Ganglioside induces caveolin-1 redistribution and interaction with the epidermal growth factor receptor

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Summary

Although caveolin-1 is thought to facilitate the interaction of receptors and signaling components, its role in epidermal growth factor receptor (EGFR) signaling remains poorly understood. Ganglioside GM3 inhibits EGFR autophosphorylation, and may thus affect the interaction of caveolin-1 and the EGFR. We report here that endogenous overexpression of GM3 leads to the clustering of GM3 on the cell membrane of the keratinocyte-derived SCC12 cell line and promotes co-immunoprecipitation of caveolin-1 and GM3 with the EGFR. Overexpression of GM3 does not affect EGFR distribution, but shifts caveolin-1 to the detergent-soluble, EGFR-containing region; consistently, caveolin-1 is retained in the detergent-insoluble membrane when ganglioside is depleted. GM3 overexpression inhibits EGFR tyrosine phosphorylation and receptor dimerization, and concurrently increases both the content and tyrosine phosphorylation of EGFR-associated caveolin-1, providing evidence that tyrosine phosphorylation of caveolin-1 inhibits EGFR signaling. Consistently, depletion of ganglioside both increases EGFR phosphorylation and prevents the EGF-induced tyrosine phosphorylation of caveolin-1. GM3 also induces delayed serine phosphorylation of EGFR-unassociated caveolin-1, suggesting a role for serine phosphorylation of caveolin-1 in regulating EGFR signaling. These studies suggest that GM3 modulates the caveolin-1/EGFR association and is critical for the EGF-induced tyrosine phosphorylation of caveolin-1 that is associated with its inhibition of EGFR activation.
Introduction

The plasma membrane of eukaryotic cells is not uniform, but instead is comprised of microdomains or rafts, highly dynamic lateral assemblies composed of glycosphingolipids and cholesterol. One form of these rafts, caveolae, has been distinguished ultrastructurally by its invaginated shape and by the presence of caveolin-1, the major structural protein of caveolae. These caveolin-containing rafts are insoluble in both nonionic detergents, such as Triton X-100, and in sodium carbonate at alkaline pH, and can be isolated as low-density fractions by sucrose density gradient. Although caveolae have been implicated as sites at which receptors and signaling molecules interact (1, 2), signaling also occurs at non-invaginated rafts, devoid of caveolin-1 but enriched in glycosphingolipids (3-5). In addition, caveolin-1 may also exist in lipid rafts without caveolae (6). The distinction between lipid rafts and caveolae, and the attribution of specific biologic functions and signaling pathways to one or the other remains confusing in the literature (7).

Caveolae have been proposed to be the site of epidermal growth factor receptor (EGFR)\(^1\) signaling, including EGFR autophosphorylation, based largely on the demonstration that caveolin-1 and EGFR both co-localize to low density, carbonate-insoluble membrane regions (8-11). However, caveolin-1 and EGFR can be separated by differences in solubility in nonionic detergent, with EGFR restricted to the detergent-soluble high-buoyancy, low-density membrane regions (12), and are rarely able to be co-immunoprecipitated when co-localized in carbonate-

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\(^1\) DMEM/F12, Dulbecco’s Modified Eagle’s Medium /F12 (1:1) mixture; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; pcDNA, SCC12 cells stably transfected with pcDNA3 vector; PPPP, threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidinopropan-1-ol HCl; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SSIA, SCC12 cells stably transfected with ganglioside-specific human sialidase cDNA; TLC, thin-layer chromatography.
insoluble membrane (12). Immunoelectron microscopy localization studies show only a small proportion of the EGFR in caveolae (13), further suggesting that EGFR is largely localized in noncaveolar membrane domains.

Yet, the evidence for an interaction between caveolin-1 and the EGFR that suppresses EGFR activation is strong. Depletion of caveolin-1 has been associated with tumor proliferation (14), psoriasis, a hyperproliferative skin disorder, and stimulation of mitogen activated protein kinase signaling (15). In addition, re-expression of caveolin-1 in oncogenically transformed cells and in human breast cancer cells inhibits cell growth (16,17). The “scaffolding domain” of caveolin-1 has been shown to co-immunoprecipitate with and bind directly to the binding sequence motifs in the kinase domain of the EGFR (10, 18). A regulatory role of caveolin-1 in EGFR signaling is further suggested by the suppression of cell proliferation and EGF-induced migration when caveolin-1 binds to the EGFR (19). These studies suggest a role for caveolin-1 in maintaining the EGFR in an inactive state, with dissociation from caveolin-1 promoting EGFR activation.

