Suppression of Epidermal Growth Factor Receptor Signaling by Protein Kinase C-α Activation Requires CD82, Caveolin-1, and Ganglioside

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Abstract

Activation of protein kinase C (PKC)-α decreases normal and neoplastic cell proliferation by inhibiting epidermal growth factor receptor (EGFR)-related signaling. The molecular interactions upstream to PKC-α that influence its suppression of EGFR, however, are poorly understood. We have found that caveolin-1, tetraspanin CD82, and ganglioside GM3 enable the association of EGFR with PKC-α, ultimately leading to inhibition of EGFR signaling. GM3- and CD82-induced inhibition of EGFR signaling requires PKC-α translocation and serine/threonine phosphorylation, which eventually triggers EGFR Thr^{654} phosphorylation and receptor internalization. Within this ordered complex of signaling molecules, the ability of CD82 to associate with PKC-α requires the presence of caveolin-1, whereas the interaction of caveolin-1 or PKC-α with EGFR requires the presence of CD82 and ganglioside GM3. Disruption of the membrane with methyl-β-cyclodextrin dissociates the EGFR/GM3/caveolin-1/CD82/PKC-α complex and prevents the inhibitory effect of PKC-α on EGFR phosphorylation, suggesting that caveolin-1, CD82, and ganglioside interact with EGFR and PKC-α within intact cholesterol-enriched membrane microdomains. Given the role of these membrane molecules in suppressing EGFR signaling, up-regulation of GM3, caveolin-1, and CD82 function may be an effective adjunctive therapy for treating epithelial cell malignancies. [Cancer Res 2007;67(20):9986–95]

Introduction

Epidermal growth factor receptor (EGFR) signaling critically regulates cell proliferation and migration in normal and neoplastic cells. The demonstration that EGFR number and activity are increased in many malignancies has promoted the development as cancer therapy of anti-EGFR antibodies and small molecules that target EGFR activity. However, the mechanisms that regulate EGFR signaling at the membrane level are incompletely understood. Much attention has focused on ligand-induced EGFR signaling and EGFR transactivation, but little attention has been paid to negative feedback on EGFR signaling by protein kinase C (PKC)-α activation (1). Activated PKC-α translocates from the nucleus to the membrane, where it phosphorylates EGFR at its Thr^{654} site and down-regulates EGFR signaling in both normal and neoplastic cells (2). The membrane events that enable the PKC-α association with EGFR, however, remain unclear.

In vitro, both EGFR and PKC-α are able to bind to caveolin-1, a structural protein that localizes to lipid raft domains where signaling molecules cluster (3, 4). Raft domains are cholesterol-dependent sites for signal transduction, membrane trafficking, cholesterol homeostasis, and the regulation of cell survival and motility (5). The interaction of caveolin-1 with EGFR inhibits its tyrosine phosphorylation and kinase activity (3) and suppresses tumor cell proliferation and metastasis (6, 7).

Tetraspanin transmembrane adaptor proteins may also facilitate membrane associations. Most tetraspanin proteins (including CD82 and CD9) inhibit cell proliferation, migration, and invasiveness; high levels of expression correlate with a more differentiated histologic appearance, low metastatic potential, and good prognosis of several tumors (8). CD82 coimmunoprecipitates with EGFR in primary keratinocytes, small cell lung carcinoma cells, and HB2 mammary epithelial cells with increased CD82 (HB2/CD82 cells; ref. 9) and suppresses EGFR dimerization and EGFR tyrosine phosphorylation (10). The mechanism by which CD82 and EGFR associate to decrease EGFR dimerization is unknown. Similarly, the interaction of tetraspanins and PKC isoforms has received little attention. CD9 and CD82 both associate with conventional PKC isoforms and recruit them into membrane complexes with integrins (11). Activation of conventional PKC isoforms by phorbol ester increases the expression of CD82 (12) and also inhibits EGFR signaling in both normal and neoplastic cells. However, the mechanism by which CD82 activates PKC and the role of CD82 in PKC-regulated EGFR signaling have not been investigated.

Gangliosides, sialylated membrane glycosphingolipids, are primarily thought to localize to cholesterol-containing membrane domains (13). In these lipid raft domains, gangliosides are well positioned to influence ligand-induced signaling and cross-talk between signaling molecules (14–21). Ganglioside GM3, the predominant ganglioside of epithelial cells, has long been known to inhibit tyrosine phosphorylation of the EGFR (22). The inhibition involves (*a*) direct binding of GM3 to the EGFR, an interaction that requires glycosylation of the EGFR and sialylation of GM3 (19); (*b*) GM3-induced decrease in the availability of EGFR for binding to its ligands (18); (*c*) GM3-induced prevention of EGFR dimerization (20); and (*d*) GM3-induced suppression of cross-talk between EGFR and integrin (21). We have shown GM3 to coimmunoprecipitate with EGFR and caveolin-1 in epithelial cell membranes. Increases in the membrane content of GM3 shift caveolin-1 from the Triton X-100–insoluble caveolar domains to the higher density membrane

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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domains with EGFR, whereas depletion of GM3 retains caveolin-1 in caveolar domains, apart from EGFR (20).

Recent evidence also suggests a relationship between gangliosides and tetraspanins. GM3 facilitates the association of tetraspanin CD9 with integrin (14, 15) and enables CD9 and CD82 to inhibit laminin-332–dependent cell motility and induce apoptosis (16, 23). In bladder cancer cells, increased GM3 promotes integrin α_3 to complex with CD9 in a membrane microdomain that includes GM3, suppressing cell invasiveness (14). Although CD82 and GM3 both suppress EGFR signaling, their potential association in regulating EGFR signaling has not been explored.

In this study, we provide evidence that GM3, CD82, and caveolin-1 associate in an ordered manner at the membrane to regulate the communication of PKC-α with EGFR. Blocking the function of any of these key intermediate molecules abrogates the ability of PKC-α to inhibit EGFR signaling.

