Gangliosides are implicated in regulating cell adhesion and migration on fibronectin by binding with the $\alpha_{5}\beta_{1}$ subunit of integrin. However, the effects of gangliosides on cell spreading and related signaling pathways are unknown. Increases in gangliosides GT1b and GD3 inhibited spreading on fibronectin, concurrent with inhibition of Src and focal adhesion kinase. Although antibody blockade of GT1b or GD3 function and gene-modulated ganglioside depletion stimulated spreading and activated Src and focal adhesion kinase, the augmented spreading by disruption of GT1b function, but not by disruption of GD3 function, was inhibited by blockade of Src and focal adhesion kinase activation. In contrast, inhibitors of protein kinase C prevented the stimulation of spreading by GD3 functional inhibition, but not by GT1b functional blockade. Modulation of either GT1b or GD3 content affected phosphoinositol 3-kinase activation, and inhibition of this activation reversed the stimulation of cell spreading by anti-GD3 antibody, anti-GT1b antibody, and ganglioside depletion, suggesting that phosphoinositol 3-kinase is an intermediate in both the FAK/Src and protein kinase C pathways that lead to cell spreading. These studies demonstrate that epithelial cell ganglioside GT1b modulates cell spreading through $\alpha_{5}\beta_{1}$/FAK and phosphoinositol 3-kinase signaling, whereas GD3-modulated spreading appears to involve phosphoinositol 3-kinase-dependent protein kinase C signaling.

Cell adhesion to the extracellular matrix triggers a cascade of intracellular biochemical signals regulated by the integrin family of receptors. Engagement and clustering of these integrins lead to the formation of focal adhesions, through which integrins are linked to intracellular cytoskeletal complexes, allowing cell spreading and migration (for review, see Ref. 1). One component of focal contact sites is focal adhesion kinase (FAK), a nonreceptor tyrosine kinase that interacts with and is activated by the $\beta_{1}$ subunit of integrin. Activation of FAK involves autophosphorylation at its Tyr$^{1076}$ site, generating a high affinity binding site for the Src homology 2 domain. FAK is also known to interact directly with the $\beta_{5}$ subunit of phosphoinositol 3-kinase (PI3K), enabling signal transduction. Activation of FAK, Src kinase, and PI3K has all been associated with stimulation of cell attachment, spreading, and migration (for review, see Ref. 1). Up-regulation of protein kinase C (PKC) also enhances cell adhesion, spreading, and migration on a fibronectin (FN) matrix by a mechanism that does not involve FAK phosphorylation (2–4).

Gangliosides are membrane glycosphingolipids that modulate several biologic processes of keratinocytes and keratinocyte-derived cell lines in vitro, including cell proliferation, adhesion, migration, differentiation, and apoptosis, at least in part by affecting transmembrane signaling (5–11). Keratinocytes and the keratinocyte-derived SCC12 cell line share three major gangliosides, GM3, GD3, and GT1b (8), all components of the “b” pathway of ganglioside biosynthesis. This content of membrane gangliosides is dictated by the expression of enzymes that promote synthesis and metabolism, particularly glycosyltransferases and ganglioside-specific sialidase. The effect of gangliosides and ganglioside depletion on cell spreading and on signaling that may impact on spreading, particularly FAK and Src signaling, has received little attention. We have manipulated ganglioside expression exogenously by pharmacologic addition or anti-ganglioside antibody blockade of function, and endogenously by overexpression of genes that promote depletion of gangliosides or synthesis of the complex ganglioside GT1b. These studies show that depletion of specific gangliosides stimulates cell spreading, whereas increased membrane content of gangliosides inhibits it. The cell spreading inhibited by GT1b overexpression or induced by anti-GT1b antibody is mediated exclusively by the FAK/Src/PI3K pathway, whereas the stimulation of spreading induced by anti-GD3 antibody appears to involve the PRC pathway as well.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and SCC12 Cell-derived Stable Transfectants—Human keratinocyte-derived SCC12F2 (SCC12) cells, a generous gift from Dr. James Rheinwald, Boston, MA, were stably transfected with a human plasma membrane ganglioside-specific sialidase cDNA (GenBank accession no. AB008185, courtesy of Dr. T. Miyagi, Tokyo, Japan) (12) in a pcDNA3 vector using LipofectAMINE reagent (Invitrogen), following the techniques described for stable transfection of cytosolic hamster sialidase cDNA (8). Gene expression in the resultant “SSIA cells” was monitored using 9-O-AcGD3, NeuAcO−2→8 NeuAcO−2→3Gal-O-Acβ-1→4Glcβ-1-Cer; GT1b, NeuAcO−2→3Galβ-1→3GalNAcβ-1→4NeuAcO−2→8 NeuAcO−2→3Galβ-1→4Glcβ-1-Cer; P13K, phosphatidylinositol 3-kinase; PIPES, 1,4-piperazinediethanesulfonic acid; PKC, protein kinase C; RU486, mifepristone; SSIA, SCC12 cells stably transfected with ganglioside-specific human sialidase cDNA.

**Footnotes**

1 The abbreviations used are: FAK, focal adhesion kinase; $\beta$-gal, $\beta$-galactosidase; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin; GD2, GalNAcβ-1→4(NeuAcO−2→8 NeuAcO−2→3Galβ-1→4Glcβ-1-Cer; GD3, NeuAcO−2→8 NeuAcO−2→3Galβ-1→4Glcβ-1-Cer; GM1, Galβ-1→3GalNAcβ-1→4NeuAcO−2→3Galβ-1→4Glcβ-1-Cer; GM2, GalNAcβ-1→4(NeuAcO−2→3Galβ-1→4Glcβ-1-Cer; GT1b, NeuAcO−2→3Galβ-1→4Glcβ-1-Cer; PI3K, phosphatidylinositol 3-kinase; PIPES, 1,4-piperazinediethanesulfonic acid; PKC, protein kinase C; RU486, mifepristone; SSIA, SCC12 cells stably transfected with ganglioside-specific human sialidase cDNA.

