Published February 1, 1986

Variants of BALB/c 3T3 Cells Lacking Complex Gangliosides Retain a Fibronectin Matrix and Spread Normally on Fibronectin-coated Substrates

Susanne L. Griffiths, Robert M. Perkins, Charles H. Streuli, and David R. Critchley

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, England. R. M. Perkins' present address is Beecham Pharmaceutical Biosciences Research Centre, Epsom, Surrey, KT18 5XQ England. C. H. Steuli's present address is Imperial Cancer Research Fund Laboratories, London, WC2A 3PX England.

Abstract. Evidence has accumulated that di- and trisialogangliosides are involved in the interaction of cells with fibronectin. We have therefore tested the ability of variants of BALB/c 3T3 deficient in such gangliosides to organize a fibronectin matrix and to spread on fibronectin-coated substrates. Whereas BALB/c 3T3 cells contained gangliosides GM3, GM1, and GD1a, direct chemical analysis showed that five out of six variants isolated contained no detectable GD1a. By the overlaying of thin layer chromatograms of cellular gangliosides with ^{125}I-cholera toxin, these variants were also found to lack ganglioside GM1. In contrast, the sialogalactoprotein profile of these cells, analyzed using an ^{125}I-ricin/SDS polyacrylamide gel overlay technique, was similar to that of the parent cell line. All variants organized an extensive fibronectin matrix comparable to that of BALB/c 3T3, as shown using either immunofluorescence or lactoperoxidase-catalyzed iodination. The variants could also spread on fibronectin-coated substrates and adopt a morphology similar to that of BALB/c 3T3 cells, with little or no difference in the concentration of fibronectin required for 50% cell spreading. Cell spreading of the variants was accompanied by the formation of focal contacts and microfilament bundles, in a manner closely resembling that seen with BALB/c 3T3 cells. Treatment of BALB/c 3T3 cells with neuraminidase, which converts much of the cellular GD1a to GM1, did not affect cell spreading on fibronectin. The results clearly demonstrate that complex gangliosides are not essential for retention of a fibronectin matrix or for spreading on fibronectin-coated substrates.

CELL adhesion to and spreading on substrates coated with the extracellular matrix glycoprotein fibronectin is thought to proceed through the interaction of a cell surface receptor with a cell binding domain in fibronectin (21, 45). The molecular features of this domain that are important for cell attachment have recently been determined (34). Characterization of the receptor has however been hampered by the apparent low affinity of soluble fibronectin for its receptor. Nevertheless reports implicating glycoproteins (3, 9, 24, 30, 35, 43), proteoglycans (26, 31), and gangliosides (23, 38, 46, 47) as the fibronectin receptor have accumulated.

Interest in a possible role for gangliosides in this context originated from experiments in which di- and trisialogangliosides were shown to be effective inhibitors of cell attachment and spreading on fibronectin-coated substrates (23, 32, 47). Despite a number of doubts concerning the specificity of this effect (33, 36), the observation that gangliosides can mediate the retention of fibronectin at the surface of a ganglioside-deficient mouse L cell variant (NCTC 2071A) has renewed speculation that gangliosides are in some way involved in the interaction of cells with fibronectin (38, 46). However, as not all cells that interact with fibronectin synthesize the more complex gangliosides, such a role must be limited to certain cells or cell types. BALB/c 3T3 cells synthesize ganglioside GM3[1] as well as the more complex gangliosides GM1 and GD1a, and also synthesize a fibronectin-containing extracellular matrix and can spread on fibronectin-coated substrates. We have recently isolated variants of BALB/c 3T3 cells deficient in complex ganglioside biosynthesis (39), and we now report on the further characterization of these variants and their ability to interact with fibronectin.

Materials and Methods

Cells and Culture Conditions

Subclones of BALB/c 3T3 cells (clone A31) were isolated, and the one with growth characteristics and ganglioside composition most like those of the parent cell line was used for subsequent studies (S24 cells). Variants defective in synthesis of gangliosides more complex than GM3 were isolated from S24 cells exposed to ethane methane sulfonate using a cholera toxin, antitoxin, and complement selection procedure, as described elsewhere (39). Variants M1-3

[1] Ganglioside nomenclature is according to Svennerholm (42). GM3 = NeuAcα2→3Galβ1→4Glc→Cer; GM2 = GalNacβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer; GM1 = Galβ1→3GalNacβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer; GD1b = Galβ1→3GalNacβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer); GT1b = NeuAcα2→3Galβ1→3GalNacβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glc→Cer.

© The Rockefeller University Press, 0021-9525/86/02/0469/08 $1.00

Published February 1, 1986

and M5-7 were cloned from populations surviving five or six rounds of selection. One variant, V4, was isolated from S24 cells not treated with mutagen. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DME),[2] supplemented with 10% heat-inactivated newborn calf serum, plus antibiotic/antimycotic solution (Biocult, Gibco Laboratories, Grand Island, NY).

Ganglioside Purification

Lipids were extracted from cell pellets (5–10 mg of protein) with 20 vol chloroform/methanol (2:1 then 1:2 vol/vol). The extract was dried under N_2, redissolved in chloroform/methanol (2:1 vol/vol), and partitioned against 0.2 vol water. The organic phase was washed three times with chloroform/methanol water (3:48:47 vol/vol), and the combined aqueous phases were dried, then desalted on Sephadex G.25 columns (0.75 g) equilibrated and eluted with chloroform/methanol/water (60:30:4.5 vol/vol). Gangliosides were separated by thin layer chromatography (TLC) on precoated Silica gel G plates (E. Merck, Darmstadt FRG) with the solvent chloroform/methanol/water (60:35:8 vol/vol), and detected by spraying with the resorcinol reagent (41). Standard gangliosides were purchased from Supelco, Inc., Bellefonte, PA.

Detection of Ganglioside GM1 in BALB/c 3T3 Cells Using a TLC, ^{125}I-labeled Cholera Toxin Overlay Procedure

Cellular gangliosides were separated on plastic-backed silica gel G TLC plates (Eastman Kodak Co., Rochester, NY) using the solvent chloroform/methanol/0.2% aqueous $CaCl_2$ (5:4:1 vol/vol), and ganglioside GM1 was detected using a modification of the procedure described by Magnani et al. (27). In brief, the dried chromatogram was immersed in 0.1% (wt/vol) polyisobutylmethacrylate (Polysciences, Inc., Warrington, PA) for 1 min, dried, sprayed, then soaked in 0.15 M NaCl, 50 mM Tris, pH 7.4, containing 0.2% gelatin for 1 h at 4°C. Plates were overlayed with iodinated cholera toxin (0.5×10^6 cpm/ml) in the same buffer for 2 h at 4°C, then washed over a 15-min period with five changes of buffer before being dried and autoradiographed. Cholera toxin was iodinated to a specific activity of 10 μCi/μg as previously described (40).