The role of phosphorylation of caveolin-1 in EGFR signaling is unknown. EGF is able to tyrosine phosphorylate caveolin-1 in A-431 cells (20), although not in adipocytes (20) or fibroblasts (21). The effect of this tyrosine phosphorylation of caveolin-1 on EGFR activity, however, is controversial (20), and the effect of ganglioside on caveolin-1 tyrosine phosphorylation has not been explored. Caveolin-1 also has 12 putative sites for serine phosphorylation that are highly conserved (22). Of these sites, only mutation at Ser 80 blocks its serine phosphorylation. Schlegel et al. have recently suggested that the phosphorylation site at Ser 80 is required for endoplasmic reticulum retention and caveolin-1 secretion, and for homo-oligomerization (22); other roles for serine phosphorylation of caveolin-1, including in signaling, have not been determined.
Gangliosides are sialylated 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 (23-29). Studies from our laboratory and others have shown that GM3, the predominant ganglioside of the membranes of both normal cultured keratinocytes and the keratinocyte-derived SCC12 cell line (26, 30), inhibits cell proliferation by suppressing EGFR activation (31) and downstream signaling (26). The mechanism for this ganglioside-specific inhibition appears to involve both binding of GM3 directly to the EGFR that requires receptor glycosylation (28) and direct post-binding effects on signal transduction (26, 28). Consistently, endogenous depletion of gangliosides, including GM3, by stable transfection of SCC12 cells with sialidase cDNA leads to cell hyperproliferation and stimulation of EGFR signaling, especially in response to EGFR ligands (26). Although ganglioside GM1 is considered a marker for caveolae (32-35), and GD3 (36) also likely localizes to caveolar domains, GM3 has been localized predominantly to carbonate- and detergent-insoluble low-density “glycosphingolipid-rich membrane” regions (4). Its relationship with caveolin-1 is unclear (5, 37-40).

Taking advantage of the distinct localizations of EGFR and caveolin-1 after detergent separation of membrane domains, we investigated the effects of endogenous modulation of ganglioside GM3 expression on caveolin-1 distribution, phosphorylation, and interaction with the EGFR. Our studies show that endogenous overexpression of GM3 promotes transport of caveolin-1 from the Triton X-100 insoluble, low-density membrane regions to the soluble, high-density domains in which EGFR predominates. GM3 overexpression increases both the association of caveolin-1 with EGFR and the tyrosine phosphorylation of caveolin-1, which may contribute to suppression of both EGFR dimerization and autophosphorylation. GM3 also induces the later phosphorylation of serine residues on caveolin-1 that is unassociated with EGFR. These results suggest a possible mechanism by which GM3 regulates the suppression of EGFR activation.
Experimental Procedures

*Cells*----Human keratinocyte-derived cell line SCC12F2 cells, a generous gift from Dr. James Rheinwald, Boston, MA, were maintained in Dulbecco’s Modified Eagle’s Medium /F12 (DMEM/F12) (Life Technologies) (1:1) medium 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*----To endogenously increase the membrane content of GM3 on SCC12 cells, SCC12 cells were pretreated with antisense oligodeoxynucleotides of both GM2/GD2 synthase and GD3 synthase, leading to blockade of synthetic pathways downstream of GM3 (Fig. 1A). The synthetic oligodeoxynucleotides were generated complementary to either the sense or the antisense strand of the 21 nucleotides at the 5’ end encoding human GM2/GD2 synthase or GD3 synthase (41), including the initiator codon ATG. These included 5’-GACAGGATGTGGCTGGGCCGC-3’ (GM2/GD2 synthase sense oligodeoxynucleotide), 5’-GCGGCCCAGCCACATCCTGTC-3’ (GM2/GD2 synthase antisense oligodeoxynucleotide), 5’-AGAGGGGCCATGGCTGTACTG-3’ (GD3 synthase sense oligodeoxynucleotide), and 5’-CAGTACAGCCATGGCCCCTCT-3’ (GD3 synthase antisense oligodeoxynucleotide). Cells were incubated with DMEM/F12 medium containing 40 µM of both GM2/GD2 synthase and GD3 synthase oligodeoxynucleotides at 37°C for 30 min. FBS was then added to a final concentration of 10%. The oligodeoxynucleotides were refreshed every other day for 3-7 days.

*Depletion of Membrane Gangliosides*----Gangliosides, including GM3, were depleted both by stable gene transfection and biochemically. SCC12 cells were stably transfected with human plasma membrane ganglioside-specific sialidase cDNA (GenBank accession number AB008185, courtesy of Dr. T. Miyagi, Tokyo, Japan) (42) in a pcDNA3 vector using LipofectAMINE reagent (43), following the techniques described for stable transfection of cytosolic hamster
sialidase cDNA (26) (Fig. 1B). Gene and protein expression in the resultant “SSIA cells” were demonstrated by Northern blot, sialidase activity measurements and ganglioside depletion shown by thin layer chromatography (TLC) immunostaining as described (43), in comparison with parental SCC12 cells and the pcDNA3 mock transfectant control (pcDNA). Four SSIA cell lines (SSIA3, SSIA6, SSIA 12 and SSIA25), and 2 mock transfected pcDNA cell lines were studied.

Synthesis of glycosphingolipids was also inhibited by treating SCC12 cells with 2 μM of racemic threo-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol, HCl (PPPP, Calbiochem, La Jolla, CA) for 5 days. PPPP inhibits the activity of glucosylceramide synthase, preventing the formation of glucosylceramide, a precursor for GM3 (Fig. 1A), but in contrast to 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) does not significantly increase ceramide content (44). Overexpression and depletion of GM3 was confirmed using ganglioside ELISA, TLC immunostaining and immunofluorescence microscopy.