2 X.-Q. Wang, P. Sun, and A. S. Paller, unpublished data.
Gangliosides Modulate Cell Adhesion and Spreading

The time course of gene expression after stimulation with 

\[ \text{RU486} \]

and the leakiness using this inducible system were tested by 

\[ \text{β-gal} \]

staining (β-gal assay kit, Invitrogen) after gene expression induced by incubation with 100 nM RU486 for 6–72 h using p17x4-tkA/β-gal-pGL-VP cells. GM2/GD2 synthase expression in p17x4-tkA/GM2/GD2-pGL-VP cells stimulated for 24–48 h with 100 nM RU486 was examined by Northern assays using a digoxigenin-labeled 640-bp GM2/GD2 synthase cDNA probe and a digoxigenin-labeled 316-bp fragment of the constitutively expressed human glyceraldehyde-3-phosphate dehydrogenase cDNA as a control, followed by detection with a DIG-high IIA antibody (Roche Molecular Biochemicals). Ganglioside expression was further confirmed by ganglioside ELISAs (see below). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-12 medium (Invitrogen) (1:1) supplemented with 10% heat-inactivated fetal bovine serum without antibiotics in 5% CO₂ at 37 °C.

Ganglioside ELISAs—Parental SCC12 cells, mock transfected (p17x4-tkA-pGL-VP cells), and p17x4-tkA/GM2/GD2 synthase-pGL-VP cells treated with or without 100 nM RU486 for 48 h were trypsinized, and suspended single cells were plated into 96-well microtiter plates. Cells were grown in the presence or absence of 100 nM RU486 for an additional 6–8 h. Cells were then fixed with 4% formalin, followed by incubation with 10–30 μg/ml anti-ganglioside antibodies as used for TLC studies for 1 h and horseradish peroxidase-labeled anti-mouse IgM (Sigma antibodies; Ganglioside expression was further confirmed by ganglioside ELISAs (see below). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/12 medium (Invitrogen) (1:1) supplemented with 10% heat-inactivated fetal bovine serum without antibiotics in 5% CO₂ at 37 °C.

Ganglioside ELISAs—Parental SCC12 cells, mock transfected (p17x4-tkA-pGL-VP cells), and p17x4-tkA/GM2/GD2 synthase-pGL-VP cells treated with or without 100 nM RU486 for 48 h were trypsinized, and suspended single cells were plated into 96-well microtiter plates. Cells were grown in the presence or absence of 100 nM RU486 for an additional 6–8 h. Cells were then fixed with 4% formalin, followed by incubation with 10–30 μg/ml anti-ganglioside antibodies as used for TLC studies for 1 h and horseradish peroxidase-labeled anti-mouse IgM (Biodesign International, Saco, Maine) for 45 min. Ganglioside expression was detected by applying BM Blue POD substrate (Roche Molecular Biochemicals), and the absorbance was read at 450 nm in a UVmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). In each case, uncoated wells, omission of the anti-ganglioside antibody, and use of purified mouse IgM in place of anti-ganglioside antibody served as negative controls. Results of treatment with the blue substrate alone were subtracted from the readings for final data.

Ganglioside Modulation—To increase the membrane content of gangliosides, cells were incubated with or without gangliosides (1 μM GTb1, 1 μM GD3, or as controls 50 μM GM3 or 50 μM GM2) in DMEM/F-12 medium containing 2% fetal bovine serum for 48 h. Following ganglioside function, cells were incubated with or without 10 μg/ml anti-GTb1 or anti-GD3 or as controls anti-GM3, or anti-GM2 antibody for 30 min in DMEM/F-12 medium containing 6 mg/ml bovine serum albumin. Established cell lines with endogenously modified gangliosides by sialidase or GM2/GD2 synthase overexpression are described above. Four SSIA cell lines (SSIA3, SSIA6, SSIA12, and SSIA25), four GM2/GD2 synthase cell lines (GM2/GD2-1, -5, -11, and -26) with and without RU486 induction, and two of each mock transfected cell line (pcDNA or p17x4-tkA-pGL-VP) were studied.

Cell Adhesion Assays—96 well plates were coated with or without 5 μg/cm² cell-binding fragment of FN or with 10 μg/cm² poly-l-lysine in phosphate-buffered saline (pH 7.4) overnight at 4 °C. After removing unbound matrix solution, the plate was air dried at room temperature. After washing three times with phosphate-buffered saline, plates were incubated with 1% bovine serum albumin and phosphate-buffered sa-
line for 1 h at 37 °C to block unbound sites. SCC12 cells pretreated with or without gangliosides for 30 h, GM2/GD2 transfectants were pretreated for 30 h with 100 mM RU486 or vehicle, or SSIA cells were starved of fetal bovine serum and EGF to synchronize cycling for the final 18 h of incubation with ganglioside or RU486. In other studies, cells were starved for the 18 h and, during the final 30 min of starvation, were treated with anti-ganglioside antibodies to block ganglioside function. Cells were then transferred to the treated plates. Cell adherence was detected by staining with Rose Bengal dye, and the absorbance was read at 540 nm as described previously (6). All experiments were performed at least three times in triplicate.

Cell Spreading Assays—Eight-well plates were treated as described for adhesion assays above. SCC12 cells treated with or without gangliosides or anti-ganglioside antibodies, and expressed as the mean ± S.D. of studies performed at least six times and assessed each time by three different individuals.