Separation of Cell Proteins by SDS PAGE and Detection of Galactoproteins with ^{125}I-labeled Ricin

Total cell proteins were separated in SDS polyacrylamide gels (25), and the proteins were transferred electrophoretically to nitrocellulose sheets (44), which were then overlaid with ^{125}I-labeled ricin (1×10^5 cpm/ml in 0.15 M NaCl/50 mM Tris, pH 7.4, containing 1.0% bovine serum albumin) for 3 h at 20°C. The sheets were washed with buffer, and the ricin binding proteins were detected by autoradiography. Ricin (RCA_{60}, Miles Laboratories Ltd., Slough, England) was iodinated to a specific activity of 4 μCi/μg as described by Burridge (8).

Surface Labeling Procedures

Sialoglycoconjugates were labeled using the IO_4/B^3H_4 method (13), and surface proteins were labeled by lactoperoxidase-catalyzed iodination (19).

Immunofluorescent Staining for Fibronectin and Actin

Cells grown either on glass coverslips or directly on tissue culture plastic (35-mm dishes) were fixed in 3.8% (vol/vol) formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature and washed three times with PBS. For fibronectin staining, cells were incubated for 40 min with a rabbit anti-human plasma fibronectin serum (diluted 1:100 in PBS), washed three times with PBS, and then incubated for 30 min with a 1:50 dilution of rhodamine-labeled goat anti-rabbit Ig (Tissue Culture Service Ltd., Slough, England). After extensive washing over a 30-min period, coverslips were mounted in PBS/50% glycerol. Cells stained in culture dishes were covered with a 25-mm-diam coverslip (thickness No. 1) using PBS/glycerol as mountant. Actin was visualized in fixed and permeabilized (0.5% Triton X-100 in PBS for 5 min) cells using nitrobenzoxadiazole-phallicidin (Molecular Probes Inc., Junction City, OR), as previously described (32).

Preparations were viewed using a Zeiss Standard 16 microscope equipped with epifluorescence. Photographs were taken on Ilford (Knutsford, Cheshire, England) HP5 35-mm film (400 ASA) uprated to 1,600 ASA during processing.

Cell Spreading on Ligand-coated Substrates

Cell spreading on fibronectin or cholera toxin B-subunit-coated substrates was assayed as previously described (32). To visualize focal contacts, cells (5×10^5/ml in DME) were plated onto glass coverslips derivatized with fibronectin as described by Aplin and Hughes (2). After varying periods, coverslips were mounted in DME onto a glass slide using a rubber O ring as a spacer. Cells were viewed using a Zeiss Standard 16 microscope equipped with epifluorescence, polarizing and analyzer filters, plus a × 63 antiflex objective, essentially as described by Abercrombie and Dunn (1).

[2] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; TLC, thin layer chromatography.

Results

Characterization of BALB/c 3T3 Variants Deficient in Complex Gangliosides

We have recently described the isolation of variants of BALB/c 3T3 cells (clone A31, subclone S24) defective in complex ganglioside biosynthesis (39). The selection procedure was based on the known specificity of cholera toxin for ganglioside GM1, and on the ability to lyse cells containing GM1 in the presence of toxin, antitoxin, and complement. The ganglioside profile of these variants, as detected by spraying TLC plates with resorcinol, is shown in Fig. 1. The major ganglioside in the parent cell line was GM3, and there was less GD1a. Ganglioside GM1 was barely detectable by this method. In contrast, variants M1, M3, and M5-7 showed little if any ganglioside GD1a. That levels of ganglioside GM1 were also dramatically reduced in these variants was shown by overlaying chromatograms of the separated gangliosides with ^{125}I-labeled cholera toxin. When this sensitive procedure was used, ganglioside GM1 was readily detected in the parent cell line but was shown to be absent from variants M1, M3, and M5-7 (Fig. 2). The results are in complete agreement with the marked reduction in binding of ^{125}I-labeled cholera toxin by these cells and the failure to incorporate [1-^{14}C]palmitate into gangliosides more complex than GM3 (39).

Variant V4, isolated from a nonmutagenized population of cells, retained both gangliosides GD1a (Fig. 1) and GM1 (Fig. 2), but at reduced levels. The observation is consistent with the finding that toxin binding by this variant was reduced by only 70%, as compared with a >95% reduction displayed by other variants, and that V4 incorporated a significant amount of [1-^{14}C]palmitate into ganglioside GD1a (39). Further investigation of toxin binding by variant V4 using indirect immunofluorescence showed it to be heterogenous, ~20% of

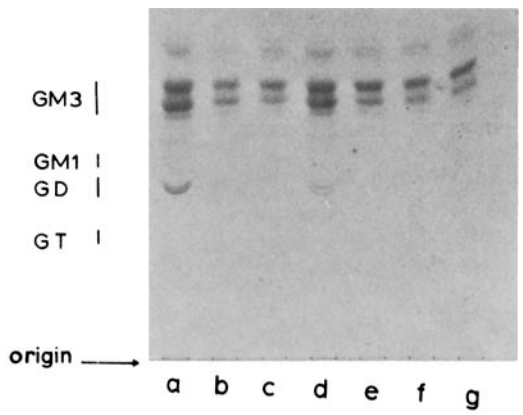

Figure 1. Gangliosides of BALB/c 3T3 cells and variants. Gangliosides purified from BALB/c 3T3 cells (clone A31, subclone S24) and variants (M1, M3, V4, M5-7) were separated by TLC on silica gel G-coated plates and were detected by spraying with resorcinol reagent as described in Materials and Methods. Lane *a*, BALB/c 3T3 cells (clone A31, subclone S24) and variants (lane *b*) M1, (lane *c*) M3, (lane *d*) V4, (lane *e*) M5, (lane *f*) M6, and (lane *g*) M7. Each lane contains the gangliosides extracted from 2×10^6 cells. The mobility of ganglioside standards is shown to the left of the figure.

Published February 1, 1986

the cells retaining toxin-binding activity. All other variants were uniformly negative for toxin binding as detected by this method (data not shown).