**Ganglioside ELISA’s**——SSIA cells, mock pcDNA transfectants, and parental SCC12 cells were treated with or without either oligodeoxynucleotides or PPPP. Cells were transferred to 96-well plates, grown for 6-8 h, then fixed for ganglioside detection as described below in the legend for Figure 2.

**Effect of Endogenous GM3 Expression on Dimerization of the EGFR**——To examine whether endogenous accumulation of GM3 modulates EGFR dimerization as a means to influence EGFR signaling, SCC12 cells were treated with or without sense or antisense oligonucleotides, then stimulated with 10 nM EGF for 10 min, followed by treatment with sulphosuccinimidyl 4-[p-maleimidophenyl] butyrate (S-SMPB) (Pierce, Rockford, IL), a crosslinker that is membrane-impermeable and hence does not crosslink the receptor's cytoplasmic domain and its interacting signaling molecules within the cell. EGFR dimerization was determined by immunoblotting using whole cell lysate.
Isolation of membrane fragments----Membranes were extracted with carbonate by the method of Song et al. (45) or with detergent by the method of Waugh et al. (12). In brief, cells reaching to 90% confluency were scraped into either 2 ml of 500 mM sodium carbonate, pH 11.0 (45) or 2 ml of 10 mM Tris-HCl, pH 7.4 buffer containing 1% Triton X-100 (12) in the presence of protease inhibitors and incubated for 30 min at 4°C. The cells in suspension were then disrupted by Dounce homogenization and sonication. Nuclei and large cellular debris were removed by centrifugation at 1,200 g for 5 min. The postnuclear supernatant was subjected to sucrose gradient centrifugation as previously described (12, 45). The opaque low-density fragments and clear high-density fragments were separated as insoluble and soluble fragments. All procedures were conducted at 4°C. Equal amounts of total protein from either soluble or insoluble fragments were mixed with an equal volume of Laemmli buffer and separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) mini-gel, followed by immunoblotting.

Immunoprecipitation----Cells were lysed in buffer (1% SDS, 10 mM Tris-HCl, pH 7.4, 1.0 mM Na₃VO₄), boiled, and protein was collected by centrifugation. Soluble protein was mixed with 5 μg of an anti-EGFR monoclonal antibody (Calbiochem) that specifically recognizes the extracellular domain or with anti-caveolin-1 polyclonal antibody (Santa Cruz Biotechnology), and then 50% protein A:agarose (Transduction Labs) was added as previously described (27).

Immunoblotting----Immunoblotting was carried out as described (27) using enhanced chemiluminescence. Protein from either immunoprecipitates or cell lysates was treated with Laemmli buffer and loaded onto SDS-PAGE mini-gels. After transfer to polyvinylidene fluoride or nitrocellulose membranes, the separated proteins were detected by immunoblotting with an anti-EGFR monoclonal antibody that specifically recognizes the extracellular domain (NeoMarkers, Fremont, CA), -phospho EGFR, -caveolin-1, -phosphotyrosine (PY20) (Transduction Labs) or -phosphoserine (Calbiochem) antibodies. Blots were reprobed as previously described (29) with anti-actin antibody to confirm equal loading. Bound antibody was
detected using enhanced chemiluminescence and band density was analyzed by the Storm 800 PhosphorImager.

Statistics----Quantitative data were expressed as mean ± standard deviation. All data were analyzed statistically by Student’s t-test, with p < 0.05 considered significant.
Results

Endogenous Overexpression of GM3 by Antisense Treatment Leads to GM3 Clustering in Both Cytoplasm and on the Membrane----TLC immunostaining and ganglioside ELISA were employed to detect ganglioside expression after SCC12 cells were treated with or without sense or antisense oligodeoxynucleotides of both GM2/GD2 synthase and GD3 synthase. GM3 expression in SCC12 cells was increased approximately 2-fold by antisense oligodeoxynucleotide treatment, which also prevented the synthesis of downstream gangliosides, in comparison with untreated and sense treated cells (Figs. 2A, B) (p<0.01). Overexpression of a human plasma membrane ganglioside-specific sialidase cDNA totally depleted SCC12 cell membrane gangliosides as detected by TLC immunostaining (Fig. 2A, lane 5). Alterations in the pattern of GM3 expression were observed by immunofluorescence microscopy. SCC12 cells subjected to ganglioside-specific sialidase transfection (Fig. 2C-c) or PPPP treatment (not shown) showed no detectable GM3 expression. On the other hand, immunofluorescence examination of SCC12 cells treated with the antisense oligodeoxynucleotide revealed clustering of GM3 at the membrane (Fig. 2C-a, arrows) and intracellularly in comparison with the homogeneous pericellular staining of untreated (Fig. 2C-b, arrows) and sense oligomer-treated (not shown) controls.