Immunoblotting—Immunoblotting was carried out as described (11) using an enhanced chemiluminescence (ECL) detection system (PerkinElmer Life Sciences) with whole cell lysates. Cells were lysed in boiling lysis buffer, and the insoluble material was removed by centrifugation. Total cell protein in the whole cell lysates was determined through a colorimetric assay (Bio-Rad) to ensure equal loading. Eight to 20 μg of total protein from the whole cell lysate was boiled in Laemmli buffer (18) and loaded onto SDS-PAGE mini-gels. After transfer to polyvinylidene difluoride or nitrocellulose membranes, the separated proteins were detected by immunoblotting with anti-phosphotyrosine, -p85 of PI3K, -FAK (Transduction Labs, Lexington, KY), -Src, or -Src phosphorylated in vitro with polyglutamic acid:polytyrosine (4:1) for 45 min at 20 °C. The membrane was re-probed with mouse anti-human FAK polyclonal antibody ( provided by Dr. D. Schlaepfer, Scripps Research Institute, La Jolla, CA), used at 1:1000 dilution, and then immunoblotted with anti-FAK monoclonal antibody ( Upstate Biotechnology). Phosphatidylinositol was resolved by TLC in chloroform, methanol, 28% ammonium hydroxide, and water (v/v/v, 86:76:10:45) for 10 min. The phosphatidylinositol-containing plates were stained with 1% ethanolic phosphomolybdic acid:phosphotungstic acid (v/v) and lyophilized. Phosphatidylinositol was resolved by TLC in chloroform, methanol, 28% ammonium hydroxide, and water (v/v/v/v, 86:76:10:45) for 10 min. The phosphatidylinositol-containing plates were stained with 1% ethanolic phosphomolybdic acid:phosphotungstic acid (v/v) and lyophilized.

Immunoprecipitation—After starvation of serum, FN, and EGF overnight, cells were stimulated with 10 μg/ml cell-binding fragment of FN (Upstate Biotechnology) for 30 min at 37 °C. FAK was immunoprecipitated from whole cell lysates using rabbit anti-human FAK polyclonal antibody, and the total reaction volume was adjusted to 1 ml in the immunoprecipitation buffer (18). After incubation with the antibodies for 2 h at 4 °C, protein A-agarose was added and incubated for an additional 1 h at 4 °C. Immunoprecipitates were washed, and the kinase assay (PI3K expression was determined by separation of 15 μg of Src kinase protein from whole cell lysate by 10% SDS-PAGE mini-gel and immunoblotting with anti-FAK monoclonal antibody.

Src Kinase Expression, Activity, and Phosphorylation—Endogenous c-Src was immunoprecipitated from cells pretreated with or without gangliosides or anti-ganglioside antibodies. The immunoprecipitated c-Src kinase was resuspended in 0.25 M Tris (pH 7.5) containing 2.5 μg of phosphatidylinositol, 15 μg of Src kinase buffer (0.25 M PIPES (pH 7.0), 10 mM MgCl2, 1 mM dithiothreitol) containing 2.5 μg of protein) were applied to a 10% SDS-PAGE mini-gel. The enolase band was visualized by autoradiography on Kodak X-Omat film developed at ~70 °C overnight and quantified by a Storm 800 Fluorescence PhosphorImager (Molecular Dynamics, Inc.). Src kinase activity was also determined by measuring the phosphorylation of a Src substrate peptide (KVEKIGEGTGYVYKK) using immunoprecipitated c-Src with a Src kinase assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. The experiment was performed three times in triplicate.

To determine the expression and phosphorylation of c-Src kinase, cells were pretreated with either gangliosides or anti-ganglioside antibodies, washed with DMEM/F-12 medium, and stimulated with 10 μg/ml cell-binding fragment of FN for 30 min at 37 °C. Whole cell lysates (25 μg of protein) were applied to a 10% SDS-PAGE mini-gel and then immunoblotted with anti-Src-phosphotyrosine 416 polyclonal antibody to assess c-Src phosphorylation. Gels were reprobed with anti-Src monoclonal antibody to measure c-Src expression as described before (11).

PI3K Expression and Activity—To determine PI3K expression and activity, cells were treated as described above. SCC12 cells were pretreated with either gangliosides or anti-ganglioside antibodies, washed with DMEM/F-12 medium, and stimulated with 10 μg/ml cell-binding fragment of FN for 30 min at 37 °C. Whole cell lysates (25 μg of protein) were applied to a 10% SDS-PAGE mini-gel and then immunoblotted with anti-Src-phosphotyrosine 416 polyclonal antibody (Upstate Biotechnology) to assess c-Src phosphorylation. Gels were reprobed with anti-Src monoclonal antibody to measure c-Src expression as described before (11).

Modulation of FAK Expression and Activity by Gene Transfection—SCC12 cells were transiently transfected with either wild-type FAK or Y397K mutated FAK cDNA (Y397K) in a pcDNA vector (both a generous gift from Dr. D. Schlaepfer, Scripps Research Institute, La Jolla, CA) using Lipofectin reagent (Invitrogen) according to the manufacturer’s instructions. Expression of the cDNAs was assessed by immunoblotting with anti-hemagglutinin and antibody (Upstate Biotechnology). To assess the function of FAK on cell spreading, untreated cells and cells transiently transfected with either wild type FAK or Y397K mutated FAK cDNA were harvested after treatment with either gangliosides or anti-ganglioside antibodies as indicated above, and spreading assays were performed.