The variant phenotype is perhaps most readily explained by a failure to synthesize one or more of the glycosyltransferase(s) involved in complex ganglioside biosynthesis. Were the same glycosyltransferase(s) also involved in addition of sugars to glycoproteins, the variants described might also be defective in glycoprotein biosynthesis. We have therefore examined both the sialo- and galactoprotein profiles of the variants and compared them with the parent cell line. Fig. 3 shows an experiment in which total cell proteins were separated by SDS PAGE and the proteins transferred to nitrocellulose sheets, which were then overlaid with ^{125}I-labeled ricin, a galactose-specific lectin. There was no evidence for any reduction in levels or molecular weights of the major ricin-binding galactoproteins in any of the variants tested. Prior treatment of the cells with neuraminidase produced a marked increase in binding of ^{125}I-labeled ricin to the major galactoprotein in all cells, showing that most of the galactose residues are subterminal to sialic acid residues and that there is no generalized defect in sialylation of glycoproteins in the variants (Fig. 3). Similarly, there was no evidence for any reduction in levels or molecular weights of the sialoglycoproteins detected by IO_4/B^3H_4 labeling or the proteins detected by lactoperoxidase catalyzed iodination (data not shown). Overall, the results suggest that the variants are specifically defective in synthesis of the more complex gangliosides and that there is no major reduction in glycosylation of glycoproteins in these cells.

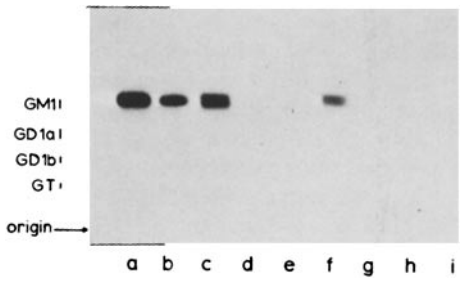

Figure 2. Detection of ganglioside GM1 in BALB/c 3T3 cells and variant cells by overlaying thin layer chromatograms with ^{125}I-labeled cholera toxin. Purified gangliosides (equivalent to 2×10^4 cells) were separated by TLC on plastic-backed silica gel G plates, and the chromatograms were overlaid with ^{125}I-cholera toxin as described in Materials and Methods. After they were washed, the sheets were dried and autoradiographed. Lanes *a* and *b*, bovine mixed brain gangliosides (Sigma Chemical Co.), 100 ng (lane *a*) and 20 ng (lane *b*). Lanes *c–i*, gangliosides of (lane *c*) BALB/c 3T3 cell (clone A31, subclone S24), and variants (lane *d*) M1, (lane *e*) M3, (lane *f*) V4, (lane *g*) M5, (lane *h*) M6, and (lane *i*) M7.

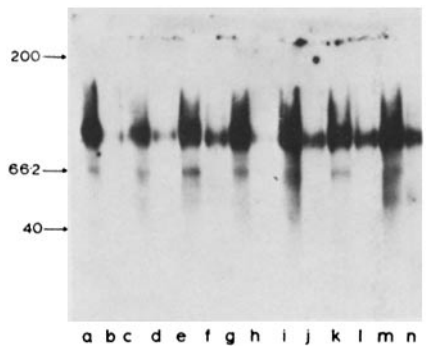

Figure 3. Detection of the galactoproteins of BALB/c 3T3 cells and variants using SDS PAGE and ^{125}I-labeled ricin. Total cellular proteins were separated by SDS PAGE (7% gel) and transferred electrophoretically to nitrocellulose sheets, which were then overlaid with ^{125}I-ricin (RCA_{60}) as described in Materials and Methods. Sheets were then washed, dried, and autoradiographed. Lanes *b*, *d*, *f*, *h*, *j*, *l*, and, *n* show ^{125}I-ricin binding to BALB/c 3T3 cells (clone A31, subclone S24) and variants M1, M3, V4, and M5-7, respectively. Lanes *a*, *c*, *e*, *g*, *i*, *k*, and *m* as above except that the cells were pretreated with neuraminidase (Behringwerke A.G., Marburg, FRG, 0.02 U/ml, 60 min at 37°C) before electrophoresis. Each lane contains 50 μg cell protein. The molecular weights ($\times 10^{-3}$) of marker proteins are shown to the left of the figure.

Interaction of Ganglioside-deficient Variants with Fibronectin

Evidence has recently been presented that di- and trisialogangliosides may act as the fibronectin receptor (23, 38, 46, 47), and it was therefore of particular interest to establish whether the variants described here could organize a fibronectin-containing matrix. Immunofluorescence detection of fibronectin showed that the parent cell line and all of the variants displayed an extensive extracellular matrix of fibronectin (Fig. 4). The relative levels of fibronectin in the matrix of the different cell lines were essentially similar as detected by labeling cell monolayers by lactoperoxidase-catalyzed iodination (Table I).

Interaction between fibroblasts and fibronectin deposited on a substrate triggers the formation of intracellular actin-containing microfilament bundles and cell spreading. The possibility that the more complex gangliosides play an impor-

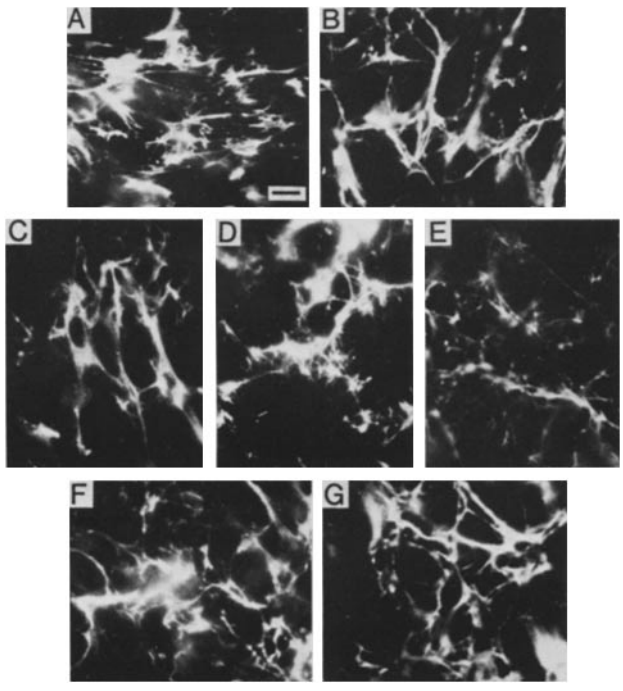

Figure 4. Localization of fibronectin on the surface of BALB/c 3T3 cells and variants by indirect immunofluorescence. Cells were grown to near confluence on 35-mm tissue culture dishes, fixed with 3.8% formaldehyde in PBS, and stained for fibronectin using a rabbit anti-human plasma fibronectin and rhodamine-labeled goat anti-rabbit Ig. (*A*) BALB/c 3T3 cells (clone A31, subclone S24) and variants (*B*) M1, (*C*) M3, (*D*) V4, (*E*) M5, (*F*) M6, and (*G*) M7. Bar, 20 μm.

