cells as well as previous work (20) demonstrate that complex gangliosides mediated the retention of fibronectin by these ganglioside-deficient cells and its organization into a fibrillar network. Other plasma membrane components may also be part of the receptor for fibronectin and they may act in concert with, or in lieu of, gangliosides in different cell types. The difficulty in identifying the native receptor(s) for fibronectin appears to result from the fact that soluble fibronectin does not bind readily to cells (1–3, 19) and appears to interact weakly with potential receptor components (19, 35–37; Akiyama, K., and K. M. Yamada, manuscript submitted for publication). It appears, however, that the fibrillar form of fibronectin is recognized by the cell surface with a higher affinity than the soluble form. In this regard, our ability to demonstrate a direct binding of fluorescent gangliosides to the fibronectin matrix of NCTC 2071A cells as well as confluent human fibroblasts (21) may be highly relevant and provide a potential assay for identifying the fibronectin receptor.

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