FAK Expression, Activity, and Phosphorylation—Immunoprecipitated FAK was incubated with 50 μl of buffer containing 50 mM HEPES (pH 7.4), 10 mM MnCl2, and 50 μg of polyglutamic acid:polytyrosine (4:1) for 10 min at 25 °C, then 5 μl ATP and 5 μCl of [γ-32P]ATP (3,000 Ci/mmol) were added to initiate the reaction. Incubation was terminated 10 min later by adding 50 μl of 20% trichloroacetic acid, and the immunoprecipitated GST was removed by adding 1 ml of buffer containing 50 μg/ml of rabbit anti-human GST monoclonal antibody onto microcentrifuge filter paper. After washing three times with 10% trichloroacetic acid, the filter paper loaded with the product was air dried, placed in scintillation fluid, and γ-[32P]-labeled substrate-polyglutamic acid:polytyrosine (4:1) was measured in a Beckman LS 6000 scintillation counter. FAK activity assays were performed three times in triplicate.

To determine FAK expression and phosphorylation, SCC12 cells were pretreated with either gangliosides or anti-ganglioside antibodies, then stimulated with 10 μg/ml cell-binding fragment of FN (Upstate Biotechnology) for 30 min at 37 °C. FAK was immunoprecipitated from whole cell lysates using rabbit anti-human FAK polyclonal antibody, and the total reaction volume was adjusted to 1 ml in the immunoprecipitation buffer (18). After incubation with the antibodies for 2 h at 4 °C, protein A-agarose was added and incubated for an additional 1 h at 4 °C. Immunoprecipitates were washed, and the kinase assay (PI3K expression was determined by separation of 15 μg of Src kinase protein from whole cell lysate by 10% SDS-PAGE mini-gel and immunoblotting with anti-FAK monoclonal antibody.

Src Kinase Expression, Activity, and Phosphorylation—Endogenous c-Src was immunoprecipitated from cells pretreated with or without gangliosides or anti-ganglioside antibodies. The immunoprecipitated c-Src kinase was resuspended in 0.25 M Tris (pH 7.5) containing 2.5 μg of phosphatidylinositol, 15 μg of Src kinase buffer (0.25 M PIPES (pH 7.0), 10 mM MgCl2, 1 mM dithiothreitol) containing 2.5 μg of protein) were applied to a 10% SDS-PAGE mini-gel. The enolase band was visualized by autoradiography on Kodak X-Omat film developed at ~70 °C overnight and quantified by a Storm 800 Fluorescence PhosphorImager (Molecular Dynamics, Inc.). Src kinase activity was also determined by measuring the phosphorylation of a Src substrate peptide (KVEKIGEGTGYVYKK) using immunoprecipitated c-Src with a Src kinase assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. The experiment was performed three times in triplicate.

To determine the expression and phosphorylation of c-Src kinase, cells were pretreated with either gangliosides or anti-ganglioside antibodies, washed with DMEM/F-12 medium, and stimulated with 10 μg/ml cell-binding fragment of FN for 30 min at 37 °C. Whole cell lysates (25 μg of protein) were applied to a 10% SDS-PAGE mini-gel and then immunoblotted with anti-Src-phosphotyrosine 416 polyclonal antibody (Upstate Biotechnology) to assess c-Src phosphorylation. Gels were reprobed with anti-Src monoclonal antibody to measure c-Src expression as described before (11).

PI3K Expression and Activity—To determine PI3K expression and activity, cells were treated as described above. SCC12 cells were pretreated with either gangliosides or anti-ganglioside antibodies, washed with DMEM/F-12 medium, and stimulated with 10 μg/ml cell-binding fragment of FN for 30 min at 37 °C. Whole cell lysates (25 μg of protein) were applied to a 10% SDS-PAGE mini-gel and then immunoblotted with anti-Src-phosphotyrosine 416 polyclonal antibody (Upstate Biotechnology) to assess c-Src phosphorylation. Gels were reprobed with anti-Src monoclonal antibody to measure c-Src expression as described before (11).
Gangliosides Modulate Cell Adhesion and Spreading

40413

and immunoblotting with anti-Pi3K p85 antibody. Experiments were performed four times.

Blockade of Specific Signaling Pathways with Inhibitors—After pre-

treatment with or without ganglioside or anti-ganglioside antibody in the presence or absence of inhibitors as indicated below, cells were plated onto FN or poly-L-lysine-coated plates as described above for spreading assays. During the 1.5-h incubation on the plates, cells were treated continuously with or without 20 μM LY294002 (PI3K inhibitor) (Calbiochem), 3 μM [14-aminom-1-tert-butyl-3-1-naphthyl]pyrazolo[3,4-
di]pyrimidine] (PP1, Src inhibitor) (Alexis, San Diego), or 20 μg/ml anti-β1 integrin blocking antibody (Chemicon International, Temecula, CA), and spreading was measured as described above. To explore the effect on ganglioside-modulated spreading of PKC inhibition, SCC12 cells were treated with or without PKC inhibitors, 100 nM bisindolylmaleimide I or 1 μM calphostin C (Calbiochem) for 3 h in DMEM/F-12 medium containing 6 mg/ml bovine serum albumin. During the final 30 min of incubation, cells were treated with 10 μg/ml anti-ganglioside antibodies, including anti-GD3, anti-GT1b and, as a control, anti-GM2 antibody before cell replating and assessment of spreading. 100 μg/ml purified mouse IgM also served as a negative control.