Published February 1, 1986

tant role in mediating this response was investigated by studying the ability of the variants M1, M3, M6, and M7 to spread on substrates coated with fibronectin (Fig. 5). All of the variants tested could spread on fibronectin-coated substrates (10 μg/ml) and adopt a morphology similar to that of the parent cell line, although their morphologies were somewhat different at lower fibronectin concentrations. Spreading of variants as a function of fibronectin concentration is shown in Fig. 6. Variants M1, M3, and M7 showed a concentration dependence for spreading indistinguishable from that of the parent cells, with 50% cell spreading occurring at 2 μg/ml of fibronectin. Variant M6 was marginally different from the others and required ~3 μg/ml of fibronectin to induce 50% cell spreading.

Table I. Quantitation of Fibronectin Associated with the Surface of BALB/c 3T3 Cells and Variants Using Lactoperoxidase-catalyzed Iodination

Cell line	^{125}I-labeled fibronectin
	(% *total incorporation*)
BALB/c 3T3 cells (clone A31, subclone S24)	3.1
Variant	
M1	6.2
M3	6.4
V4	3.6
M5	4.4
M6	3.9
M7	6.3

Confluent cell monolayers (6-cm dishes) were labeled by lactoperoxidase-catalyzed iodination exactly as described by Hynes (20), and labeled proteins were separated on SDS polyacrylamide (7%) gels. Fixed gels were sliced (1 mm width) and counted in a Beckman 5500 gamma counter (Beckman Instruments Inc., Palo Alto, CA). The counts per minute incorporated into the region of the gel corresponding to an authentic fibronectin standard were expressed as a percentage of total incorporation. The results shown are from a single experiment, but essentially identical results were obtained in a separate experiment. Autoradiographic analysis of duplicate gels confirmed that BALB/c 3T3 cells and variants showed a prominent band exactly co-migrating with a fibronectin standard.

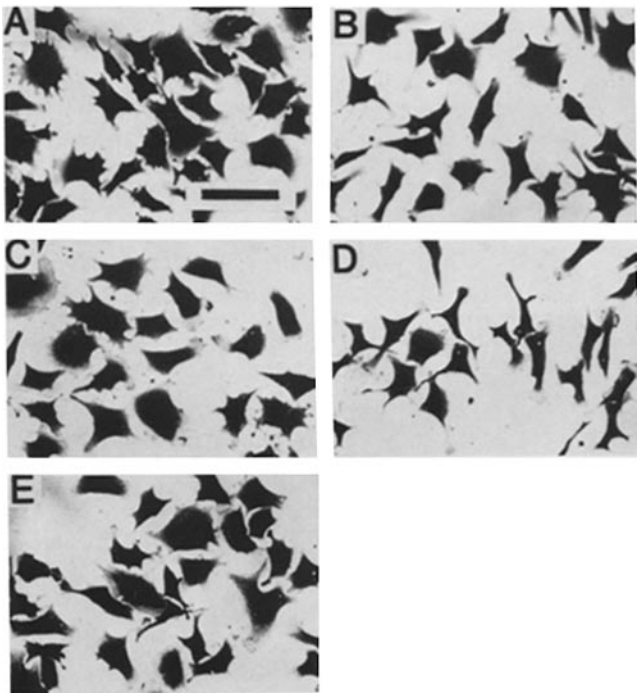

Figure 5. Morphology of BALB/c 3T3 cells and variants on fibronectin-coated substrates. Microtiter wells were coated with 10 μg/ml of human plasma fibronectin (2 h, 37°C) and then treated with 10 mg/ml bovine serum albumin for 1 h at 37°C. Wells were washed twice with PBS, 1×10^4 trypsinized cells were added in 100 μl DME, and the cells were allowed to spread for 1.5 h at 37°C in a CO_2 incubator. Cells were fixed in 3.8% formaldehyde and stained with crystal violet (0.02% wt/vol). (*A*) BALB/c 3T3 cell (clone A31 subclone S24) and variants (*B*) M1, (*C*) M3, (*D*) M6, and (*E*) M7. Bar, 100 μm.

Spreading of the variants and the parent cell line on fibronectin-coated substrates was accompanied by the formation of actin-containing microfilament bundles (Fig. 7), although there was considerable heterogeneity in the size and extent of the bundles formed in different cells within the same population. Spreading of the parent cell line was also accompanied by the formation of focal contacts, as seen by interference reflection microscopy, but again there was considerable heterogeneity in the size and number of focal contacts found in cells within the same population (Fig. 8, *A* and *B*). Interestingly, the focal contacts formed by these cells under normal culture conditions were generally larger and more clearly defined than those observed during spreading on fibronectin (data not shown). All of the variants were similarly able to form focal contacts both when spreading on fibronectin-

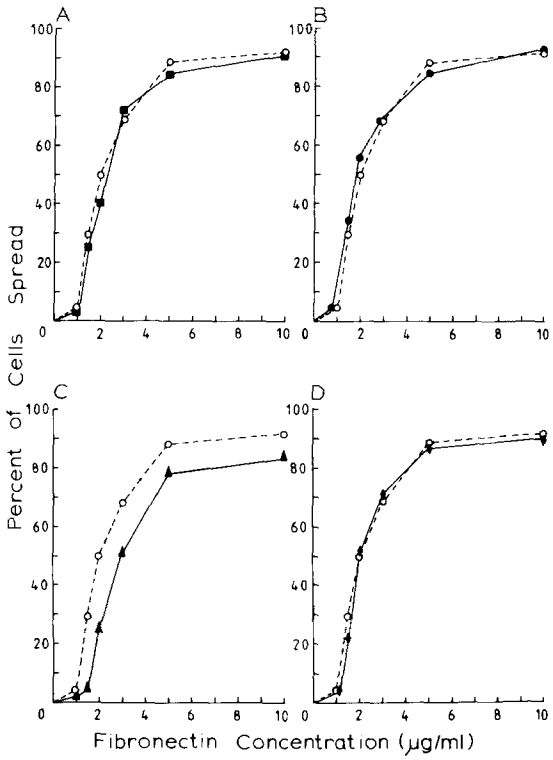

Figure 6. Quantitation of the ability of BALB/c 3T3 cells and variants to spread on fibronectin-coated substrates. Cells were plated onto microtiter wells coated with varying concentrations of fibronectin (0–10 μg/ml) as described in Fig. 5 and allowed to spread for 2 h at 37°C. Cells were fixed and stained, and spreading was quantitated by counting the number of spread cells in from three to six fields of view (80–100 cells/field), which was then expressed as a percentage of the total number of attached cells. Duplicate wells were used for each concentration of fibronectin. The spreading of variants (*A*) M1 (■), (*B*) M3 (●), (*C*) M6 (▲), and (*D*) M7 (▼) is in each case compared with that of BALB/c 3T3 cells (clone A31, subclone S24) (○).

