Ganglioside GM3 Blocks the Activation of Epidermal Growth Factor Receptor Induced by Integrin at Specific Tyrosine Sites*

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The epidermal growth factor receptor (EGFR) can be activated by both direct ligand binding and cross-talk with other molecules, such as integrins. This integrin-mediated cross-talk with growth factor receptors participates in regulating cell proliferation, survival, migration, and invasion. Previous studies have shown that ligand-dependent EGFR activation is inhibited by GM3, the predominant ganglioside of epithelial cells, but the effect of GM3 on ligand-independent, integrin-EGFR cross-talk is unknown. Using a squamous carcinoma cell line we show that endogenous accumulation of GM3 disrupts the ligand-independent association of the integrin β1 subunit with EGFR and results in inhibition of cell proliferation. Consistently, endogenous depletion of GM3 markedly increases the association of EGFR with tyrosine-phosphorylated integrin β1 and promotes cell proliferation. The ligand-independent stimulation of EGFR does not require focal adhesion kinase phosphorylation or cytoskeletal rearrangement. Stimulation of EGFR and mitogen-activated protein kinase signaling by GM3 depletion involves the phosphorylation of EGFR at tyrosine residues 845, 1068, and 1148 but not 1086 or 1173. The specific blockade of phosphorylation at Tyr-845 with Src family kinase inhibition and at Tyr-1148 with phosphatidylinositol 3-kinase inhibition suggests that GM3 inhibits integrin-induced, ligand-independent EGFR phosphorylation (cross-talk) through suppression of Src family kinase and phosphatidylinositol 3-kinase signaling.

Integrins are cell surface-adhesive receptors formed by α and β subunits, which bind to extracellular matrix proteins. Integrin-mediated adhesion to extracellular matrix triggers intracellular signaling pathways to modulate cell proliferation, shape, migration, invasion, and survival (for review see Refs. 1 and 2). Integrin signaling is mediated by intracellular molecules, such as c-Src, small GTPases, adaptor molecules such as Shc, the protein-tyrosine phosphatases SHP-2, and phosphatidylinositol 3-kinase (3–5). Integrins can also cross-communicate with growth factor receptors, enabling growth factor receptor signaling upon extracellular matrix binding of the interacting integrin in the absence of growth factors (1) and culminating in enhanced cell mitogenesis and oncogenesis. Although the mechanism for this cross-talk is poorly understood, this cross-talk does not require cytoskeletal mobility or focal adhesion kinase (FAK)1 activation (6), suggesting interaction at the membrane level, proximal to FAK and cytoskeletal activation. In fact, certain growth factor receptors have been shown to interact physically with specific integrins, suggesting that the formation of complexes at the membrane level is required for the convergence of signaling pathways (6–11). EGFR, for example, forms a complex with β1 integrin after cells attach to fibronectin (6, 12), both platelet-derived growth factor receptor-β and vascular endothelial cell growth factor receptor 2 show association with α5β1 integrin (13, 14), and ErbB2 associates with α5β1 or α6β4 (15).

Gangliosides, sialylated membrane glycosphingolipids, have recently been shown to modulate epithelial cell proliferation, adhesion, migration, differentiation, and survival (16–24). GT1b, the highly sialylated ganglioside of keratinocytes and the keratinocyte-derived squamous carcinoma cell line SCC12, strongly inhibits adhesion, spreading, and migration of cultured epithelial cells specifically when cells are plated on a fibronectin (FN) matrix (17, 19, 23, 25). GT1b has been shown to bind specifically to the α5 subunit of α5β1 integrin, leading to competitive inhibition of the interaction of this integrin with FN and explaining the specificity of the GT1b inhibitory effect (19). GM3, the major ganglioside of epithelial cells (19), predominantly plays a role in regulating mitogenesis through inhibition of EGFR phosphorylation (18, 26), a process that requires the presence of EGFR ligand (18, 20, 24, 26). GM3 is also able to regulate the adhesion and migration of several carcinoma and other tumor cell lines grown on FN (27–30); however, GM3 is unable to bind directly to either the α5 or β1 subunits of integrin (19). We have recently shown that endogenous accumulation of GM3 suppresses SCC12 cell migration and invasion through inhibition of extracellular matrix-promoted matrix metalloproteinase-9 activation and disrupts the association of matrix metalloproteinase-9 with integrin α5β1 (31). Consistently, depletion of GM3 facilitates activation of matrix metalloproteinase-9 through a mechanism that requires phosphorylation of the EGFR (31), suggesting that GM3