RESULTS

Generation of Stable Inducible GM2/GD2 Synthase Transfectants—Preliminary studies with RU486-inducible expression of β-gal showed strong expression of the β-gal, peaking at 24–48 h after exposure to 100 nM RU486, totally reversible by elimination of the RU486 within 72 h. 29 monoclonal cell lines of GM2/GD2 synthase-expressing cells were generated. Northern analyses showed a 3.6–5.9-fold increase in expression of GM2/GD2 synthase in cells transfected with both the transactivator and the GM2/GD2 synthase constructs when induced by RU486 (Fig. 2A). Expression of the GM2/GD2 synthase in transfected cells without RU486 induction and in parental SCC12 cells and mock transfected cells with and without RU486 induction were equivalent. TLC immunostaining (not shown) and ganglioside ELISA (Fig. 2B) showed increased expression of GT1b and de novo expression of GD2 in transfected cells induced by RU486, whereas expression of gangliosides GM3 and GD3 was unchanged. Transfected cells did not express GM2 or GM1 de novo. Control cells (parental SCC cells, mock transfected cells with and without induction, transfected cells without the RU486 induction, and cells treated with vehicle only) showed no change in ganglioside expression. Four SSIA cell lines (SSIA3, 6, 12, and 25), four GM2/GD2 synthase lines (clones 1, 5, 11, and 26) with and without RU486 induction were plated on FN or poly-L-lysine-coated plates as described above for spreading assays. During the 1.5-h incubation on the plates, cells were treated continuously with or without PKC inhibitors, 100 nM bisindolylmaleimide I or 1 μM calphostin C (Calbiochem) for 3 h in DMEM/F-12 medium containing 6 mg/ml bovine serum albumin. During the final 30 min of incubation, cells were treated with 10 μg/ml anti-ganglioside antibodies, including anti-GD3, anti-GT1b and, as a control, anti-GM2 antibody before cell replating and assessment of spreading. 100 μg/ml purified mouse IgM also served as a negative control.

RESULTS

Generation of Stable Inducible GM2/GD2 Synthase Transfectants—Preliminary studies with RU486-inducible expression of β-gal showed strong expression of the β-gal, peaking at 24–48 h after exposure to 100 nM RU486, totally reversible by elimination of the RU486 within 72 h. 29 monoclonal cell lines of GM2/GD2 synthase-expressing cells were generated. Northern analyses showed a 3.6–5.9-fold increase in expression of GM2/GD2 synthase in cells transfected with both the transactivator and the GM2/GD2 synthase constructs when induced by RU486 (Fig. 2A). Expression of the GM2/GD2 synthase in transfected cells without RU486 induction and in parental SCC12 cells and mock transfected cells with and without RU486 induction were equivalent. TLC immunostaining (not shown) and ganglioside ELISA (Fig. 2B) showed increased expression of GT1b and de novo expression of GD2 in transfected cells induced by RU486, whereas expression of gangliosides GM3 and GD3 was unchanged. Transfected cells did not express GM2 or GM1 de novo. Control cells (parental SCC cells, mock transfected cells with and without induction, transfected cells without the RU486 induction, and cells treated with vehicle only) showed no change in ganglioside expression. Four SSIA cell lines (SSIA3, 6, 12, and 25), four GM2/GD2 synthase lines (clones 1, 5, 11, and 26) with and without RU486 induction, and two of each mock transfected cell lines were studied.
Gangliosides Modulate Cell Adhesion and Spreading

Results were consistent among transfected cell lines in each experiment.

Gangliosides Modulate Cell Adhesion on FN Matrix—Consistent with our previous studies that showed GT1b to inhibit adhesion of normal undifferentiated cultured keratinocytes to FN (6), addition of 1 μM GT1b significantly inhibited attachment of the SCC12 cells to FN (p < 0.001) (Fig. 3A). 1 μM GD3 also inhibited adhesion to FN, although slightly less than the inhibition by GT1b. Adhesion to FN was not impacted by treatment with 50 μM GM3 or GM2. Gangliosides had no effect on SCC12 cell adhesion to poly-l-lysine or plastic (not shown). Blockade of functional ganglioside resulted in increased adherence to FN with anti-GT1b (p < 0.05), anti-GD3 (p < 0.05), and the combination of anti-GT1b and anti-GD3 (p < 0.01) antibodies (Fig. 3B). No significant change in adhesion was induced by antibodies directed against other gangliosides of the SCC12 cell (anti-GM3 and anti-9-O-acetyl-GD3 antibodies), or when cells were plated on poly-l-lysine. Endogenous ganglioside modification by RU486-induced overexpression of GM2/GD2 synthase (increases GT1b without significantly decreasing GD3 or GM3) decreased adhesion to FN in contrast to parental SCC12 cells, vector controls, and transfected cells without RU486 stimulation (p < 0.01) (Fig. 3C; SSIA cells (overexpression of ganglioside-specific sialidase and depletion of all gangliosides) showed significantly increased adherence to FN (p < 0.001) compared with parental SCC12 cells and pcDNA vector controls (Fig. 3C). Neither induced GM2/GD2 synthase overexpressors nor SSIA cells showed any change in adherence to a poly-l-lysine matrix. Consistent with the time-limited effect of adding GT1b to keratinocytes plated on FN (6), the significant increase in binding of SSIA cells to FN was an early phenomenon, with a maximal difference between SSIA cells and controls at 90 min but no difference between binding of SSIA cells and controls after 120 min (Fig. 3D).

Gangliosides Modulate Cell Spreading on FN Matrix—To assess the effect of keratinocyte plasma membrane gangliosides on cell spreading, keratinocyte-derived SCC12 cells were pretreated with or without gangliosides or anti-ganglioside antibodies. In addition, ganglioside-deficient SSIA cells, their mock pcDNA cell controls, GM2/GD2 synthase overexpressors with or without RU486 induction, or their vector controls were allowed to attach and spread on FN matrix in the presence of soluble cell-binding fragment of FN for 1.5 h. Ganglioside depletion by sialidase overexpression (Fig. 4, A and E) or blockade of specific keratinocyte gangloside function with anti-GT1b (Fig. 5, D and G) or -GD3 antibody (Fig. 5, F and G) increased cell spreading on a FN matrix by 2.5-fold, 2.2-fold, and 2.0-fold, respectively, compared with parental SCC12 cells (Figs. 4, A and E; and 5G), pcDNA control cells (Fig. 4E), or cells treated with control anti-ganglioside antibodies (Fig. 5, B and G). In contrast, pharmacological addition of ganglioside GT1b (Fig. 5, C and H) or GD3 (Fig. 5, E and H) or overexpression of GM2/ GD2 synthase (Fig. 4, D and E) inhibited cell spreading on a FN matrix by 2.9-fold, 2.4-fold, and 2.7-fold, respectively, compared with parental SCC12 cells, GM2/GD2 synthase transfected cells without RU486 induction (Fig. 4, C and E), or control gangliosides (Fig. 5, A and H). None of the ganglioside modulations affected cell spreading on either poly-l-lysine (Fig. 4E and 5, G and H) or the uncoated plastic surface (not shown).