Published February 1, 1986

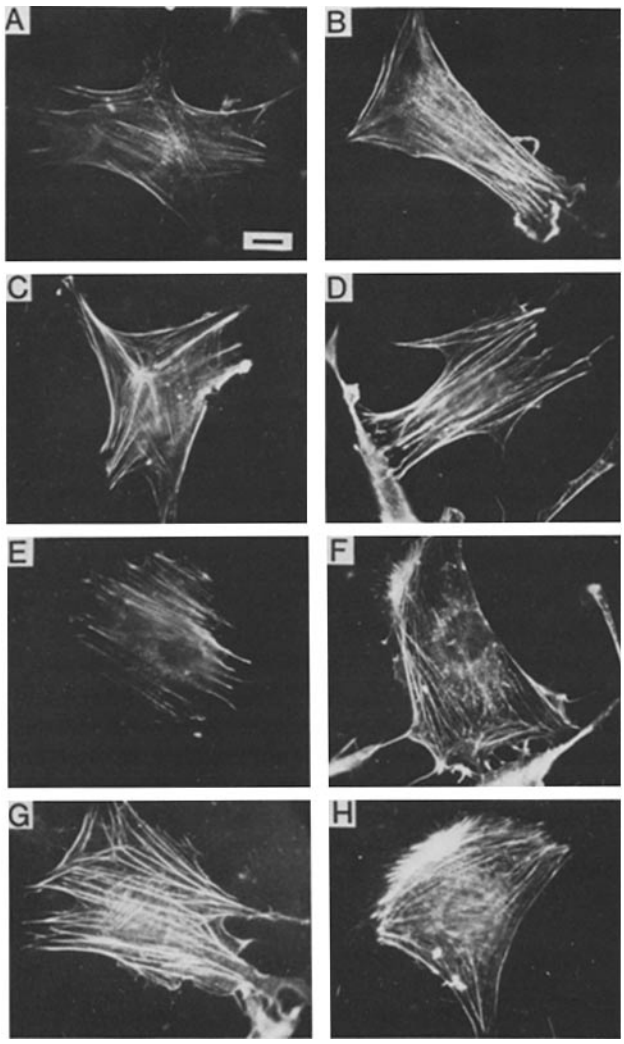

Figure 7. Organization of actin in BALB/c 3T3 cells and variants after plating onto fibronectin-derivatized coverslips. Cells (5×10^4 cells in 100 μl DME) were plated onto 12-mm glass coverslips (in 35-mm dishes) derivatized with fibronectin (200 μg/ml). After 15 min at 37°C (in a CO_2 incubator), coverslips were flooded with DME and incubated for a further 4–6 h at 37°C. Cells were fixed, permeabilized with Triton X-100, and stained for actin with nitrobenzoxadiazole phallicidin, as described in Materials and Methods. (*A* and *B*) BALB/c 3T3 cells (clone A31, subclone S24) and variants (*C*) M1, (*D*) M3, (*E*) V4, (*F*) M5, (*G*) M6, and (*H*) M7. Bar, 20 μm.

coated substrates (Fig. 8, *C–H*) and when grown under normal culture conditions, although there was some tendency for variants to form more extensive close contacts than the parent cell line. Cultures of variant V4 contain some cells that still synthesize complex gangliosides. Experiments in which variant V4 was examined both by interference reflection microscopy and immunofluorescence showed that there was no correlation between the ability of cells to form focal contacts on fibronectin-coated substrates and the ability to bind cholera toxin, a marker for cells containing the more complex gangliosides (data not shown).

The Effect of Neuraminidase Treatment of BALB/c 3T3 Cells on Cell Spreading

To clarify further the importance of sialic acid–containing glycoconjugates in cell spreading, we examined the effect of neuraminidase treatment on the ability of BALB/c 3T3 cells to spread on fibronectin-coated substrates. Neuraminidase has previously been shown to convert much of the disialoganglioside GD1a in BALB/c 3T3 cells to GM1 (11, 12), as well as removing sialic acid residues from sialoglycoproteins (Fig. 3). However, neuraminidase had absolutely no effect on the amount of fibronectin required to induce 50% spreading of BALB/c 3T3 cells, although it did increase the ability of cells to adopt a partially spread morphology on substrates coated with the ganglioside GM1-specific ligand cholera toxin (Fig. 9), in agreement with previous results (32).

Discussion

Tumor virus transformation of animal cells in culture invariably leads to an alteration in glycolipid metabolism (16). In mouse cells, transformation results in a failure to synthesize gangliosides more complex than GM3 (e.g. GM2, GM1 and GD1a) (5, 15), apparently as a consequence of a marked decrease in the activity of a single glycosyltransferase in the biosynthetic pathway (4). In an attempt to clarify the significance of this phenomenon, we have recently isolated variants of BALB/c 3T3 cells with a similar defect in ganglioside biosynthesis (39). We have previously shown that these cells, which were selected for resistance to lysis in the presence of cholera toxin, antitoxin, and complement, express a marked reduction in toxin binding as compared with the parent cell line and also fail to incorporate [1-^{14}C]palmitate into gangliosides more complex than GM3. In the present study we have confirmed by direct chemical analysis that the variants have a simplified ganglioside composition and have also shown by overlaying thin layer plates of separated gangliosides with ^{125}I-cholera toxin that the cells (apart from V4) lack any detectable ganglioside GM1. We have found no evidence, however, for any alteration in glycoprotein composition. Thus the level(s) and molecular weight(s) of the major ricin-binding galactoprotein, and the sialoglycoproteins detected by IO_4/B^3H_4 labeling, were similar in variants and the parent cell line. These results are consistent with the notion that the variants are specifically defective in synthesis of sialic acid– and galactose-containing glycolipids, and that glycoprotein synthesis has not been affected in these cells. The ability to isolate such variants can be explained by the known specificity of cholera toxin for ganglioside GM1 (12, 14) and the fact that similar carbohydrate sequences to that in GM1 do not appear to be common in glycoproteins. Mutants isolated using ligands with a less restricted specificity, e.g. ricin and wheat germ agglutinin, have been found to show alterations in both glycolipid and glycoprotein biosynthesis (7, 16).