Endogenous Overexpression of GM3 Blocks EGFR Autophosphorylation and Dimer Formation--Endogenous accumulation of GM3 inhibited EGFR autophosphorylation. (Fig. 3A, bottom row, lane 4) and blocked EGFR dimerization (Fig. 3B, lane 3) in comparison with untreated cells (Fig. 3A, bottom row, lane 2; B, lane 1) and cells treated with sense oligodeoxynucleotides (Fig. 3A, bottom row, lane 3; B, lane 2). GM3 accumulation had no effect on EGFR expression (Fig. 3A, top row).

Endogenous Overexpression of GM3 Facilitates the Interaction of GM3 and Caveolin-1 with EGFR---- Immunoprecipitation of membrane EGFR from whole cell lysates with GM3 overexpression showed a doubling of caveolin-1 (p<0.01) (Fig. 4, middle row, lane 3), and a 4-
fold increase in GM3 (p<0.001) in association with the EGFR (Fig. 4, bottom row, lane 3), in contrast with untreated (Fig. 4, middle and bottom rows, lane 1) and sense oligomer-treated (Fig. 4, middle and bottom rows, lane 2) controls, indicating that overexpressed GM3 complexes with EGFR and caveolin-1. GM3 overexpression did not affect either EGFR or caveolin-1 expression (not shown).

Endogenous Overexpression of GM3 Shifts Caveolin-1 from Detergent Insoluble Membrane Microdomain into Detergent Soluble Membrane Microdomain without Affecting EGFR Expression or Distribution---- The relative localizations of ganglioside GM3, caveolin-1 and EGFR within cell membranes were detected using sodium carbonate and detergent separation techniques. Results indicated that GM3, caveolin-1 and EGFR all localized to the carbonate-insoluble membrane, regardless of GM3 content (Fig. 5A). However, treatment of membranes with detergent allowed separation of caveolin-1 and EGFR. Membrane EGFR was found only in Triton X-100 soluble membrane microdomains (Fig. 5B, C, top rows) and the alteration of GM3 expression had no effect on either the expression (not shown) or distribution of the EGFR (Fig. 5B, top row, lanes 3, 4, 7, 8; C, top row). In contrast, caveolin-1 shifted from the Triton X-100 insoluble membrane microdomains into soluble membrane microdomains that contained the EGFR when GM3 was overexpressed (Fig. 5B, middle row, lanes 3, 7). Consistently, endogenous ganglioside depletion by treatment with PPPP (Fig. 5B, middle row, lanes 4, 8) or sialidase overexpression (Fig. 5C, middle row) retained caveolin-1 in the insoluble membrane domain, away from effective interaction with the EGFR. In untreated and sense oligomer-treated cells, expression of GM3 was limited to the detergent insoluble membrane domains (Fig. 5B, bottom row, lanes 1, 2, 5, 6); however, overexpression of GM3 in the SCC12 cells led to increased GM3 content in both the detergent soluble and insoluble membrane regions (Fig. 5B, bottom row, lanes 3, 7). Treatment with PPPP or overexpression of sialidase depleted GM3 (Fig. 5B, bottom row, lanes 4, 8; Fig. 5C, bottom row).
GM3 Modulates Caveolin-1 Phosphorylation to Regulate EGFR Function----

Endogenous overexpression of GM3 strongly promoted the tyrosine phosphorylation of caveolin-1 in association with EGFR by 10 min after exposure to EGFR ligand (Fig. 6A, 4th row, lane 7), increasing it by 4.2-fold in comparison with untreated or sense oligomer-treated cells (p<0.001) (Fig. 6A, 4th row, lanes 5,6). Concurrently, overexpression of GM3 inhibited the autophosphorylation of EGFR by 4.1-fold (Fig. 6A, 2nd row, lane 7) in comparison with untreated or sense oligomer-treated cells (p<0.001) (Fig. 6A, 2nd row, lanes 5, 6) after stimulated with either 10 nM EGF or 12 nM TGF-α (not shown). Ligand stimulation had no effect on the sustained GM3-induced doubling of the amount of caveolin-1 associated with EGFR (Fig. 6A, 3rd row, lanes 4, 7, 10, 13). However, without exposure to EGF, caveolin-1 was not tyrosine phosphorylated, regardless of the increase in GM3 (Fig. 6A, 4th row, lane 4). When ganglioside was depleted by sialidase overexpression (Fig. 6B, 4th row, lanes 7, 10, 13) or PPPP treatment (not shown), EGF was unable to stimulate tyrosine phosphorylation of caveolin-1 in association with the EGFR. Ganglioside depletion also diminished the co-immunoprecipitation of caveolin-1 with EGFR in both sialidase overexpressors (Fig. 6B, 3rd row, lanes 4, 7, 10, 13) and PPPP treated cells (not shown) by almost 2-fold (p<0.01), regardless of ligand stimulation. Under these conditions, EGFR autophosphorylation was increased by 3.8-fold within 10 min after stimulation with either 10 nM EGF (Fig. 6B, 2nd row, lane 7) or 12 nM TGF-α (not shown), in comparison with parental SCC12 cells and mock pcDNA transfected cells (Fig. 6B, 2nd row, lanes 5, 6; p<0.001). Endogenous overexpression of GM3 had no effect on the weak tyrosine phosphorylation of caveolin-1 unassociated with the EGFR (Fig. 7A, middle row). Consistently, decreasing GM3 expression also had no effect on the weak tyrosine phosphorylation of caveolin-1 that was not associated with the EGFR (Fig. 7B, middle row).