Inhibition of Spreading by Specific Gangliosides Is Pathway-dependent—To explore the molecular mechanism of the effect of gangliosides on keratinocyte spreading, the impact of modulation of integrin β3, subunit, c-Src, FAK, and PI3K function were examined. The effect of FAK function on inhibition of cell spreading by gangliosides was evaluated by transient transfection of wild type FAK cDNA into the SCC12 cells; the effect of FAK function on stimulation of spreading by specific or global ganglioside depletion was assessed by transient transfection of mutant FAK cDNA (Y397F) or incubation with FAK antisense oligodeoxynucleotide. The transfection efficiency of the wildtype FAK cDNA was 49.2%, and that of the Y397F FAK cDNA was 42.7%. As shown in Fig. 6A, blockade of FAK function by either overexpression of FAK mutant at the critical Y397F phosphorylation site or incubation of cells with FAK antisense oligodeoxynucleotide decreased cell spreading on a FN matrix by 3.1-fold and 4.1-fold, respectively (Fig. 6A) compared with parental SCC12 cells. Conversely, overexpression of wild type FAK increased cell spreading by 2.1-fold. The stimulatory effect of wild type FAK overexpression on cell spreading was maintained in the presence of GT1b, but it could not reverse the inhibition of spreading induced by GD3 (Fig. 6B). Treatment of SCC12 cells with FAK antisense oligodeoxynucleotides or by transient transfection with Y397F mutant FAK cDNA markedly suppressed cell spreading (Fig. 6A) and reversed the stimulatory effect of global ganglioside depletion induced by...

![Fig. 4. Endogenous modulation of ganglioside expression by stable gene transfection of SCC12 cells affects cell spreading on a FN matrix.](image-url)
sialidase overexpression or specific GT1b depletion by anti-GT1b antibody treatment (Fig. 6C). Blockade of FAK function was unable to reverse the stimulation of cell spreading triggered by treatment with anti-GD3 antibody (Fig. 6C).

Inhibition of β1 integrin function with anti-β1 antibody, of c-Src with [4-amino-1-tert-butyl-3-(1’-naphthyl)pyrazolo[3,4-d]pyrimidin]), and of PI3K with LY294002 also markedly diminished spreading of untreated SCC12 cells (Fig. 7A) and SCC12 cells treated with GM2 control ganglioside (Fig. 7B). Although inhibition of β1 integrin, c-Src, and PI3K function reversed the stimulation of spreading by anti-GT1b antibody and sialidase overexpression (Fig. 7, C and E), only inhibition of PI3K function was able to reverse the spreading induced by anti-GD3 antibody (Fig. 7D).

**Gangliosides Inhibit Phosphorylation and Activity of FAK, Src, and PI3K, but Not Their Expression**—Blockade of specific ganglioside function by anti-GT1b or anti-GD3 antibody increased the phosphorylation of FAK at tyrosine 397 site by 2.6-fold and 2.5-fold, respectively (Fig. 8A, bottom row), and increased FAK activity by 1.9-fold and 1.7-fold, respectively (Fig. 8B). FAK phosphorylation at the tyrosine 397 site of SSIA cells was increased 3.4-fold, and activity was increased 2.3-fold compared with parental SCC12 and pcDNA controls (Fig. 8, A, bottom row, and B). Consistently, pharmacological addition of ganglioside GT1b or GD3 decreased FAK phosphorylation at tyrosine 397 site and activity (Fig. 8A, bottom row, and B). Ganglioside GM2 treatment or functional blockade with anti-9-O-acetyl-GD3 antibody did not affect either FAK phosphorylation or activity (Fig. 8). FAK expression was not altered by ganglioside supplementation or functional blockade (Fig. 8A, top row). The effect of gangliosides on FAK phosphorylation specifically at the 397 site was similar to that on phosphoryl-
Blockade of FAK signaling reverses the stimulatory effects of anti-GT1b antibody and global ganglioside depletion by sialidase overexpression but not that of anti-GD3 antibody. SCC12 cells were either transiently transfected with FAK cDNA (wild type FAK or mutant FAK Y397F) or treated with FAK antisense or sense oligodeoxynucleotides to modulate FAK expression and function (A). SCC12 cells were transiently transfected with wild type FAK to increase FAK expression, then cells were treated with or without 1 μM GT1b, 1 μM GD3, or as a control, 50 μM GM2 (B). Parental SCC12 cells, SSIA cells, and SCC12 cells transiently transfected with mutant FAK Y397F or treated with FAK antisense oligodeoxynucleotide were treated with or without 10 μg/ml anti-GT1b, -GD3, or as a control, anti-GM2 antibody (C). The effect on cell spreading was determined as the percentage of spread cells/total cells and is expressed as the means ± S.D. as described under “Experimental Procedures.” All studies were performed six times in triplicate with three different individuals. **, p < 0.01; ***, p < 0.001.