Materials and Methods

Cells. The human squamous carcinoma SCC12F2 cell line (SCC12; courtesy of Dr. James Rheinwald, Harvard University, Department of Dermatology, Brignam and Women's Hospital, Harvard Institutes of Medicine, Boston, MA) was maintained in DMEM/F12 (1:1, v/v; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum without antibiotics in 5% CO_2 at 37°C. For all studies, cells were starved of serum and growth factors overnight and treated with or without 10 nmol/L EGF for 10 min before analysis.

Ganglioside expression modulation. GM3 in SCC12 cells was increased by pharmacologic addition of purified GM3 (Sigma) or blocking the metabolism of GM3 to more complex gangliosides with antisense oligodeoxynucleotide (20). GM3 was decreased by treating cells with 2 μmol/L *threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidinopropan-1-ol HCl (PPPP; Matreya) for 5 days (20) or stable transfection of plasma membrane ganglioside-specific sialidase (17). PPPP prevents the formation of glucosylceramide (GM3 precursor) without accumulating ceramide (24, 25). GM3 expression after modulations was detected by ganglioside ELISA (20) and TLC immunostaining (19).

Immunoblotting. Immunoblotting was done as described previously (26). Cells were starved of serum and growth factors overnight and then treated with or without antibody directed against CD82 or GM3 for 0.5 to 1.5 h in the presence or absence of 500 nmol/L Go6976 (Calbiochem), a conventional PKC inhibitor (PKC-α is the only conventional PKCs in SCC12 cells). After stimulation with or without 10 nmol/L EGF (Sigma) for 10 min, immunoprecipitated protein or total protein isolated from whole-cell lysate was applied for immunoblotting with antibodies directed against EGFR, phosphotyrosine EGFR, and caveolin-1 (BD Biosciences); CD82, PKC-α, and phosphothreonine PKC-α at its 638 site (Santa Cruz Biotechnology, Inc.); or phosphothreonine EGFR at its 654 site (BioDesign).

Immunoprecipitation. Cells prepared as above were harvested and lysed in cold immunoprecipitation buffer and the EGFR, CD82, or PKC-α was immunoprecipitated with antibodies (see Immunoblotting; ref. 27).

PKC activity assay. After starvation of serum and growth factors, cells with increased or decreased GM3 content were treated with or without 500 nmol/L Go6976 and antibody directed against GM3, CD82, 9-*O*-acetyl GD3, or CD9 in serum-free medium for 30 min followed by stimulation with 10 nmol/L EGF for 10 min. After terminating EGF stimulation with cold PBS (pH 7.4), cells were lysed in cold lysis buffer (27) and PKC-α was immunoprecipitated from the whole-cell lysate. PKC kinase activity was assessed using immunoprecipitated PKC-α as described previously (28). The γ-^{32}P-labeled product from the PKC-α activity assay was measured in a Beckman LS 6000 liquid scintillation counter. Kinase activity assays were done in triplicate three times.

CD82 and caveolin-1 overexpression. The 823-bp coding domain of the full-length *CD82* gene (bp 249–1,052; accession number NM-002231) was amplified by reverse transcription-PCR from the human carcinoma cell line H1098. *CD82* coding domain cDNA was ligated into a pcDNA4/V5-His (Invitrogen) vector at the *Nhe*I/*Xho*I restriction site. Sequence analysis showed incorporation of the entire human *CD82* coding sequence. After transfection of *CD82* cDNA into SCC12 cells and selection with 40 μg/mL zeocin for 10 days, CD82 expression was detected by immunoblotting with anti-V5 antibody (Invitrogen) and anti-CD82 antibody. The human *caveolin-1* cDNA construct was a gift from Dr. Heidi Karpen (Baylor College of Medicine, Houston, TX).

CD82, CD9, caveolin-1, and PKC-α knockdown. Four potential small interfering RNA (siRNA) oligonucleotides within the coding sequence of *CD82* were designed with siRNA Target Finder software and commercially generated (Invitrogen). Oligonucleotides were initially transfected into the CD82-overexpressing SCC12 cells using LipofectAMINE 2000 (Invitrogen) to determine which most efficiently decreased CD82 expression. A scrambled oligonucleotide (5′-AACGGGCTGCAGGAATTCGAT-3′) was transfected in tandem as a control. Sequence 5′-AAGAGCAGTTTCATCTCTGTC-3′ (bp 360–380) provided the greatest decrease in CD82 expression; this oligonucleotide was cloned into the pSilencer4.1-CMVhygro vector (Ambion) to generate siRNA plasmid. CD9 siRNA was prepared as described previously (14). Sense and antisense oligomers, scrambled control sense and antisense oligomers, were inserted into the pSilencer4.1-CMVhygro vector as described for CD82 siRNA. Caveolin-1–specific sense and antisense siRNAs and their scrambled controls were prepared as described for CD82 siRNA using previously described oligonucleotides (29). siRNA for human PKC-α was a gift from Dr. Mitchell Denning (Loyola University, Maywood, IL; ref. 30).

The siRNA expression plasmids for caveolin-1, CD82, PKC-α, and controls were transfected into SCC12 cells using LipofectAMINE 2000 reagent. Two days after transfection, hygromycin (50 μg/mL; for CD82, caveolin-1, CD9, and their controls) or puromycin (10 μg/mL; for PKC-α and its control) was added for 10 days for selection. Expression of CD82, PKC-α, CD9, or caveolin-1 in the transfected cells was detected by immunoblotting.

Cholesterol depletion and repletion. Cholesterol was depleted by incubating cells with methyl-β-cyclodextrin (MβCD; 5%, w/v, in DMEM/F12; Sigma) for 30 min at 37°C (31). Cholesterol was repleted by adding 80 μg/mL of soluble cholesterol (Sigma) for 1 h at 37°C (31, 32). Cholesterol content was detected using an Amplex Red Cholesterol Assay kit (Invitrogen). Trypan blue staining was used to determine cell viability.