* This work was supported by National Institutes of Health Grant R01 AR44619 and the Fujisawa Healthcare, Inc. Research Endowment. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: FAK, focal adhesion kinase; EGFR, epidermal growth factor (EGF) receptor; FBS, fetal bovine serum; FN, fibronectin; GD2, GalNAcβ1→4[NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1-Cer; GD3, NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1-Cer; GD5, NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1-Cer; GT1b, NeuAcα2→3Galβ1→4Glcβ1-Cer; GT1b, NeuAcα2→3Galβ1→4Glcβ1-Cer; GM2, GalNAcα1→4[NeuAcα2→8Galβ1→4Glcβ1-Cer; GM3, NeuAcα2→3Galβ1→4Glcβ1-Cer; MAPK, mitogen-activated protein kinase; pcDNA, SCC12 cells stably transfected with pcDNA3 vector; RU-486, mifepristone; SSIA, SCC12 cells stably transfected with ganglioside-specific human sialidase cDNA; PPL1, /4-amino-1-tert-butyl-3-(1’-napthyl)pyrazole[3,4-d]pyrimidine]; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
is also able to modulate ligand-independent, integrin-dependent EGFR signaling.

Using genetic manipulation of GM3 content, we have investigated the effect of ganglioside modulation on EGFR-integrin cross-talk. To further examine the molecular mechanism of ligand-independent, integrin-promoted EGFR activation modulated by GM3, we have evaluated the effects of GM3 on the integrin association. The specific strategies for the antibody-mediated inhibition that GM3 expression affects EGFR phosphorylation at specific residues. These studies show that endogenous alterations in GM3 expression influence the association of the EGFR and integrin β1 subunit and the resultant activation of the EGFR promoted by FN or type I, IV, and VII collagen, all triggers of integrin-1 subunit and the resultant activation of the EGFR and integrin β1 subunit. Furthermore, these investigations provide evidence that phosphorylation of specific tyrosine residues on the EGFR is impacted by GM3 content modulation, including tyrosine residues that require Src kinase and phosphatidyl-inositol 3-kinase signaling for phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Cells**—The human keratinocyte-derived SCC12F2 cell line (SCC12), a generous gift from Dr. James Reinwald (Harvard, Boston, MA), was maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) without antibiotics in 5% CO2 at 37 °C. Overexpression of GM3 by Treatment with Antisense Oligodeoxynucleotides—Membrane content of GM3 on SCC12 cells was endogenously increased as described previously (24) by treatment concurrently of SCC12 cells with antisense oligodeoxynucleotides of both GM2/GD2 synthase and GD3 synthase, leading to blockade of synthetic pathways downstream of GM3.

**Total Depletion of Membrane Gangliosides**—Gangliosides were depleted by stable gene transfection. SCC12 cells were stably transfected with human plasma membrane ganglioside-specific sialidase cDNA (GenBank accession number NM138185, courtesy of Dr. T. Miyagi, Tokyo, Japan) (32) in a pcDNA3 vector using LipofectAMINE reagent (18, 22). Gene and protein expression in the resultant SSIA cells were demonstrated by Northern blot and sialidase activity measurements. Ganglioside depletion was shown by thin layer chromatography (TLC) immunostaining (18, 20, 22). Four SSIA cell lines (SSIA3, SSIA6, SSIA12, and SSIA25) and 2 mock-transfected pcDNA cell lines were studied. Overexpression and depletion of GM3 was confirmed using ganglioside enzyme-linked immunosorbent assay (23), TLC immunostaining (18, 20, 22), and immunofluorescence microscopy (24).

**Proliferation Assays**—Proliferation was assessed by MTT analysis. Briefly, cells were seeded in 96-well plates at 2 × 10⁴ cells per well and incubated for 6 h in 10% FBS-containing medium. After starvation of serum, growth factors, and FN for 18 h, cells were grown in the presence of either 10% FBS or 10 μg/ml human FN (cellular FN, Sigma). Medium was replaced every 24 h, and cells were stained with MTT reagent per the manufacturer’s instructions (Roche Applied Science). Absorbance was read at A₅₇₀ nm.