Blockade of specific ganglioside function using anti-GT1b or anti-GD3 antibody or of total ganglioside function by sialidase overexpression increased the phosphorylation of c-Src at tyrosine 416 by 2.1–3.6-fold (Fig. 9A, bottom row) when cells were grown in the presence of FN; pharmacological addition of ganglioside GT1b or GD3 decreased Src kinase phosphorylation at tyrosine 416 by 2.1-fold or 1.5-fold, respectively (Fig. 9A, bottom row). Treatment with anti-9-O-acetyl-GD3 control anti-ganglioside antibody or GM2 control ganglioside, and transfection with the pcDNA vector, had no effect on Src phosphorylation (Fig. 9A, bottom row). Ganglioside modulation did not alter Src kinase expression (Fig. 9A, top row). As measured by in vitro phosphorylation of acid-denatured elonase, Src kinase activity was increased dramatically in both ganglioside-depleted SSIA cells by 4.5-fold (Fig. 9B, lane 3) and by functional blockade of ganglioside GT1b or GD3 by 3.1-fold and 1.8-fold, respectively (Fig. 9B, lanes 4 and 5), compared with parental SCC12 cells (Fig. 9B, lane 1), mock pcDNA transfectant (Fig. 9B, lane 2), and cells with functional blockade of ganglioside 9-O-acetyl-GD3 (Fig. 9B, lane 6). Pharmacological addition of ganglioside GT1b or GD3 decreased Src kinase activity by 3.6-fold and 2.1-fold, respectively (Fig. 9B, lanes 8 and 9), whereas GM2 treatment had no effect on Src kinase activity (Fig. 9B, lane 7). Similar results were noted by measuring the phosphorylation of Src substrate peptide using a Src kinase assay kit (Fig. 9C).

Blockade of GT1b or GD3 function increased the activity of PI3K by 3.3-fold and 2.9-fold, respectively (Fig. 10B, top row). The inhibition of PI3K activity by gangliosides was comparable with the inhibition by treatment with anti-β1 integrin antibody (2.1-fold) but was less than that induced by specific inhibition of PI3K activity with LY294002 (8.2-fold) (data not shown). Neither functional inhibition of 9-O-acetyl-GD3 nor addition of GM2 modulated PI3K activity (Fig. 10). PI3K expression was not affected by the addition or blockade of any ganglioside (Fig. 10, A and bottom rows of B). Blockade of PKC Signaling Prevents the Stimulation of Spreading by Depletion of GD3 Function but Not of GT1b Function—Treatment of SCC12 cells with the PKC inhibitors calphostin C (Fig. 11) or bisindolylmaleimide I (not shown) significantly reversed the effect of stimulation of cell spreading by anti-GD3 antibody, but it had no effect on the stimulation of cell spreading induced by anti-GT1b antibody in the presence of FN.

DISCUSSION

Our previous studies have demonstrated an inhibitory role of the more highly sialylated keratinocyte gangliosides, GT1b and GD3, in cell adhesion and migration specifically on a FN matrix (6, 23). The mechanism of this inhibition, at least in part, relates to the specific ability of GT1b and to a lesser extent...
GD3, but not other tested gangliosides, to bind directly to the $\alpha_5\beta_1$, the primary receptor by which keratinocytes and keratinocyte-derived cell lines bind to FN, and the resultant suppression of the $\alpha_5\beta_1$-FN interaction (9). Through this interaction with integrin, GT1b is also able to trigger cell apoptosis when keratinocytes and keratinocyte-derived SCC12 cells are plated on FN (23, 33) by a mechanism that involves inhibition of the integrin-linked kinase/protein kinase B/Akt signaling pathway (11, 13). GD3, although also able to induce keratinocyte and keratinocyte-derived cell apoptosis through a mechanism that promotes mitochondrial cytochrome c release (11), does not require cell exposure to FN and has no effect on integrin-linked kinase/protein kinase B signaling (11). These studies of apoptosis provide evidence of two independent mechanisms by which GT1b and GD3 influence signaling in keratinocytes.

Here, we show that both GT1b and GD3, but not other keratinocyes or keratinocyte-derived SCC12 cell gangliosides, are able to inhibit cell spreading on a FN matrix, and we have delineated the signaling mechanisms responsible for this inhibition. The increase in cell spreading observed when cells were treated with anti-GT1b and anti-GD3 antibodies, but not with antibodies directed against other gangliosides, further confirms the specificity of the effect on cell spreading.

Although pharmacologic addition of gangliosides increases membrane concentration of added gangliosides, and anti-ganglioside antibodies block ganglioside function, physiologically relevant shifts in membrane ganglioside content likely result from changes in expression of enzymes that deplete gangliosides or lead to the synthesis of more complex gangliosides. As a result, we also examined the effects on cell spreading of endogenous modification in gangliosides through gene overexpression of two human genes: a ganglioside-specific plasma membrane sialidase, which we have previously shown to deplete totally the membrane gangliosides of SCC12 cells (13), and GM2/GD2 synthase, which we predicted would lead to endogenous increases in GT1b content in the SCC12 cells. Consistent with the effects on spreading of exogenous manipulation, depletion of membrane gangliosides increased spreading and the increased GT1b expression after stable GM2/GD2 synthase transfection reduced cell spreading when cells were plated on FN in the absence of both serum and growth factors.