Flow cytometry to detect EGFR on the cell surface. Cells with modulated expression of ganglioside were trypsinized to form a single-cell suspension. Cells (1×10^6) in 98 μL of 2% PBS-bovine serum albumin were mixed with 2 μL of FITC-EGF (40 μg/mL; Molecular Probes) or FITC-IgG (1:64; Sigma) as an isotype control and incubated for 30 min on ice in the dark. Propidium iodide was added for the last 2 min to sort dead cells. Cells were washed and resuspended in 500 μL PBS for measurement in a Beckman Coulter Epics XL-MCL counter.

Cell surface biotinylation. Cell surface protein was biotinylated with freshly prepared NHS-ss-biotin (1.5 mg/mL) following the manufacturer's instruction (Pierce). EGFR was immunoprecipitated from total protein of the whole biotinylated cell lysate. Biotinylated EGFR was purified by incubation of immunoprecipitated EGFR with streptavidin beads (33). After separation from beads by incubation in Laemmli buffer for 5 min at 95°C, EGFR was loaded onto a 7.5% SDS-PAGE minigel for immunoblotting.

Immunofluorescence staining. To detect EGFR distribution, cells were plated onto glass slides and allowed to grow for 2 days. After cells were fixed in cold 4% paraformaldehyde for 10 min and permeabilized in 100% methanol for 5 min, cells were incubated with polyclonal anti-human EGFR antibody (Santa Cruz Biotechnology) overnight at 4°C. FITC-conjugated anti-rabbit IgG detected the distribution of EGFR with 10 μg/mL propidium iodide as a counterstain. To examine the effect of GM3 content on PKC-α translocation, SCC12 cells plated onto glass slides were treated with 10 μmol/L GM3 or its vehicle, DMSO, for 48 h (26). After starvation of serum and growth factors overnight, cells were stimulated with or without either 100 μmol/L 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 1 min (positive control) or 10 nmol/L EGF for 10 min. Stimulation was terminated before cells were fixed in cold acetone/methanol (1:1) for 10 min. After blocking

with 5% goat serum-PBS, cells were incubated with mouse anti-PKC-α antibody (Upstate) overnight at 4°C. Cells were then incubated with FITC-labeled goat anti-mouse antibody for 45 min and counterstained with 10 mmol/L 4′,6-diamidino-2-phenylindole (DAPI). Images were captured using the UV LSM 510 Meta confocal imaging system.

Results

GM3 facilitates the association of EGFR, caveolin-1, CD82, and PKC-α. To explore the effect of GM3 on the interaction of EGFR with other molecules, the membrane content of GM3 was manipulated. GM3 was increased by treating cells with 50 μmol/L purified GM3 (data not shown) or with antisense oligomers directed against both GD3 synthase (converts GM3 to GD3; ref. 34) and GM2/GD2 synthase (converts GM3 to more complex gangliosides GD2 and GT1b in SCC12 cells; refs. 35, 36) to block GM3 metabolism (Fig. 1*A* and *B*). Incubation of cells with PPPP, which prevents the synthesis of GM3, reduced GM3 content dramatically (Fig. 1*A* and *B*; refs. 24, 25). Neither treatment affected ceramide content (Fig. 1*B, top row*). When control cell lysates were incubated with anti-EGFR antibody, three strong bands of proteins coimmunoprecipitated with EGFR, as shown by separation on 10% SDS-PAGE gels and Coomassie blue staining (data not shown). Immunoblot analysis showed these bands to be caveolin-1 (at molecular weight ~21 kDa), tetraspanin CD82 (at molecular weight ~46 kDa), and PKC-α (at molecular weight ~82 kDa; Fig. 1*C*). The same group of proteins and GM3 were coimmunoprecipitated with CD82 when cell lysates were treated with anti-CD82 antibody (Fig. 1*D*), further suggesting a relationship among these molecules. Increasing the content of GM3 by antisense treatment increased the association of these molecules, regardless of whether immunoprecipitated with anti-EGFR antibody (Fig. 1*C, lane 5*) or anti-CD82 antibody (Fig. 1*D, lane 5*). In contrast, depletion of GM3 by biochemical treatment with PPPP (*lane 3*) or stable transfection of ganglioside-specific membrane sialidase (data not shown) eliminated the association of EGFR with caveolin-1, CD82, and PKC-α (Fig. 1*C*). Depletion of GM3 also prevented the association of EGFR and caveolin-1 with CD82 (Fig. 1*D*). Modulating GM3 expression did not affect the association of CD82 with PKC-α (Fig. 1*D, bottom row*).

CD82 is required for PKC-α or caveolin-1 to associate with the EGFR and inhibit its phosphorylation. To consider if CD82 is critical for the inhibitory effect of GM3 and PKC-α on EGFR signaling, the expression of CD82 was reduced by CD82 siRNA transfection. CD82 siRNA suppressed the translation of CD82 by at least 90% in comparison with its scrambled control (Fig. 2*A*). The reduction in CD82 by siRNA treatment dissociated both caveolin-1 (Fig. 2*B, middle row*) and PKC-α (*bottom row*) from EGFR. Knockdown of CD82 expression led to a marked increase in EGFR tyrosine phosphorylation (Fig. 2*C, second row*) and suppressed the PKC-α phosphorylation (*bottom row*) even in cells with increased GM3 content. Knockdown of CD82 did not significantly augment the high level of EGFR tyrosine phosphorylation or decrease PKC-α phosphorylation at Thr^{638} in cells without GM3 (*lane 6*). Disruption of CD82 did not affect the expression of either EGFR or PKC-α (Fig. 2*C, top* and *third rows*), and manipulation of GM3 content did not affect CD82 expression (Fig. 2*A*). Knockdown of CD9 expression, on the other hand, had no effect on either the association of EGFR with caveolin-1 and PKC-α or on EGFR phosphorylation (Supplementary Fig. S1). To further confirm the effects of CD82 and GM3, CD82 expression was increased by stable transfection of *CD82* cDNA into SCC12 cells, antisense-treated cells with increased GM3, and PPPP-treated cells with depleted GM3