The effect of ganglioside GM3 on serine phosphorylation of caveolin-1 was also determined using immunoblotting. Caveolin-1 in association with the immunoprecipitated EGFR was never serine phosphorylated, regardless of GM3 content (Fig. 6A, B, bottom rows). However,
caveolin-1 unassociated with the EGFR was strongly serine-phosphorylated when GM3 was endogenously overexpressed after stimulation for 30 min with either 10 nM EGF (Fig. 7A, bottom row, lane 10) or 12 nM TGF-α (not shown). Serine phosphorylation of caveolin-1 unassociated with EGFR was increased by 4.6-fold at 30 min after stimulation with 10 nM EGF (Fig. 7A, bottom row, lane 10). In contrast, the endogenous decrease in GM3 expression induced by either PPPP treatment (Fig. 7B, bottom row, lanes 7, 10, 13) or sialidase overexpression (not shown) decreased the serine phosphorylation of caveolin-1 unassociated with EGFR by 2- to 3-fold after stimulation with 10 nM EGF.
Discussion

Signaling in membranes is now thought to be facilitated by compartmentalization of interacting molecules. We have investigated the possibility that ganglioside GM3 mobilizes caveolin-1 to colocalize with the EGFR and thereby regulate receptor function. To most closely simulate endogenous shifts in ganglioside expression, gene modulation was selected as a means to manipulate ganglioside content, rather than pharmacologic addition of ganglioside. The resulting endogenous overexpression of GM3 led to clustering of GM3 at the membrane and intracellularly in comparison with the homogeneous pericellular staining of control cells, suggesting that overexpressed GM3 clusters within specific functional membrane domains. The increased co-immunoprecipitation of caveolin-1 and the EGFR with GM3 when GM3 is overexpressed provided evidence of co-clustering of these three membrane components.

The differences in the distribution of caveolin-1 and the EGFR after membrane extraction with detergent versus carbonate provide a means to investigate biochemically the effects of manipulation of ganglioside content on the relative localizations of caveolin-1 and the EGFR within membranes. Our discovery that endogenous manipulation of GM3 content leads to shifts of caveolin-1 in the membrane to allow juxtaposition of caveolin-1 with the EGFR in detergent-soluble membrane domains is consistent with the recently demonstration that GM3 shifts of caveolin-1 from detergent-insoluble to detergent-soluble membrane in association with integrins α5 and α3 by galactose-induced synthesis of GM3 in a CHO mutant cell line (46). Importantly, our finding that the caveolin-1/EGFR interaction is associated with the inhibition of EGFR phosphorylation and dimerization supports the speculation by Couet et al. that caveolin binding to the EGFR stabilizes the receptor kinase in an inactive conformation and prevents receptor dimerization (10). Previous studies with exogenous addition of GM3 have demonstrated that GM3 inhibits EGF-dependent tyrosine phosphorylation of EGFR but does not affect receptor-receptor interaction (47). Our demonstration that endogenous overexpression of GM3 inhibits
EGFR dimerization suggests a greater local concentration of endogenously overexpressed GM3 or a difference in orientation of endogenous versus exogenously added GM3, allowing inhibition of receptor dimerization. We propose that overexpression of GM3 inhibits EGFR phosphorylation by: a) binding directly to the extracellular region of the EGFR (28); b) inhibiting EGFR dimerization; and c) as suggested here, promoting the association of caveolin-1 and the EGFR. The significance of the increased caveolin-1/EGFR association in GM3-induced inhibition of the EGFR requires further investigation.

In untreated and sense oligomer-treated cells, expression of GM3 was limited to the detergent insoluble membrane domains. Endogenous overexpression of GM3 in the SCC12 cells, however, led to increased GM3 content in both the detergent soluble and insoluble membrane regions. Although undoubtedly some of the GM3 seen in the Triton X-100 soluble fraction reflects the observed intracellular accumulation, the specific co-immunoprecipitation from whole cell lysates of GM3 and caveolin-1 with anti-EGFR antibody directed against the extracellular region confirms that endogenous increases in GM3 shift GM3 into the same detergent soluble membrane domain as the EGFR and the shifted caveolin-1. The movement of GM3 with caveolin-1 into detergent soluble domains positions GM3, which binds to the EGFR through extracellular interactions (28), as a stabilizer of the caveolin-1/EGFR interaction and a facilitator of the inhibitory effect of caveolin-1. The movement of GM3 with caveolin-1 also implies that GM3 may bind directly or indirectly to caveolin-1, transporting it into proximity with the EGFR. However, interactions of other proteins with SCC12 cell gangliosides have involved extracellular carbohydrate-dependent interactions (27, 28), and caveolin-1 is not glycosylated and has no extracellular domain. Direct interaction of GM3 with caveolin-1 would require binding of the transmembrane region of caveolin-1 with the intramembrane sphingolipid moiety of GM3. Alternatively, an indirect interaction of GM3 with caveolin-1 via an adaptor molecule with an extracellular domain is another possibility. The mechanism by which GM3 content affects caveolin-1 transport may also be explained by the recent recognition that the plasma membrane
ganglioside-specific sialidase is enriched in caveolar domains in close association with caveolin-1 and binds directly to the caveolin-1 in Hela cells and COS-1 cells (48). Given that GM3 is a substrate for this plasma membrane ganglioside-specific sialidase, the binding of GM3 to this membrane sialidase may compete with caveolin-1 for binding to the sialidase, thus releasing or promoting continued attachment with the caveolin-1 depending on GM3 content.