In previous studies, we noted no effect of GM3, the predominant ganglioside of SCC12 cells, on normal keratinocyte or
Gangliosides Modulate Cell Adhesion and Spreading

Fig. 10. Ganglioside depletion or functional blockade of ganglioside GT1b or GD3 activates PI3K activity, whereas exogenously increased GT1b or GD3 inhibits PI3K activity. Ganglioside-depleted SSIA cells (four cell lines), mock transfected pcDNA cells (two cell lines), and SCC12 cells pretreated with or without either gangliosides or anti-ganglioside antibodies as described above were starved for 18 h in serum-free DMEM/F-12 medium. Cells were lysed after stimulation with 10 μg/ml soluble cell-binding fragment of FN for 30 min. PI3K activity was measured using phosphatidylinositol as a substrate with immunoprecipitated PI3K as described under “Experimental Procedures,” shown in the top rows in both A and B. Expression of PI3K was determined using 15 μg of total protein from whole cell lysate and as shown in the bottom rows of both A and B. The SSIA cell and pcDNA phosphorylations shown in A are representative of the studied cell lines.

SCC12 cell adhesion to FN (6), in contrast to evidence that adhesion to FN of mouse mammary carcinoma cells requires GM3 (24). Here we provide further evidence of a lack of GM3 effect on spreading, FAK phosphorylation, and Src activation in keratinocyte-derived cells. Although the difference in effects of GM3 on adhesion may reflect cell specificity, the previously described effects of GM3 on adhesion may also be mediated via EGFR signaling and cross-talk with the FAK signaling pathway (25, 26); our studies were purposely performed in the absence of both growth factors and serum to address specifically the role of FAK/Src/PI3K signaling without the influence of autophosphorylation of EGFR. Furthermore, the direct relationships demonstrated between both GT1b and GD3 are

![Image](https://via.placeholder.com/150)

Fig. 11. Inhibition of PKC signaling reverses the stimulatory effect of anti-GD3 antibody on SCC12 cell spreading on a FN matrix. SCC12 cells were incubated with either 100 nM bisindolylmaleimide I (not shown) or 1 μM calphostin C for 3 h, and anti-ganglioside antibodies were added in the final 30 min. Cells were then harvested and plated onto eight-well cell culture plates precoated with 5 μg/cm² soluble cell-binding fragment FN. Cell spreading was measured as described under “Experimental Procedures,” and results are expressed as the mean ± S.D. *, p < 0.05.

In contrast, we have shown inhibition of Src kinase phosphorylation by a ganglioside in the SCC12 keratinocyte-derived cell line under these conditions in the presence of FN, suggesting both ganglioside and cell specificity of the effect on Src activation. PI3K-dependent, FAK-independent mechanisms for epithelial cell adhesion have been described previously (28), as has the role of activation of PKC for cell attachment and spreading (29). Although PI3K and PKC pathways appear to be distinct in several studies, recent investigations suggest a relationship of early PI3K activity as a requisite for the activation of certain isoforms of PKC (1), such as PKC-ε (30) and PKC-ζ (31). Our preliminary data suggest that GD3 is able to inhibit PKC activation via a PI3K-dependent cascade support a common pathway in epithelial cell spreading. The isoforms of PKC which are involved in this GD3-mediated effect on cell spreading and the time course of this activation deserve further investigation.

How do gangliosides GT1b and GD3 inhibit nonreceptor FAK and Src kinase signaling? Although gangliosides have a lipid component in the membrane that putatively could interact with FAK (cytoplasmic) and Src-kinase (located on the inner leaflet of the plasma membrane), the discrepant influences on spreading and FAK or Src signaling of specific gangliosides that differ only in their sialic acid and carbohydrate chains suggest a direct ganglioside interaction external to the membrane with a receptor upstream of these signaling molecules. Furthermore, the direct relationships demonstrated between both GT1b and αβ1 (9) and GM3 and the EGFR (10) require intact carbohydrate groups on both proteins and the gangliosides, providing further evidence that the external component of gangliosides is required, rather than the lipid intramembrane component. The recent recognition that signaling occurs in specific membrane domains that are enriched in cholesterol and glycosphingolipids provides a potential explanation for the separate signaling pathways inhibited by specific gangliosides and induced by their depletion. We propose that gangliosides GT1b and GD3 reside in distinct membrane microdomains with their specific signaling molecules, consistent with the recent demonstration by Vyas et al. (32) that GM1 and GD3 are “packaged” in different membrane regions. Thus, GT1b may be packaged with αβ1 integrin, FAK, Src kinase,
and PI3K, whereas GD3 is complexed within membranes with PKC and PI3K, allowing differential inhibition of signaling pathways, leading to apoptosis and inhibition of spreading by different mechanisms.

REFERENCES
1. Giancotti, F. G., and Ruoslahti, E. (1999) Science 285, 1028–1032
2. Vuori, K., and Ruoslahti, E. (1999) J. Biol. Chem. 268, 21459–21462
3. Masson-Gadais, B., Salers, P., Songrand, P., and Lisitsky, J. C. (1997) Exp. Cell Res. 236, 238–247
4. Miranti, C. K., Ohno, S., and Brugge, J. S. (1999) J. Biol. Chem. 274, 10571–10581
5. Paller, A. S., Arnsmeier, S. L., Fisher, G. J., and Yu, Q. C. (1995) Exp. Cell Res. 217, 118–124
6. Wang, X. Q., Sun, P., O’Gorman, M., Tai, T., and Paller, A. S. (2001) J. Biol. Chem. 276, 42782–42792
7. Hakomori, S.-i. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 225–226
8. Danilkovitch, A., Skeel, A., and Leonard, E. J. (1999) Exp. Cell Res. 248, 575–582
9. Disatnik, M. H., Boutet, S. C., Lee, C. H., Mochly-Rosen, D., and Rando, T. A. (2002) J. Cell Sci. 115, 2151–2163
10. Balciunaite, E., and Kazlauskas, A. (2001) Biochem. J. 382, 241–250
11. Sung, C. C., Gutierrez-Steinl, C., Wroe-Smith, T., Nickoloff, B. J., and Paller, A. S. (1997) J. Invest. Dermatol. 108, 662 (abstr.)