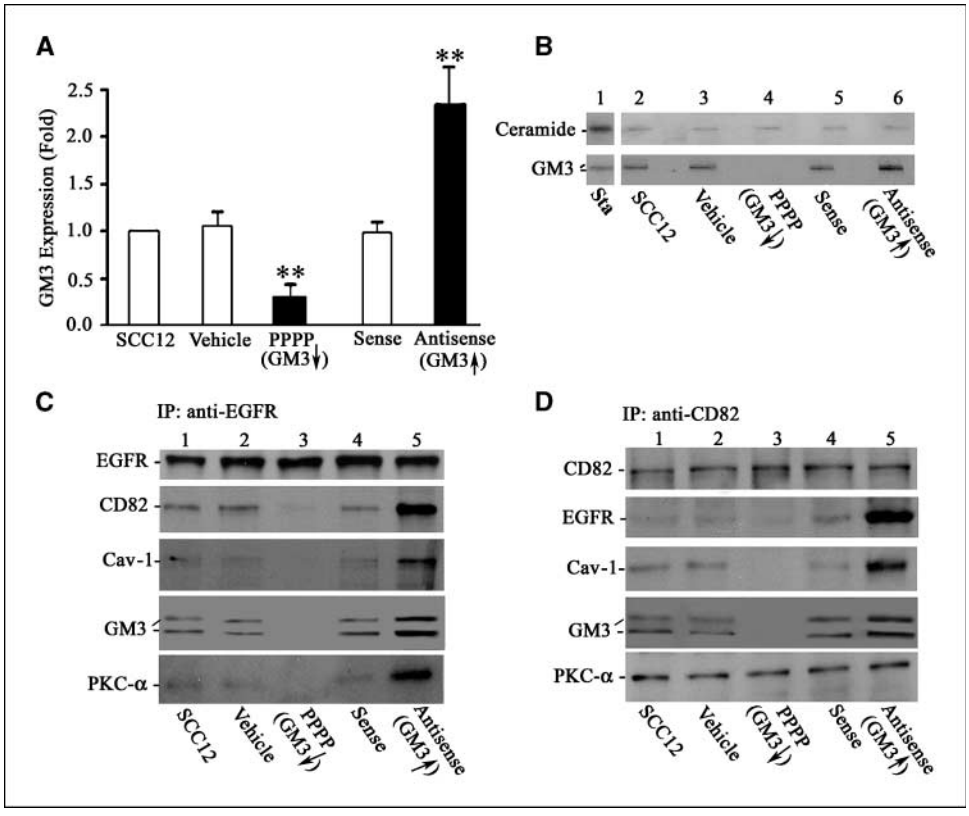

Figure 1. GM3 facilitates the association of EGFR, caveolin-1, CD82, and PKC-α. GM3 was depleted by treatment of cells with PPPP or increased by treatment of cells with antisense oligomers to both GM2/GD2 synthase and GD3 synthase (20). The expression of GM3 was confirmed by both ganglioside ELISA (*A*; **, $P < 0.01$; ref. 36) and TLC immunostaining (*B, bottom row*; ref. 19). *B, top row,* the content of ceramide, a precursor of GM3, was detected by TLC immunostaining. After starvation of serum and growth factor overnight, cells were stimulated with 10 nmol/L EGF for 10 min before cells were lysed. EGFR (*C*) and CD82 (*D*) were immunoprecipitated from the cell lysate with antibody directed against EGFR or CD82, respectively. The coimmunoprecipitated caveolin-1 (*Cav-1*; *third row*), PKC-α (*bottom row*), CD82 (*C, second row*), or EGFR (*D, second row*) was detected by immunoblotting an aliquot of the immunoprecipitate. The coimmunoprecipitated GM3 (*fourth row*) was detected by TLC immunostaining. *SCC12,* untreated cell control; *Vehicle,* DMSO-treated cells/control for PPPP; *PPPP,* GM3-depleted cells; *Sense,* cells treated with sense oligomers to both GM2/GD2 synthase and GD3 synthase, control for antisense-treated cells; *Antisense,* GM3-overexpressing cells.