**Immunoblotting**—Immunoblotting was carried out as described (19, 21) using protein extracted from either whole cell lysates or immunoprecipitates and an enhanced chemiluminescence detection system (PerkinElmer Life Sciences). In brief, cells were treated with or without antisense oligodeoxynucleotides or were stably transfected with either sialidase cDNA in a pcDNA3 vector or GM2/GD2 synthase cDNA using an RU-486-inducible system (33, 34) as previously described (18, 22–24). Cells were plated onto 6-well cell culture plates precoated with or without 5% FBS-containing medium (FBS) without antibiotics in 5% CO2 at 37 °C.

**RESULTS**

**Induction of Endogenous Changes in Ganglioside Content**—Ganglioside GM3 was increased 1.9-fold after SCC12 cells were treated with antisense oligodeoxynucleotides of both GM2/GD2 synthase and GD3 synthase as detected by TLC immunostaining and ganglioside enzyme-linked immunosorbent assays (24). No detectable ganglioside was found on the cell membrane after cells were stably transfected with human plasma ganglioside-specific sialidase (22, 24).

**Endogenous Modulation of GM3 Regulates Proliferation of Cells**—By 72 h after incubation in serum-containing medium, statistically significance differences (p < 0.05) in proliferation were noted between both sialidase overexpressers and their vector control cells and GM3 overexpressors (antisense oligomer-treated cells) and their control SCC12 cells (Fig. 1A). When cells were grown in the absence of serum or growth factors but in the presence of FN, a statistically significant increase in proliferation of ganglioside-depleted cells versus control cells was noted within 24 h (Fig. 1B); the discrepancy in growth was further accentuated by 72 h of incubation (p < 0.001). Similarly, proliferation of GM3-overexpressing cells was significantly suppressed by 72 h incubation in the presence of FN (Fig. 1B).

**Endogenous Modulation of GM3 Regulates Ligand-independent EGFR and MAPK Phosphorylation**—Expression of FN promoted ligand-independent EGFR phosphorylation (Fig. 2, middle row, lane 2), which was inhibited by endogenous accumulation of GM3 (Fig. 2, middle row, lane 6) and facilitated by ganglioside depletion (Fig. 2, middle row, lane 8). Modulation of ganglioside GM3 content did not affect EGFR expression (Fig. 2, top row). Blockade of integrin β1 with anti-β1-blocking antibody prevented EGFR phosphorylation in the presence of FN, including in ganglioside-depleted cells (Fig. 2, lane 4).
GM3 overexpression by antisense treatment inhibits cell proliferation, whereas ganglioside-depletion facilitates cell proliferation. To accumulate GM3 endogenously, SCC12 cells in Dulbecco's modified Eagle's medium/F-12 with 10% FBS were treated with antisense oligodeoxynucleotides of both GM2/GD2 synthase (synthesizes GM2 from GM3) and GD3 synthase (synthesizes GD3 from GM3), effectively blocking GM3 metabolism (24). Other SCC12 cells were stably transfected with human plasma ganglioside-specific sialidase gene to eliminate membrane ganglioside as described before (22, 24). Parental SCC12 cells, sense-treated cells, and vector-transfected cells served as controls. Cell proliferation was detected by MTT assay as described under “Experimental Procedures.” In brief, cells were plated on 96-well plates and starved of serum, growth factors, and FN overnight before incubation in Dulbecco's modified Eagle's medium/F-12 medium with either 10% FBS (A) or 10 μg/ml FN (B). The absorbance of each cell line was read at A₅₆₀ nm after staining with MTT reagents. Oligodeoxynucleotides were added daily throughout the study.

To determine the specificity of the effects of GM3 on ligand-independent, integrin-dependent EGFR activation, the effects of modulation of GT1b, another epidermal ganglioside that affects specifically FN-induced integrin activation, were compared with the effects of changes in content of GM3. Control cells plated on FN (Fig. 3A) or on types I (Fig. 3B), IV, or VII collagen (not shown), but not on poly-lysine (not shown), showed EGFR phosphorylation between 10

![Graph A](image1.png)

![Graph B](image2.png)
and 30 min (Fig. 3, A and B, second and third rows). Endogenous accumulation of GM3 by antisense treatment inhibited EGFR phosphorylation in the face of either FN or collagen I (Fig. 3, A and B, lane 3 of the second and third rows) in comparison with untreated or sense-treated control SCC12 cells (Fig. 3, A and B, lanes 1 and 2 of the second and third rows).
GM3 Disrupts Integrin-induced EGFR Activation

![Graph](image)

**Fig. 4.** The increased phosphorylation of p42/p44 MAPK by GM3 depletion is partially ablated by blockade of EGFR signaling.