In analyzing the possible relationship between altered ganglioside metabolism and other aspects of the transformed phenotype, we have been particularly interested in reports suggesting that di- and trisialogangliosides might play a role in the interaction of cells with the large external transformation-sensitive glycoprotein, fibronectin. Thus fibronectin-mediated attachment of Chinese hamster ovary cells to collagen was inhibited by gangliosides GT1 ≃ GD1a > GM1 ≫ GM3, and the inhibitory activity was partially retained by the oligosaccharide moiety (23). Spreading of baby hamster kidney cells on fibronectin (32, 47), and the fibronectin-mediated hemagglutination of erythrocytes (47) was similarly inhibited by gangliosides, although the specificity of the effect is brought into question by the observation that gangliosides also inhib-

Published February 1, 1986

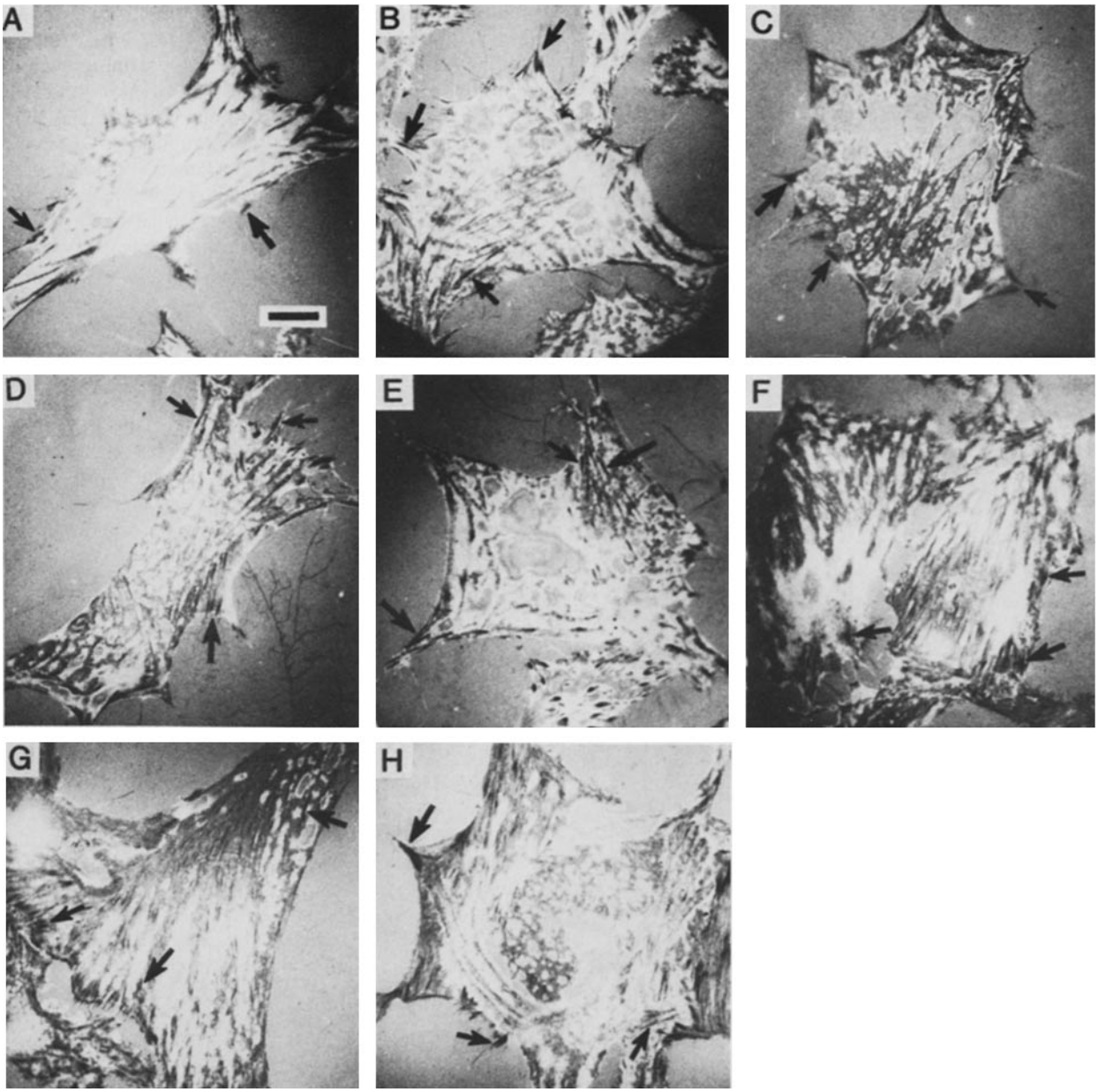

Figure 8. Formation of focal contacts by BALB/c 3T3 cells and variants during spreading on fibronectin-derivatized coverslips. Cells were plated onto glass coverslips derivatized with fibronectin exactly as described in the legend to Fig. 7 and viewed by interference reflection microscopy as outlined in Materials and Methods. (*A* and *B*) BALB/c 3T3 cells (clone A31, subclone S24) and variants (*C*) M1, (*D*) M3, (*E*) V4, (*F*) M5, (*G*) M6, and (*H*) M7. Bar, 20 μm.

ited spreading on the mannose-specific lectin concanavalin A (33, 36). However, experiments with a mouse L cell variant (NCTC 2071A) provide strong additional support for a role of gangliosides in organizing fibronectin at the cell surface (33, 38, 46). These cells, which are almost completely lacking in gangliosides, synthesize but fail to retain fibronectin at the cell surface. The addition of gangliosides to the growth medium leads to a dramatic increase in fibronectin at the cell surface and the formation of an extensive extracellular fibronectin-containing matrix. Di- and trisialogangliosides were particularly effective in this respect. Whereas fibronectin in solution binds only weakly to immobilized gangliosides (32), fluorescently labeled gangliosides have recently been shown to bind to the fibronectin matrix formed by human fibroblasts (37). Were di- and trisialogangliosides to act as the fibronectin receptor the variants described in this study would be expected to show loss of surface-associated fibronectin. However, the variants we have isolated displayed levels of fibronectin indistinguishable from those in the parent cell line, as shown by indirect immunofluorescence and lactoperoxidase-catalyzed iodination. The results clearly show that gangliosides more complex than GM3 are not required by BALB/c 3T3 cells for organization of a fibronectin-containing pericellular matrix. This conclusion is consistent with the known ability of cells that lack any substantial amounts of the more complex gangliosides (e.g., Chinese hamster ovary, baby hamster kidney cells [11]) to organize a fibronectin matrix. It remains possible that ganglioside GM3, when present at sufficiently high levels,