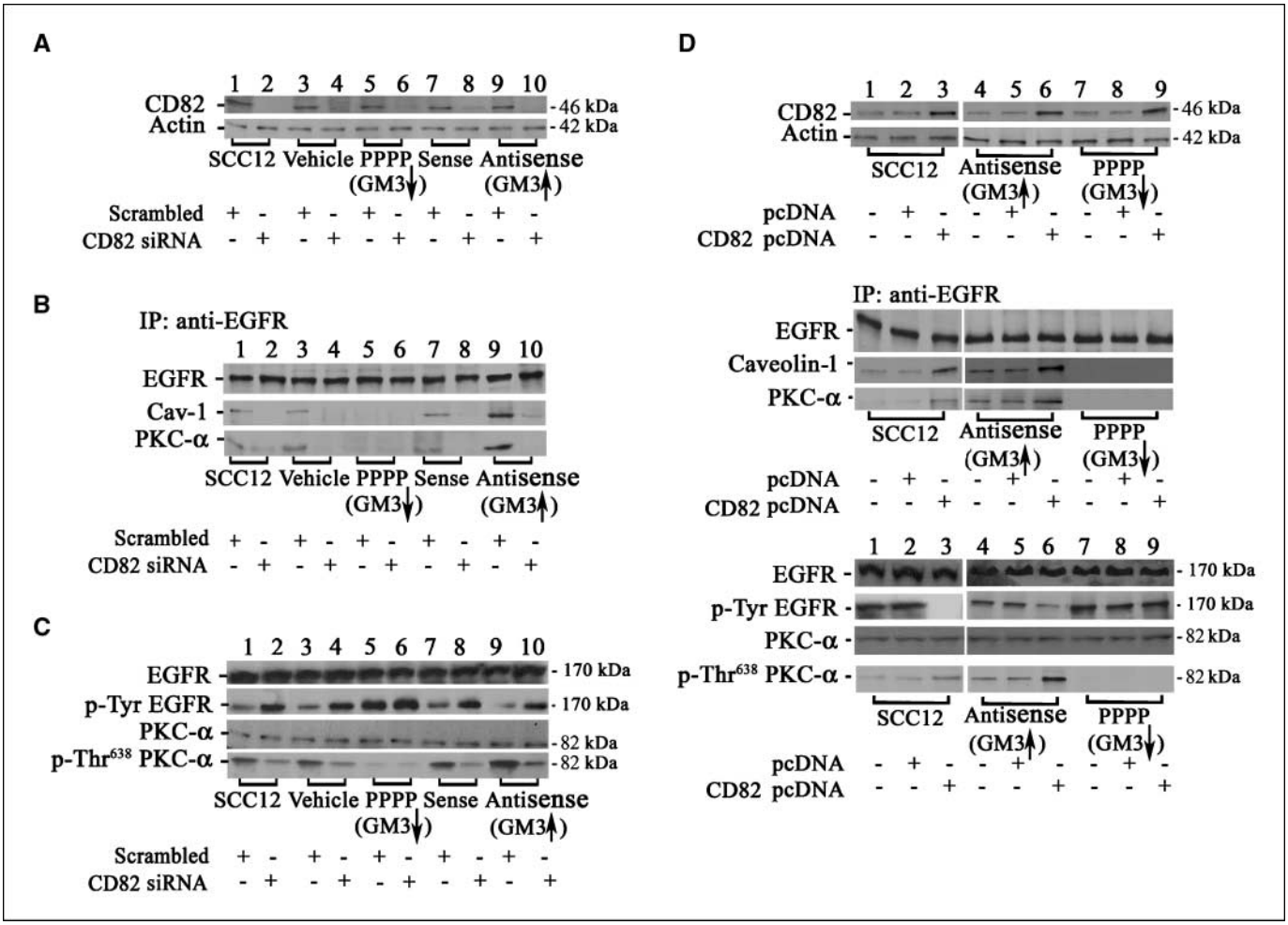

Figure 2. CD82 is required for PKC-α or caveolin-1 to associate with the EGFR and inhibit its phosphorylation. Cells were prepared as indicated in Fig. 1. The expression of CD82 was either disrupted by transfection of human CD82 siRNA or increased by stable transfection of human *CD82* cDNA. Cells were starved overnight of serum and EGF and stimulated with 10 nmol/L EGF for 10 min. *A* and *D, top row,* CD82 expression was detected by antibody directed against CD82 in total protein from the whole-cell lysates; *second row,* equal loading was confirmed by probing the same membrane with anti-actin antibody. To assess the association of EGFR with caveolin-1 and PKC-α, EGFR (*B, top row* and *D, third row*) was immunoprecipitated from cells with anti-EGFR antibody. The coimmunoprecipitated caveolin-1 (*B, second row* and *D, fourth row*) and PKC-α (*B, third row* and *D, fifth row*) were detected using an aliquot of immunoprecipitate. To detect EGFR and PKC-α phosphorylation (*C* and *D*), total protein from the whole-cell lysate was used. The expression (*C, top row* and *D, sixth row*) and tyrosine phosphorylation (*C, second row* and *D, seventh row*) of EGFR were detected by antibody directed against EGFR or tyrosine-phosphorylated EGFR, respectively. The expression (*C, third row* and *D, eight row*) and phosphorylation (*C* and *D, bottom rows*) of PKC-α were detected by anti-PKC-α antibody or anti-phosphorylated PKC-α antibody that specifically identifies the Thr^{638} phosphorylation site.

(Fig. 2*D, top row*). CD82 elevation (*lane 3*) enhanced the association of EGFR with caveolin-1 (*fourth row*) and PKC-α (*fifth row*), increased PKC-α phosphorylation at Thr^{638} (*bottom row*), and prevented EGFR tyrosine phosphorylation (*seventh row*). Accumulation of GM3 accentuated (*lane 6*) the effects on the association and signaling of CD82 overexpression. Importantly, depletion of ganglioside prevented both the association of EGFR with caveolin-1 and PKC-α and EGF-related activation of PKC-α by CD82 overexpression, arresting to the critical role of GM3.

Caveolin-1 and cholesterol-enriched membrane are critical for the inhibitory effect of PKC-α on EGFR. To address the importance of caveolin-1 in the interactions that allow PKC-α–induced inhibition of EGFR tyrosine phosphorylation, caveolin-1 expression was reduced by treatment with caveolin-1 siRNA (Fig. 3*A*) or antisense oligomers (Fig. 3*C, top row*). Decreased expression of caveolin-1 profoundly suppressed the association of both CD82 and PKC-α with EGFR (Fig. 3*B, a*) and EGFR and PKC-α with CD82 (Fig. 3*B, b*) even in the presence of increased GM3 (*lane 10*). In contrast, overexpression of caveolin-1 by stable transfection of *caveolin-1* cDNA (Fig. 3*A, lane 13*) increased these associations (Fig. 3*B, lane 13*). Disruption of caveolin-1 expression by siRNA (data not shown) or antisense oligomers enhanced EGFR tyrosine phosphorylation (Fig. 3*C, fourth row*) and suppressed PKC-α Thr^{638} phosphorylation (*bottom row*) even in the presence of increased GM3 (*lane 10*). Disruption of caveolin-1 expression did not affect PKC-α threonine phosphorylation or EGFR tyrosine phosphorylation in GM3-depleted cells. Caveolin-1 modulation likewise did not alter the expression of EGFR or PKC-α in whole-cell lysates (Fig. 3*C, third* and *fifth rows*).

MβCD treatment decreased cell cholesterol levels by 65% as detected fluorometrically without altering cell viability (98% viable by trypan blue staining; data not shown). Cholesterol depletion eliminated the association of EGFR with CD82, caveolin-1, and PKC-α even in the presence of increased GM3 (Fig. 3*D, lane 14*). Associations were restored by adding exogenous cholesterol to MβCD-treated cultures. Consistently, treatment with MβCD

eliminated the inhibition of EGFR tyrosine phosphorylation by GM3, and cholesterol supplementation restored this inhibition (data not shown).