Cells prepared as indicated in Fig. 1 were treated with or without the EGFR inhibitor, 250 nM AG1478, as described under “Experimental Procedures.” After starvation of serum, FN, and growth factors overnight in the continued presence of AG1478, cells were plated onto 6-well cell culture plates precoated with 5 μg/cm² FN and incubated for 10 min. Cells were then treated with booted lysis buffer as described in Fig. 2 for 10 min, and insoluble cell debris was removed by brief centrifugation at 1000 rpm for 5 min. Twenty μg of total protein from the whole cell lysate was applied onto 12% SDS-PAGE mini-gel, and MAPK phosphorylation was detected with anti-EGFR MAPK polyclonal antibody.

|       | Sense | Antisense | pcDNA | SSIA |
|-------|-------|-----------|-------|------|
| 1     | +     | -         | -     | -    |
| 2     | +     | -         | -     | -    |
| 3     | +     | -         | -     | -    |
| 4     | +     | -         | -     | -    |
| 5     | +     | -         | -     | -    |
| 6     | +     | -         | -     | -    |
| 7     | +     | -         | -     | -    |
| 8     | +     | -         | -     | -    |
GM3 Disrupts Integrin-induced EGFR Activation

Fig. 5. Cytochalasin D disrupts integrin-dependent phosphorylation of FAK but not EGFR. A and B, cells were prepared as described in Fig. 1. After starvation of serum, FN, and growth factors overnight in the presence of oligodeoxynucleotides, cells were treated with 0.4 μM cytochalasin for 10 min to 4 h; 10 μg/ml FN was added in the final 10 min. Cells were then treated with boiled lysis buffer as described in Fig. 2 for 10 min, and insoluble cell debris was removed by centrifugation at 1000 rpm for 5 min. Ten (top rows) to 15 μg (bottom rows) of total protein from the whole cell lysate was applied onto 7.5% SDS-PAGE mini-gels for EGFR detection (A) or 10% SDS-PAGE mini-gels for FAK detection (B). The expression of EGFR or FAK was detected with anti-EGFR (A, top row) or anti-FAK (B, top row) monoclonal antibody. The phosphorylation of EGFR or FAK was detected with anti-phospho-EGFR (A, bottom row) or anti-phospho-397-FAK (B, bottom row) antibody. C, cells were incubated with antisense oligodeoxynucleotides of FAK to block the function of FAK as described before (23). In brief, synthetic oligodeoxynucleotides were generated complementary to either the sense or the antisense strand of the 20 nucleotides encoding human FAK at the 5’ end (68), including the initiator codon ATG (5’-AGGCAAGCTCATTTAATAAATG-3’), FAK sense oligodeoxynucleotide; 5’-TCAAGTAAAGGCAGTCCTG-3’; FAK antisense oligodeoxynucleotide). Cells grown in 6-well cell culture plates were incubated with 30 μM sense or antisense oligodeoxynucleotides of FAK in serum-free Dulbecco’s modified Eagle’s medium/F-12 medium for 30 min before 10% FBS was added. The oligodeoxynucleotides were refreshed every other day for 5 days. Cells were then trypsinized and transferred into new 6-well cell culture plates precoated with 5 μg/cm² FN. After 10 min of incubation with FN in the continued presence of sense or antisense oligodeoxynucleotides of FAK, cells were treated with boiled lysis buffer as described in Fig. 2, and 20 μg of post-nuclear lysates were applied for immunoblotting. Phosphorylation of FAK (top row) and EGFR (bottom row) were determined as described for A and B.

Src and PI3 Kinase Signaling Differentially Impact the Phosphorylation of Specific EGFR Phosphorylation Sites Induced by Ganglioside Depletion—Blockade of Src kinase activity with 3 μM PP1 dramatically decreased ganglioside depletion-facilitated EGFR phosphorylation at the 845 residue (Fig. 9, top row), whereas blockade of PI3 kinase activity with 20 μM LY294002 significantly inhibited ganglioside depletion-promoted EGFR phosphorylation at the 1148 residue (Fig. 9, bottom row). Neither Src nor PI3 kinase activation was required for ganglioside depletion-promoted, ligand-independent EGFR phosphorylation at the 1068 residue (Fig. 9, middle row).