The role of gangliosides as shuttle molecules within the membrane to inhibit growth factor receptor phosphorylation has recently been suggested for ganglioside GM1. Similar to the effect of GM3 on EGFR function, GM1 inhibits phosphorylation (49) and dimerization (50) of the platelet-derived growth factor receptor. However, overexpression of GM1 by gene transfection with GM2/GD2 synthase and GM1 synthase cDNA’s in Swiss 3T3 cells shifts the platelet-derived growth factor receptor itself from the “glycosphingolipid-enriched domains” of the Triton X-100-insoluble region to the heavier, detergent-soluble “non-GEM” domains (51) without affecting the distribution of caveolin-1. In contrast, our studies show that GM3 overexpression shifts caveolin-1 into EGFR-rich domains, without affecting the expression or distribution of the EGFR itself.

Having shown that GM3 affects caveolin-1 distribution and association with the EGFR, we then evaluated the effect of modulation of ganglioside content on caveolin-1 tyrosine phosphorylation. Caveolin-1 phosphorylation is stimulated by EGF in A431 cells, but not in other cells (20). Our studies confirm the stimulation of tyrosine phosphorylation of caveolin-1 by EGF in SCC12 cells and implicate GM3 as a cofactor in the EGF-induced tyrosine phosphorylation of caveolin-1. Of importance, our data with ganglioside depletion strongly suggest that ganglioside is critical for EGF-stimulated caveolin phosphorylation in general, since ganglioside depletion eliminates EGF-stimulated caveolin-1 phosphorylation. The concordance of these results of ganglioside depletion with both sialidase overexpression and PPPP treatment eliminates the possibility that sialidase itself is an inhibitor of EGF-induced tyrosine phosphorylation of caveolin-1. Our data also implicate tyrosine phosphorylation of caveolin-1 as a means to strengthen the interaction of...
caveolin-1 with the EGFR and increase the suppressive effect of caveolin-1 on EGFR phosphorylation, given the suppressive effect on EGFR activation of caveolin-1 binding.

The role of serine phosphorylation of caveolin-1 in signaling is unknown. We found no evidence of serine phosphorylation of the caveolin-1 in association with the immunoprecipitated EGFR at any time, regardless of GM3 content. However, GM3 induced serine phosphorylation of caveolin-1 that is not associated with the EGFR as a late phenomenon after EGF stimulation, concurrent with downregulation of caveolin-1 tyrosine phosphorylation. The role of serine phosphorylation of caveolin-1 in EGFR signaling deserves further investigation.

In conclusion, we provided evidence for a role of tyrosine phosphorylated caveolin-1 as an inhibitor of EGFR signal transduction outside of caveolar domains, and have shown that membrane GM3 content regulates the localization of caveolin-1 relative to the EGFR, possibly facilitating the GM3-modulated caveolin-1 induced receptor inhibition. In addition, ganglioside appears to be a critical co-factor in allowing EGF-induced tyrosine phosphorylation of caveolin-1. Finally, the significant activation of serine phosphorylation of caveolin-1 that is dissociated from the EGFR by GM3 overexpression suggests a role for serine phosphorylation of caveolin-1 in modulating EGFR signaling.
GM3 modulates caveolin-1/EGFR interaction

References


Figure Legends

Figure 1. Endogenous modulation of ganglioside GM3 expression. Treatment with antisense oligodeoxynucleotides to both GM2/GD2 synthase and GD3 synthase prevented the synthesis of downstream ganglioside components GM2 and GD3, respectively, leading to accumulation of GM3 (A). Incubation of SCC12 cells with PPPP blocked the synthesis of GM3 from its precursor lactosylceramide, resulting in depletion of GM3 and downstream gangliosides (A). Overexpression of sialidase also depletes GM3 and other gangliosides by cleaving the critical sialic acid residue(s) (B).