The GM3- and CD82-induced activation of PKC-α leads to translocation of PKC-α and EGFR phosphorylation at Thr^{654}. Increases in GM3 level enhanced EGFR Thr^{654} phosphorylation (Fig. 4*A, second row, lane 3*) concomitant with increased PKC-α phosphorylation (*bottom row*) and decreased EGFR tyrosine phosphorylation (*third row*). Increases in GM3 also reduced EGFR kinase activity (data not shown). Our previous studies have found that treatment of cells with anti-GM3 or anti-CD82 antibody augments, rather than reverses, the inhibitory effects of GM3 and CD82, respectively, on EGFR signaling (data not shown). We further investigated the effects of these antibodies on EGFR signaling in cells with increased GM3. Treatment of cells with increased GM3 using anti-GM3 antibody further augmented EGFR phosphorylation at Thr^{654}, increased PKC-α phosphorylation, and decreased EGF-induced tyrosine phosphorylation of EGFR (Fig. 4*A, lane 4*). Antibody directed against 9-*O*-acetyl GD3, the second most prevalent ganglioside in SCC12 cells (27), did not further increase these effects (*lane 5*). Antibody directed against CD82 also accentuated the effects of increased GM3 on EGFR Thr^{654} and PKC-α phosphorylation to a similar extent as anti-GM3 antibody while inhibiting EGFR tyrosine phosphorylation (*lane 6*). Treatment of cells with anti-CD9 antibody did not affect the response to increased GM3 content (*lane 7*). Both anti-GM3 and anti-CD82 antibodies (but not control anti-9-*O*-acetyl GD3 and anti-CD9 antibodies) decreased activation of EGFR kinase as well (data not shown). The inability of antibodies directed against other SCC12 gangliosides or CD9 to alter PKC-α phosphorylation and EGFR Thr^{654} phosphorylation minimizes the possibility that effects result from nonspecific rearrangement of surface molecules by bivalent antibody.

GM3 depletion by either stable transfection of plasma membrane ganglioside-specific sialidase (data not shown; ref. 17) or treatment of cells with PPPP (Fig. 4*A, lane 10*) decreased phosphorylation of EGFR at Thr^{654} (*second row*), eliminated PKC-α phosphorylation (*bottom row*), and increased EGFR tyrosine phosphorylation