DISCUSSION

Cross-communication between integrins and growth factor receptors is thought to be required for maximal activation of the Ras-MAPK signal transduction pathway that drives cell proliferation. How integrin and growth factor receptor signaling are integrated proximal to MAPK is largely unknown. In this study we demonstrate a regulatory role for ganglioside GM3 in ligand-independent, matrix-dependent inhibition of EGFR and MAPK phosphorylation leading to modulation of epithelial cell proliferation. The matrix-dependent effects on EGFR phosphorylation of manipulation of GM3 expression are seen when cells are plated on a variety of matrices that activate integrin β1, suggesting modulation of β1 integrin-EGFR cross-talk.

Existing models for Src and FAK function in integrin signaling have located FAK upstream of Src kinase in integrin signaling (40). FAK autophosphorylation at Tyr-397 has been proposed to recruit Src kinase through its SH2 domain, with stabilization of the Src-FAK interaction through the SH3 domain of Src kinase. However, mutant cells lacking Src kinases show little induction of tyrosine phosphorylation of FAK after integrin stimulation, suggesting that Src kinase is upstream of FAK (41). Furthermore, sites on Src SH2 and SH3 domains, although key for binding to FAK and other proteins involved in cell motility, appear to have little effect on FAK tyrosine phosphorylation (42). Our studies show that the effect of ganglioside depletion on integrin-induced EGFR phosphorylation persists despite either lack of functional FAK or inhibition of cytoskeletal rearrangement. In contrast, blockade of EGFR kinase activation by AG1478 significantly diminishes the increased phosphorylation of MAPK induced by ganglioside depletion to the level of that of the cell without ganglioside depletion, providing further evidence that extracellular matrix-induced growth factor receptor activation plays a significant role in
adhesion-induced MAPK activation and that the activation of ERK by ganglioside depletion is EGFR-dependent. These results are consistent with the demonstration by Moro et al. (6) that treatment with cytochalasin D of ECV304 human endothelial cells plated on collagen I had no effect on adhesion-induced tyrosine phosphorylation of the EGFR, although it dramatically reduced phosphorylation of FAK.

Ganglioside depletion specifically phosphorylates and GM3 overexpression inhibits phosphorylation of three specific EGFR tyrosines in the absence of EGFR ligand. Two of these, Tyr-1068 and Tyr-1148, are major EGFR autophosphorylation sites (43–46), yet two other EGFR autophosphorylation sites (Tyr-1086 and Tyr-1173) are not impacted by modulation of GM3 content. The third site at which ligand-induced EGFR phosphorylation is regulated by ganglioside is Tyr-845, a unique site that is not phosphorylated by EGFR ligands (47–49) but is the site of binding and phosphorylation of the EGFR by Src kinase (48). Trans-activation by Src kinase of this EGFR Tyr-845 site is considered critical to the matrix-induced stimulation of mitogenesis and tumorigenesis (48–57). Consistently, the

FIG. 6. GM3 disrupts the association of EGFR and the integrin β1 subunit in the face of FN or type I collagen. Cells were prepared as described in Fig. 3. After immunoprecipitation (IP), the purity of EGFR was detected with anti-EGFR monocalon antibody (top row, A and B). After exposure to FN (A) or type I collagen (Col I; B) for 0 (second row), 10 (third row), 30 (fourth row), and 60 min (bottom row), co-immunoprecipitated integrin β1 subunit was detected with anti-integrin β1 subunit monoclonal antibody. Lane 1, SCC12 cells; lane 2, sense-treated cells; lane 3, antisense-treated cells; lane 4, pcDNA mock control cells; lane 5, SSIA 3 clone; lane 6, SSIA 6 clone; lane 7, p17 × 4-tkA/pGL-VP mock control cells; lane 8, p17 × 4-tkA/GM2/GD2-pGL-VP clone 1 cells; lane 9, p17 × 4-tkA/GM2/GD2-pGL-VP clone 1 cells treated with 100 nM RU-486 for 48 h; lane 10, p17 × 4-tkA/GM2/GD2-pGL-VP clone 26 cells treated with 100 nM RU-486 for 48 h.