Figure 2. GM3 accumulation by antisense treatment leads to GM3 clustering in both cytoplasm and on the membrane. SCC12 cells, maintained in DMEM/F12 with 10% FBS, were treated with antisense oligodeoxynucleotides of both GM2/GD2 synthase (synthesizes GM2 from GM3) and GD3 synthase (synthesizes GD3 from GM3) to cause endogenous accumulation of GM3. In other studies, SCC12 cells were stably transfected with human ganglioside-specific sialidase to deplete all SCC12 gangliosides, including GM3, as indicated in Materials and Methods. To evaluate ganglioside content, gangliosides were extracted and purified from $10^8$ cells (27), then applied to aluminum-backed TLC plates and separated in chloroform: methanol: water in 0.02% CaCl$_2$, 55:45:10, v/v/v. After migration, immunostaining was performed (A) with anti-GM3, -GM2, -GD2, -GT1b (Seikagaku Corp., Tokyo, Japan) and -GD3 (Sigma) antibodies as described (29). Lanes 2, 3 and 4 show SCC12 cells untreated (-), treated with sense oligomers (+S), and treated with antisense oligomers (+A). Lane 1 = Ganglioside standards. GM3 expression was also measured by ganglioside ELISA (B). $10^4$ cells were grown in 96-well plates in DMEM/F12 with 10% FBS for 6-8 h, fixed in 4% formaldehyde, and incubated with 10-30 µg/ml of anti-GM3, -GM1, -GM2, -GD2, -GT1b (Seikagaku Corp., Tokyo, Japan) or -GD3 (Sigma) antibodies for 1 h. Binding was detected with horseradish peroxidase labeled anti-mouse IgM and BM Blue POD substrate (Roche Molecular Biochemicals, Indianapolis, IN), and read at O.D. 450 nm in a UV$_{max}$
kinetic microplate reader. 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. The pattern of ganglioside expression as a function of GM3 content was examined by routine immunofluorescence using anti-GM3 antibody (Glycotech Corp., Rockville, MD) (C) and fluorescein-conjugated goat anti-mouse IgG. Nuclei in C-c were stained with propidium iodide. a, antisense-treated; b, untreated, c, SSIA cells without ganglioside. In parallel experiments, omission of primary antibody, or addition of purified IgG served as negative controls (not shown). Studies were performed at least 3 times in triplicate. Arrows in Fig. 2C-a indicate the clusters of GM3 on the membrane with accumulation of GM3; arrows in Fig. 2C-b show the homogenous distribution of GM3 of the SCC12 cell membrane without additional GM3.

**Figure 3.** Endogenous accumulation of GM3 inhibits both EGFR autophosphorylation and dimerization. The effect on overall EGFR autophosphorylation of endogenous accumulation of GM3 was determined by immunoblotting (30). Whole cells were treated with boiling lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4, 1.0 mM Na$_3$VO$_4$) for 10 min and centrifuged. Total protein (10 µg) from the postnuclear fraction was applied to an 8% SDS-PAGE mini-gel and immunoblotted with anti-EGFR antibody (Transduction Labs, Lexington KY) (A, top row). Blots were stripped and re-probed with anti-phospho-EGFR (Transduction Labs) (A, bottom row). To study EGFR dimerization (B), cells were treated with or without sense or antisense oligodeoxynucleotides. Cells at 90% confluence were starved of both serum and EGF for 18-24 h, stimulated with 10 nM EGF for 10 min, washed, and treated for 30 min at 4°C with 3.5 mM S-SMPB (optimal concentration based on previous studies, not shown), to stabilize dimerization after EGF stimulation. The reaction with S-SMPB was terminated by the addition of 60 mM glycine in 3 mM Tris-HCl (pH 8.5). EGFR dimer formation was assessed by immunoblotting. Lane 1, untreated; 2, sense-treated; and 3, antisense-treated SCC12 cells.
Figure 4. Endogenous accumulation of GM3 facilitates the interaction of both caveolin-1 and GM3 with EGFR. SCC12 cells were treated with antisense oligodeoxynucleotides to both GM2/GD2 synthase and GD3 synthase as indicated in Materials and Methods to endogenously accumulate GM3. Membrane EGFR was immunoprecipitated from whole cell lysates with anti-EGFR antibody (IgG2b against extracellular region amino acids 6-273). Aliquots of the immunoprecipitates were immunoblotted with anti-EGFR monoclonal antibody (top row) or anti-caveolin-1 antibody (Transduction Labs) (middle row). Gangliosides were extracted from the remaining immunoprecipitate and identified by TLC immunostaining (bottom row). 1, untreated; 2, sense-treated; 3, antisense-treated SCC12 cells.