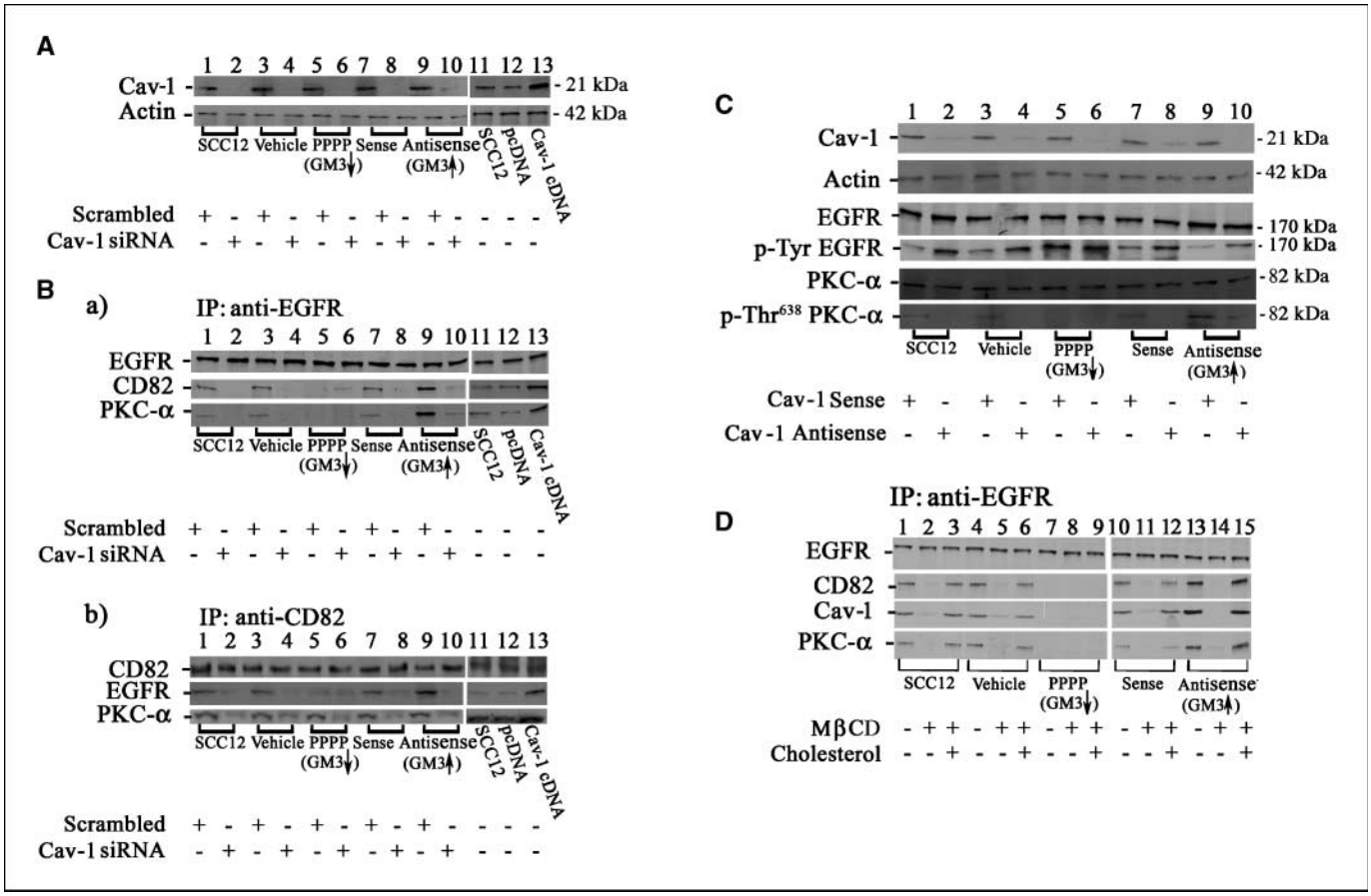

Figure 3. Caveolin-1 and cholesterol-enriched membrane are critical for the effect of PKC-α on EGFR. Cells were prepared as indicated in Fig. 1. The expression of caveolin-1 was disrupted by either stable transfection with human caveolin-1 siRNA (*A* and *B*) or transfection of caveolin-1 antisense oligomers (*C*) or was increased by stable transfection of *caveolin-1* cDNA (*A* and *B*) into SCC12 cells with or without modified ganglioside expression. Cells were starved overnight of serum and EGF and stimulated with 10 nmol/L EGF for 10 min before protein extraction. *A* and *C, top rows,* caveolin-1 expression was detected by antibody directed against caveolin-1 using total protein from the whole-cell lysates. *A, bottom row* and *C, second row,* equal loading was confirmed by probing the same membrane with anti-actin antibody. For assessing the association of these molecules, EGFR (*B, a, top row*) and CD82 (*B, b, top row*) were immunoprecipitated from cell lysate with anti-EGFR and anti-CD82 antibodies, respectively. The coimmunoprecipitated CD82 (*B, a, second row*), EGFR (*B, b, second row*), and PKC-α (*B, a* and *B, b, bottom rows*) were detected using an aliquot of the immunoprecipitate. The expression (*C, third row*) and tyrosine phosphorylation (*fourth row*) of EGFR were detected in whole-cell lysates by antibodies directed against EGFR and phosphotyrosine EGFR. The expression (*C, fifth row*) and phosphorylation of PKC-α (*bottom row*) were detected by either an anti-PKC-α antibody or anti-phosphorylated PKC-α antibody that specifically recognizes Thr^{638} phosphorylation. *D,* cholesterol was depleted by treating cells with 5 mmol/L MβCD for 30 min and repleted as described in Materials and Methods. Immunoprecipitation and immunoblotting were done to detect the association of these molecules in control cells, cells depleted of GM3, and cells with increased GM3 content.

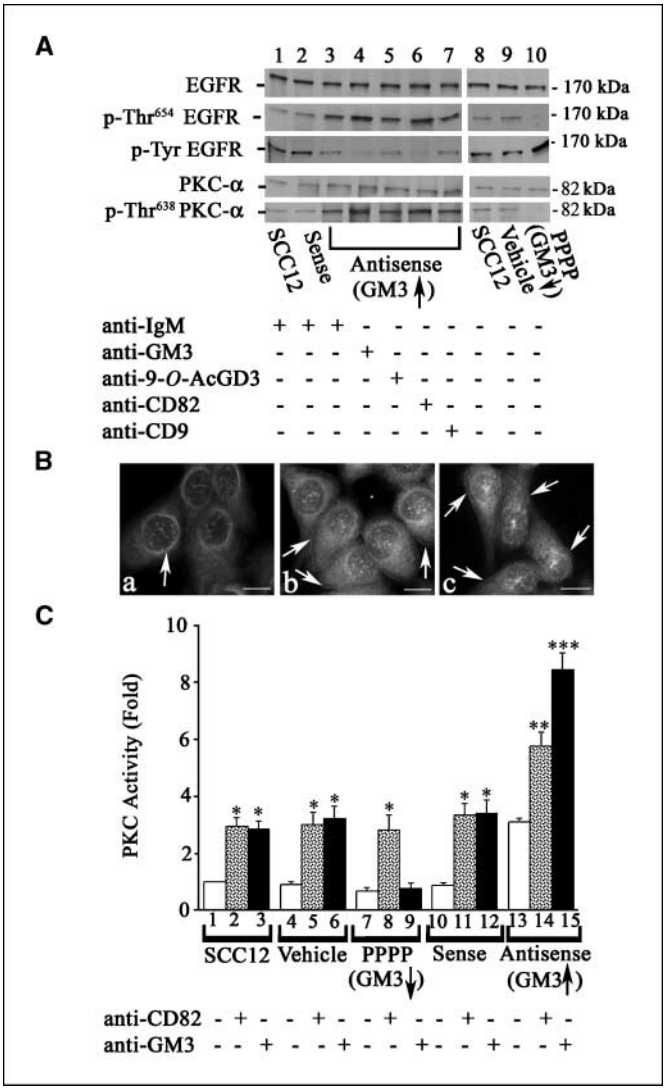

Figure 4. The GM3- and CD82-induced activation of PKC-α leads to translocation of PKC-α and EGFR phosphorylation at Thr^{654}. *A,* cells were prepared as indicated in Fig. 1 and, at 75% confluence, starved of both serum and EGF overnight. Cells were treated with EGF after incubation with or without 10 μg/mL antibody directed against GM3, 9-*O*-acetyl GD3 (*anti-9-O-AcGD3*), CD82, CD9, IgM (control for anti-GM3 and anti-9-*O*-acetyl GD3 antibodies), or IgG (control for anti-CD82 and anti-CD9 antibodies). For immunoblotting to detect EGFR and PKC-α expression and phosphorylation, total protein from the whole-cell lysate was probed with antibodies directed against EGFR (*top row*), phosphorylated EGFR at its Thr^{654} site (*second row*), phosphotyrosine EGFR (*third row*), PKC-α (*fourth row*), and phosphorylated PKC-α at its Thr^{638} site (*bottom row*) as described in Materials and Methods. Results with anti-IgM antibody-treated cells resembled those of anti-IgG antibody-treated cells (data not shown). *B,* cells treated with GM3 or DMSO for 48 h were starved overnight and then stimulated with EGF or TPA as indicated in Materials and Methods. PKC-α localization was detected with anti-PKC-α antibody and FITC-conjugated secondary antibody. Cells were counterstained with DAPI. *a,* SCC12 cells + EGF alone; *b,* SCC cells + GM3 + EGF; *c,* SCC12 cells + TPA (positive control). *Arrows,* PKC-α at the nuclear membrane (*a*) and PKC-α translocation to the cell membrane (*b* and *c*). *Bar*, 35 μm. *C,* in the presence of [γ-^{32}P]ATP, PKC-α activity was detected using immunoprecipitated PKC-α to incubate with its specific substrate as described in Materials and Methods. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(*third row*). No manipulation affected the total expression of EGFR (*top row*) or PKC-α (*fourth row*), suggesting that GM3 and CD82 have no effect on protein synthesis or stability.