FIG. 7. The association of integrin β1 and EGFR requires both integrin β1 and EGFR kinase activities. Cells that overexpress ganglioside-specific sialidase (22), pcDNA vector controls, and parental SCC12 control cells were treated with or without 0.4 μM cytochalasin D for 30 min, 20 μg/ml anti-integrin β1 blocking or stimulatory antibody for 4 h, or 250 nM AG1478 for 12 h. Integrin β1 subunit or EGFR was immunoprecipitated (IP) from undenatured cell lysate with either anti-integrin β1 subunit or anti-EGFR polyclonal antibody as described before (18). The association of integrin β1 with EGFR was detected with either anti-EGFR (A) or anti-integrin β1 subunit (B) monoclonal antibody.
absence of Src kinases profoundly suppresses matrix-induced tyrosine phosphorylation (41). Although phosphorylation by c-Src at Tyr-845 occurs with both EGF- and integrin-mediated EGFR activation (47), Tice et al. (49) has recently shown that mutation at Tyr-845 does not affect its kinase activity in response to EGF, suggesting that phosphorylation at the Tyr-845 site may play a more critical role in integrin-induced EGFR activation. Given a role for Src kinase binding to the EGFR in its ganglioside-modulated effects on EGFR phosphorylation and induction of mitogenesis, these data suggest that Src kinase functions upstream of FAK in ligand-induced EGFR activation and that Src kinase binding to FAK is not required for EGFR phosphorylation.

Adhesion to matrix induces the formation of a macromolecular complex that includes the EGFR, β1 integrin, Src kinase, and the adaptor protein p130Cas, but not FAK (10). This coimmunoprecipitation strictly requires matrix adhesion and the presence of EGFR; it is disrupted by treatment with PP1, a specific Src kinase inhibitor, and is markedly diminished in c-Src−/− fibroblasts, suggesting that Src kinase and EGFR are both required for complex formation. The activation of the EGFR by ganglioside depletion at sites that are activated by Src kinase (Tyr-845) and phosphatidylinositol 3-kinase (Tyr-1148) and the associated increase in EGFR-β1 integrin coimmunoprecipitation suggests that a complex of the EGFR and β1 integrin together with the Src kinase and phosphatidylinositol 3-kinase is required for cross-talk. Our data showing that increased GM3 expression inhibits the association of β1 integrin and the EGFR further provides evidence that ganglioside acts as a disruptor of complex formation. The lack of effect on the EGFR-β1 integrin association of treatment with cells by cytokexalasin D further suggests that cytoskeletal reorganization through FAK signaling is not necessary for EGFR-β1 integrin complex formation. The complex formation, however, requires that both β1 integrin and EGFR kinase be activated given that treatment with either anti-integrin β1 blocking antibody or AG1478 to block EGFR kinase function prevents the association of β1 and EGFR.

What is the mechanism of ganglioside inhibition of EGFR-β1 integrin cross-talk, and how is it stimulated by ganglioside depletion? The assembly of transduction complexes is thought to involve caveolin-1, a transmembrane protein that acts as a scaffolding protein to aggregate growth factor receptors, integrins, and Src kinases, thus promoting signaling. Caveolin-1 has been shown to participate in integrin-induced Shc tyrosine phosphorylation, leading to Ras-MAPK activation (58). Furthermore, depletion of caveolin-1 has been shown to disrupt the association of Src kinases with β1 integrins, resulting in loss of adhesion (59). Trans-activation of the EGFR by another stimulus, angiotensin II, has recently been shown to occur in caveolar domains together with tyrosine-phosphorylated caveolin-1 and c-Src (60). We have noted by coimmunoprecipitation studies that GM3 is able to complex with caveolin-1, EGFR, and Src in SCC12 cells. We propose that GM3 interferes with integrin-induced EGFR phosphorylation through the effect of ganglioside on caveolin-1 function and disruption of complex formation. Four means of ganglioside interference with caveolin-1 function are possible. First and least likely is that ganglioside may be able to bind to caveolin-1 or a complex component directly, thus sterically preventing signaling. Second, ganglioside GM3 may shift caveolin-1 out of its complex with EGFR, β1 integrin, and Src kinases, thus inhibiting signaling. We have previously shown that, in the face of EGF, overexpression of GM3 shifts caveolin-1 from the detergent-insoluble caveolar domains to detergent-soluble domains (24). Shifts in caveolin-1 localization and the effect of ganglioside modulation on caveolin association with other molecules in the absence of EGF, but

**Fig. 8.** GM3 modulates ligand-independent EGFR phosphorylation at 845, 1068, and 1148 residues. Cells were incubated in the presence of FN for 10 min after starvation of serum, FN, and growth factors, and total protein extracts were prepared as described in Fig. 1. Twenty μg of total protein from the whole cell lysate was applied onto 7.5% SDS-PAGE mini-gels. The phosphorylation of EGFR at specific residues was detected with anti-EGFR-phospho-845, -1068, -1086, -1148, and -1173 antibodies.