Figure 5. Endogenous accumulation of GM3 shifts caveolin-1 from Triton X-100 insoluble membrane microdomains into soluble membrane to facilitate the interaction of caveolin-1 and GM3 with EGFR without affecting EGFR expression or distribution. SCC12 cells were treated with GM2/GD2 synthase and GD3 synthase antisense oligodeoxynucleotides to accumulate GM3, 2 μM PPPPP to inhibit ganglioside synthesis, or by stable transfected with ganglioside-specific membrane sialidase to deplete gangliosides as indicated in Materials and Methods. (A) Cells were incubated with 500 mM Na₂CO₃ (pH 11.0) at 4°C according to the technique of Song et al. (46), and separated by sucrose density gradient centrifugation. Equal amount of proteins from either soluble (high-density fragments) or insoluble (low-density fragments) fractions were applied to an 8% (EGFR, top row) or 12% (caveolin-1, middle row) SDS-PAGE mini-gel and immunoblotted. Gangliosides were extracted from 5 mg soluble or insoluble protein, and GM3 was detected by TLC immunostaining with anti-GM3 antibody (bottom row). Lanes 1, 5, untreated; 2, 6, sense-treated; 3, 7, antisense-treated; 4, 8, PPPPP-treated. To further evaluate the effect of GM3 content on the distribution of EGFR and caveolin-1 in detergent-soluble and insoluble membrane, cells were treated with 1% Triton X-100 in Tris-HCl (pH 7.4) lysis buffer at 4°C with interrupted homogenization and sonication, followed by sucrose density gradient centrifugation as previously described (12). EGFR (B and C, top rows) and caveolin-1 (B and C,
middle rows) were detected by immunoblotting, and GM3 (B and C, bottom rows) was isolated and detected as described for carbonate-treated membrane. Lanes 1-8 of B corresponds to Lanes 1-8 of A. Gels and TLC’s were performed 3-6 times. Immunoblots of pcDNA and SSIA6 are representative of those from two vector- and four sialidase-transfected SCC12 cell lines (C).

Figure 6. Endogenous accumulation of GM3 upregulates tyrosine phosphorylation of caveolin-1 that is associated with the EGFR to inhibit EGFR function. SCC12 cells pretreated with or without oligodeoxynucleotides (A) or stably transfected with or without sialidase cDNA (B) were stimulated without or with 10 nM EGF for 10, 30 and 60 min after starving of both serum and growth factors. EGFR was immunoprecipitated from whole cell lysates as indicated in Figure 4. To assess EGFR-related caveolin-1 and caveolin-1 phosphorylation, immunoprecipitated EGFR was run on an 8% SDS-PAGE mini-gel to check the purity and equal loading with anti-EGFR antibody (A, B, top rows) and autophosphorylation with anti-phosphotyrosine EGFR antibody (A, B, second rows). Aliquots were run on 12% SDS-PAGE mini-gels to detect the caveolin-1 co-immunoprecipitated with EGFR using anti-caveolin-1 antibody (A, B, third rows), caveolin-1 tyrosine phosphorylation (p-tyr-Caveolin-1) with PY20 antibody (A, B, fourth rows), and caveolin-1 serine phosphorylation (p-ser-Caveolin-1) with anti-phosphoserine antibody (A, B, bottom rows). Lane 1, antibody positive control (A, B); 2, 5, 8, 11, untreated (A, B); 3, 6, 9, 12, sense-treated (A), or pcDNA vector control (B); 4, 7, 10, 13, antisense- treated (A) or SSIA cells (B). Immunoblots were performed in triplicate and are representative of the different transfected cell lines.

Figure 7. Endogenous accumulation of GM3 upregulates delayed serine phosphorylation of caveolin-1 that is unassociated with EGFR. SCC12 cells pretreated with or without oligodeoxynucleotides (A) or PPPP (B) were stimulated without or with 10 nM EGF for 10, 30 and 60 min after starving of both serum and growth factors. After immunoprecipitation of EGFR, the remaining cell lysate was incubated with polyclonal anti-caveolin-1 antibody to
immunoprecipitate caveolin-1 unassociated with the EGFR as indicated in Materials and Methods. Purity and equal loading of caveolin-1 was checked with anti-caveolin-1 monoclonal antibody (A, B, top rows). Caveolin-1 tyrosine phosphorylation was detected with PY20 antibody (A, B, middle rows), and serine phosphorylation was detected with anti-phosphoserine antibody (A, B, bottom rows). The first lane of A and B shows a positive control for antibody binding. Lanes 2, 5, 8, 11 untreated (A, B); lanes 3, 6, 9, 12 sense-treated (A) or dimethylsulfoxide 0.1% vehicle treated control (B); lanes 4, 7, 10, 13 antisense-treated (A) or PPPP treated cells (B). Immunoblots were performed in triplicate.
A

Ceramide  
\[ \text{GlcCer} \]  
\[ \text{LacCer} \]  
\[ \text{GM3} \rightarrow \text{GM2} \]  
\[ \text{GD3 synthase antisense} \]

B

\[ \text{GM3: NANA} \alpha 2 \rightarrow 3 \text{ Gal} \beta 1 \rightarrow 4 \text{ Glc} \beta 1 \rightarrow \text{CER} \]
\[ \text{Sialidase} \]
\[ \text{Gal} \beta 1 \rightarrow 4 \text{ Glc} \beta 1 \rightarrow \text{CER} \]

Figs. 1A,B
Figs. 3A,B
Figs. 5A-C
IP: anti-caveolin-1 from lysates without EGFR

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-Caveolin-1
-p-tyr-Caveolin-1
-p-ser-Caveolin-1

Figs. 7A,B
Ganglioside induces caveolin-1 redistribution and interaction with the epidermal growth factor receptor
Xiao-Qi Wang, Ping Sun and Amy S. Paller

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