Published February 1, 1986

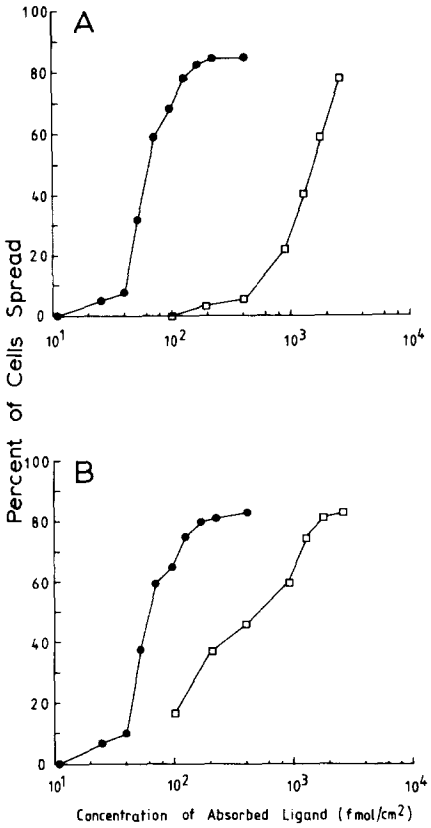

Figure 9. The effect of neuraminidase treatment on spreading of BALB/c 3T3 cells on substrates coated with fibronectin and cholera toxin. Cells were plated onto microtiter wells coated with varying concentrations of fibronectin (●) or cholera toxin (□) as described in Fig. 5 and allowed to spread for 2 h at 37°C. Cell spreading was quantitated as described in Fig. 6, and related to the levels of substrate-bound ligand, determined in parallel experiments using radiolabeled fibronectin or cholera toxin. Where indicated, cells were pretreated with 0.04 U/ml neuraminidase for 1 h at 37°C before plating into the microtiter wells. (*A*) Spreading of control BALB/c 3T3 cells (clone A31, subclone S24). (*B*) Spreading of neuraminidase-treated BALB/c 3T3 cells (clone A31, subclone S24).

can fulfill the role of the more complex gangliosides, although GM3 was relatively ineffective either in inhibiting cell attachment and spreading of cells on fibronectin (23, 32) or in supporting the formation of a fibronectin matrix in NCTC 2071A cells (46).

We have considered the idea that complex gangliosides might also influence the ability of fibronectin to initiate the transmembrane events that lead to reorganization of the cytoskeleton and to cell spreading. Since glycolipids are not thought to span the membrane it would seem unlikely that they could play a primary role in mediating such events. However, glycolipids are, for reasons that remain unclear, resistant to detergent extraction and remain associated with Triton X-100 cytoskeletons (17, 40) and have recently been reported to be present in the substrate attached material that remains after cells are removed from tissue culture dishes with EDTA (10, 29). Indirect evidence that ligand-ganglioside interactions can lead to transmembrane cytoskeletal reorganization in lymphocytes has been presented (22), although in fibroblasts the reorganization was incomplete (32). However, we have found no evidence that the variants described in this study are substantially defective in their response to fibronectin-coated substrates. The concentration of fibronectin that promoted 50% cell spreading was unaltered, and spreading was accompanied by formation of actin-containing microfilament bundles that terminated at focal contacts. Similarly, neuraminidase treatment of the parent BALB/c 3T3 cells, which effectively reduces the level of ganglioside GD1a in the cell membrane (11), did not alter the concentration of fibronectin required to support cell spreading.

In summary, we have found no evidence that complex gangliosides are absolutely required either for the organization of a fibronectin-containing extracellular matrix or for mediating the spreading response of BALB/c 3T3 cells to fibronectin. Neither would gangliosides appear to be important in modulating the activity of the fibronectin receptor as has been suggested in the case of the receptors for platelet-derived and epidermal growth factor (6). Our conclusions are in apparent conflict with the results obtained with NCTC 2071A cells, in which the more complex gangliosides can clearly influence the ability of the cells to retain fibronectin at the cell surface and to organize a fibronectin matrix (38, 46). They are, however, consistent with numerous reports that point to glycoproteins (4, 9, 24, 30, 35, 43) or proteoglycans (26, 31) as the fibronectin receptor. The lack of agreement on the precise nature of the receptor may be a reflection of differences between cell types or may be related to data that suggest that there are distinct receptors involved in cell attachment to fibronectin and the organization of a fibronectin-containing extracellular matrix (28).

This work was supported by the Medical Research Council (U. K.).

Received for publication 17 June 1985; and in revised form 27 September 1985.

References

1. Abercrombie, M., and G. A. Dunn. 1975. Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. *Exp. Cell Res.* 92:57–62.

2. Aplin, J. D., and R. C. Hughes. 1981. Protein-derivatized glass coverslips for the study of cell-to-substratum adhesion. *Anal. Biochem.* 113:144–148.

3. Aplin, J. D., R. C. Hughes, I. Jaffe, and N. Sharon. 1981. Reversible cross-linking of cellular components of adherent fibroblasts to fibronectin and lectin-coated substrata. *Exp. Cell Res.* 134:488–494.

4. Brady, R. O., and P. H. Fishman. 1974. Biosynthesis of glycolipids in virus transformed cells. *Biochim. Biophys. Acta.* 355:121–148.

5. Brady, R. O., and P. T. Mora. 1970. Alteration in glanglioside pattern and synthesis in SV40 and polyoma virus transformed mouse cell lines. *Biochem. Biophys. Acta.* 218:308–319.

6. Bremer, E. G., S. Hakomori, D. F. Bowen-Pope, E. Raines, and R. Ross. 1984. Ganglioside mediated modulation of cell growth, growth factor binding and receptor phosphorylation. *J. Biol. Chem.* 259:6818–6826.

7. Briles, E. B., E. Li, and S. Kornfield. 1977. Isolation of wheat germ agglutinin-resistant clones of Chinese hamster ovary cells deficient in membrane sialic acid and galactose. *J. Biol. Chem.* 252:1107–1116.

8. Burridge, K. 1978. Direct identification of specific glycoproteins and antigens in sodium dodecyl sulphate gels. *Methods Enzymol.* 50:54–64.

9. Chen, W. T., E. Hasegawa, T. Hasegawa, C. Weinstock, and K. M. Yamada. 1985. Development of cell surface linkage complexes in cultured fibroblasts. *J. Cell Biol.* 100:1103–1114.

10. Cheresh, D. A., J. R. Harper, G. Schulz, and R. A. Reisfeld. 1984. Localization of the gangliosides GD_2 and GD_3 in adhesion plaques and on the surface of human melanoma cells. Proc. Natl. Acad. Sci. USA. 81:5767–5771.

11. Critchley, D. R., and M. Vicker. 1977. Glycolipids as membrane receptors important in growth regulation and cell-cell interaction. *In* Aspects of Cell Surface Organization. G. Poste and G. L. Nicolson, editors. Elsevier/North-Holland, Amsterdam. 307–370.