**Fig. 9.** Src kinase or phosphatidylinositol 3-kinase activity is required to trigger GM3 depletion-promoted ligand-independent EGFR phosphorylation at 845 or 1148 residues. SCC12 cells were stably transfected with or without human plasma ganglioside-specific sialidase gene in pcDNA vector (22). Cells were treated with or without either 3 μM Src kinase inhibitor PP1 or 20 μM phosphatidylinositol 3-kinase inhibitor LY294002 as described before (23). After starvation of serum, FN, and growth factors overnight in the presence of either PP1 or LY294002, cells were stimulated with 10 μg/ml FN for 10 min. Total protein from whole cell lysates was prepared by treating cells with boiied lysis buffer as indicated in Fig. 2 for 10 min and centrifuging to remove insoluble cell debris. 20 μg of total protein from the whole cell lysate was applied onto a 7.5% SDS-PAGE mini-gel, and the phosphorylation of EGFR promoted by ganglioside depletion at specific residues was detected with anti-EGFR-phospho-845, -1068, and -1148, respectively.

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X.-Q. Wang, P. Sun, and A. S. Paller, unpublished results.
presence of matrix, have not been explored. Third, GM3 may disrupt integrin-induced EGFR phosphorylation and proliferation of both normal keratinocytes and, more so, squamous carcinoma cells (18, 20, 24, 2861–2878). The demonstration that gangliosides also control matrix-induced cell proliferation, inhibiting Src kinase-modulated phosphorylation of the EGFR, gives further impetus to the development of targeted anti-ganglioside therapy for cancers, particularly epithelial carcinomas.

REFERENCES

1. Giancotti, F. G., and Ruoslahti, E. (1999) Science 285, 1028–1032
2. Danen, E. H., and Yamada, K. M. (2001) J. Cell. Physiol. 189, 1–13
3. Clark, E. A., and Brugge, J. S. (1995) Science 266, 233–239
4. Yamada, K. M., and Miyamoto, S. (1995)Curr. Opin. Cell Biol. 7, 681–689
5. Schneller, M., Vuori, K., and Ruoslahti, E. (1997) J. Biol. Chem. 272, 1536–1542
6. Liu, Y., Bishop, A., Witucki, L., Kraybill, B., Shimizu, E., Tsien, J., Ubersax, J., Blethow, J., Morgan, D. O., and Shokat, K. M. (1999) J. Biol. Chem. (Lond.) 274, 671–678
7. Sasaki, H., Nakamura, M., Ohno, T., Matsuda, Y., Yuda, Y., and Nonomura, Y. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2026–2030
8. Casella, J. F., Flanagan, M. D., and Lin, S. (1981) Nature 289, 302–305
9. Lee, J. W., and Juliano, R. L. (2002) J. Biol. Chem. 277, 12619–12625
10. Wilson, L. K., Luttrell, D. K., Parsons, J. S., and Parsons, J. S. (1999) Mol. Cell. Biol. 19, 1249–1258
11. Waton, G. M., Chen, W. S., Rosenfeld, M. G., and Gill, G. N. (1999) J. Biol. Chem. 274, 1750–1754
12. Satoh, M., Ito, A., Nojiri, H., Handa, K., Numahata, K., Ohyama, C., Saito, S., Handa, T., and Aruffo, A. (2002) J. Biol. Chem. 277, 1544–1549
13. Liu, Y., Bishop, A., Witucki, L., Kraybill, B., Shimizu, E., Tsien, J., Ubersax, J., Blethow, J., Morgan, D. O., and Shokat, K. M. (1999) J. Biol. Chem. (Lond.) 274, 671–678
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J. Biol. Chem. 2003, 278:48770-48778.
doi: 10.1074/jbc.M308818200 originally published online September 25, 2003

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