12. Critchley, D. R., C. H. Streuli, S. Kellie, S. Ansell, and B. Patel. 1982. Characterization of the cholera toxin receptor on Balb/c 3T3 cells as a ganglioside similar to, or identical with, ganglioside GM1. No evidence for galactoproteins with receptor activity. *Biochem. J.* 204:209–219.

13. Crtichley, D. R., J. A. Wyke, and R. O. Hynes. 1976. Cell surface and metabolic labeling of the proteins of normal and transformed chicken cells. *Biochim. Biophys. Acta.* 436:335–352.

14. Fishman, P. H. 1982. Role of membrane gangliosides in the binding and action of bacterial toxins. *J. Membr. Biol.* 69:85–92.

15. Fishman, P. H., R. O. Brady, R. M. Bradley, S. A. Aaronson, and G. J. Todaro. 1974. Absence of a specific ganglioside galactosyltransferase in mouse cells transformed by murine sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 71:298–301.

16. Gottlieb, C., and S. Kornfield. 1976. Isolation and characterization of two mouse L-cell lines resistant to the toxic lectin ricin. *J. Biol. Chem.* 251:7761–7768.

17. Hagmann, J., and P. H. Fishman. 1982. Detergent extraction of cholera toxin and gangliosides from cultured cells and isolated membranes. *Biochim. Biophys. Acta.* 720:181–187.

18. Hakomori, S. 1981. Glycosphingolipids in cellular interactions, differentiation and oncogenesis. *Annu. Rev. Biochem.* 50:733–764.

19. Hynes, R. O. 1973. Alteration of cell surface proteins by viral transformation and by proteolysis. *Proc. Natl. Acad. Sci. USA.* 70:3170–3174.

20. Hynes, R. O. 1973. Alteration of cell-surface proteins by viral transformation and by proteolysis. *Proc. Natl. Acad. Sci. USA.* 70:3170–3174.

21. Hynes, R. O., and K. M. Yamada. 1982. Fibronectins: multifunctional modular glycoproteins. *J. Cell Biol.* 95:369–377.

22. Kellie, S., B. Patel, E. J. Pierce, and D. R. Critchley. 1982. Capping of cholera toxin-ganglioside GM_1 complexes in mouse lymphocytes is accompanied by co-capping of α-actinin. *J. Cell. Biol.* 97:447–454.

23. Kleinman, H. K., G. R. Martin, and P. H. Fishman. 1979. Ganglioside inhibition of fibronectin mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. USA.* 76:3367–3371.

24. Knudsen, K. A., P. E. Rao, C. H. Damsky, and C. A. Buck. 1981. Membrane glycoproteins involved in cell-substratum adhesion. *Proc. Natl. Acad. Sci. USA.* 78:6071–6075.

25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

26. Laterra, J., J. E. Siebert, and L. A. Culp. 1983. Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. *J. Cell Biol.* 96:112–123.

27. Magnani, J., M. Brockhaus, D. F. Smith, and V. Ginsburg. 1982. Detection of glycolipid ligands by direct binding of carbohydrate-binding protein to thin layer chromatograms. *Methods Enzymol.* 83:235–241.

28. McKeown-Longo, P. J., and D. F. Mosher. 1985. Interaction of the 70.000-mol. wt. amino terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts *J. Cell. Biol.* 100:364–375.

29. Okada, Y., G. Mugnai, E. G. Bremer, and S. Hakomori. 1984. Glycosphingolipids in detergent insoluble substrate attachment matrix (DISAM) prepared from substrate attachment material. *Exp. Cell. Res.* 155:448–456.

30. Oppenheimer-Marks, N., and F. Grinnell. 1984. Calcium ions protect cell-substratum adhesion receptors against proteolysis. *Exp. Cell Res.* 152:467–475.

31. Perkins, M. E., T. H. Ji, and R. O. Hynes. 1979. Cross-linking of fibronectin to sulfated proteoglycans at the cell surface. *Cell.* 16:941–952.

32. Perkins, R. M., S. Kellie, B. Patel, and D. R. Critchley. 1982. Gangliosides as receptors for fibronectin? Comparison of cell spreading on a ganglioside-specific ligand with that on fibronectin. *Exp. Cell. Res.* 141:231–243.

33. Perkins, R. M., S. Kellie, B. Patel, and D. R. Critchley. 1982. The cell surface receptor for fibronectin; the role of gangliosides in membranes and tumour growth. Developments in Cancer Research. Vol. 7. T. Galeotti, A. Cittadini, G. Neri, and S. Papa, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. 29–38.

34. Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.

35. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191–198.

36. Rauvala, H., W. G. Carter, and S. Hakomori. 1981. Studies on cell adhesion and recognition. I. Extent and specificity of cell adhesion triggered by carbohydrate-reactive proteins (glycosidases and lectins) and by fibronectin. *J. Cell. Biol.* 88:127–137.

37. Spiegel, S., J. Schlessinger, and P. H. Fishman. 1984. Incorporation of fluorescent gangliosides into human fibroblasts: mobility, fate and interaction with fibronectin. *J. Cell. Biol.* 99:699–705.

38. Spiegel, S., K. M. Yamada, B. E. Hom, J. Moss, and P. H. Fishman. 1985. Fluorescent gangliosides as probes for the retention and organisation of fibronectin by ganglioside-deficient mouse cells. *J. Cell Biol.* 100:721–726.

39. Streuli, C. H., S. L. Griffiths, and D. R. Critchley. 1985. Isolation of variants of Balb/c 3T3 cells defective in complex ganglioside biosynthesis. *Exp. Cell. Res.* 156:260–270.

40. Streuli, C. H., B. Patel, and D. R. Critchley. 1981. The cholera toxin receptor ganglioside GM1 remains associated with Triton X-100 cytoskeletons of Balb/c 3T3 cells. *Exp. Cell. Res.* 136:247–254.

41. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta.* 24:604–611.

42. Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem.* 10:613–623.

43. Tarone, G., G. Galetto, M. Prat, and P. M. Comoglio. 1982. Cell surface molecules and fibronectin-mediated cell adhesion: effect of proteolytic digestion of membrane proteins. *J. Cell Biol.* 94:179–186.

44. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

45. Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 52:761–799.

46. Yamada, K. M., D. R. Critchley, P. H. Fishman, and J. Moss. 1983. Exogenous gangliosides enhance the interaction of fibronectin with ganglioside-deficient cells. *Exp. Cell. Res.* 143:295–302.

47. Yamada, K. M., D. W. Kennedy, G. R. Grotendorst, and T. Momoi. 1981. Glycolipids: receptors for fibronectin? *J. Cell. Physiol.* 109:343–351.