GM3-induced translocation of PKC-α from the nucleus to the membrane was examined by immunofluorescence confocal microscopy (Fig. 4*B*). In contrast to negative controls (untreated cells; data not shown), cells treated with GM3 but without EGF (data not shown), and cells treated with EGF alone (Fig. 4*B, a*), cells treated with both GM3 and EGF showed clear translocation of PKC-α from the nucleus to the cytosol and membrane (Fig. 4*B, b*), equivalent to that of TPA-treated positive controls (Fig. 4*B, c*). Increases in GM3 and treatment with anti-GM3 or anti-CD82 antibody also led to EGF-induced activation of PKC-α kinase (Fig. 4*C*). Depletion of GM3 with PPPP reduced PKC-α activation (*lane 7*; $P < 0.05$) but not in the presence of anti-CD82 antibody (*lane 8*).

The expression and activation of PKC-α are required for GM3-induced inhibition of EGFR tyrosine phosphorylation and suppression of cell cycling. Inhibition of PKC-α activity (Fig. 5*A*) and PKC-α phosphorylation at Thr^{638} (Supplementary Fig. S2) by treating cells with Go6976 reversed the inhibitory effect of GM3 on EGF-induced cell cycle progression (Fig. 5*B*), suppressed phosphorylation of EGFR at Thr^{654} (Fig. 5*C, second row, lane 9*), and prevented the inhibition of EGFR tyrosine phosphorylation (*bottom row*) by GM3 even in cells with increased GM3 content and treated with anti-GM3 antibody (*lane 10*). Disruption of PKC-α expression by PKC-α siRNA similarly prevented suppression of EGFR signaling by GM3 in cells with increased GM3 (Fig. 5*D, lane 2*) even in cells treated with anti-GM3 antibody (*lane 4*). Anti-GM3 antibody treatment did not affect EGFR expression (Fig. 5*C* and *D, top rows*).

Increases in GM3 trigger EGFR internalization. Flow studies with FITC-conjugated EGF showed that supplementation of cells with GM3 (which approximately doubles membrane content; ref. 37) reduced EGFR content on the cell surface in comparison with untreated control cells, cells with decreased GM3, and cells treated with other gangliosides, including GT1b (Fig. 6*A*). Inhibition of PKC-α activity with Go6976 reversed the internalization of EGFR induced by increased GM3 content (Fig. 6*B*). Consistently, GM3 elevation decreased the content of biotinylated EGFR, particularly when cells were treated with anti-GM3 (*lane 4*) or anti-CD82 (*lane 6*) antibody but not with anti-9-*O*-acetyl GD3 or anti-CD9 antibody (Fig. 6*C*), reflecting the decreased cell surface EGFR. PPPP treatment to deplete GM3 increased the amount of biotinylated EGFR (*lane 10*). EGFR internalization was also prevented by treatment of cells with a PKC-α inhibitor, Go6976 (Fig. 6*C, lane 14*). Finally, to further assure that the reduced content of surface EGFR seen with flow and biotinylation studies reflected EGFR internalization, EGFR localization was detected by immunofluorescence imaging. Confocal analysis confirmed that GM3 elevation triggered EGFR internalization (Fig. 6*D, c* versus *a*). EGFR internalization induced by GM3 did not trigger receptor degradation (Fig. 6*C, top row*).

Discussion

Previous studies by our laboratory and others have established that PKC-α phosphorylates and down-regulates EGFR (1), GM3 induces the association of caveolin-1 with EGFR and down-regulates its signaling (20), and CD82 coimmunoprecipitates with EGFR to suppress its activation (9, 10, 38). We now show that all of these components associate in an ordered fashion: CD82 associates with EGFR and allows caveolin-1 to associate with EGFR; caveolin-1 enables PKC-α to associate with CD82 and therefore with EGFR; and all of these associations are potentiated by ganglioside GM3, which enables PKC-α translocation to the membrane and is required for PKC-α and CD82 to associate with EGFR.

The result of this cascade of associations is negative regulation of EGFR tyrosine kinase phosphorylation. The phosphorylation cascade that allows activation of PKC-α to inhibit EGFR autophosphorylation is partially understood. For its catalytic activity and translocation, PKC-α is initially phosphorylated at its Thr^{497} site in the activation loop. PKC-α is then autophosphorylated at Thr^{638} and Ser^{657} sites, which promotes maturation and stability of activated PKC-α (39). Ultimately, activated PKC-α phosphorylates EGFR at Thr^{654}, leading to EGFR internalization and down-regulation of EGFR signaling (2, 40–42). The demonstration that GM3 and CD82 promote PKC-α translocation, Thr^{638} phosphorylation, and kinase activation supports a role for GM3 and CD82 as key participants in the membrane events that precede PKC-α activation. We have previously shown that GM3 suppresses EGFR tyrosine phosphorylation at Tyr^{845}, Tyr^{1068}, and Tyr^{1148} (21); consistent with signaling through PKC-α, we now show that GM3 and CD82 enhance EGFR phosphorylation at its Thr^{654} site. Our early work showed that GM3 has no effect on EGFR expression or phosphatase activity. The new observation that GM3 leads to internalization of EGFR not only supports PKC-α activation as an intermediate step but also explains the previous demonstration of decreased availability of EGFR to its ligand in SCC12 cells with increased GM3 (18). PKC-α activation is classically accompanied by increased intercellular levels of calcium and diacylglycerol, and PKC-α is the only Ca^{2+}-dependent isoform of PKC in SCC12 cells. In preliminary studies, we have found that GM3 is unable to activate PKC-α, inhibit EGFR tyrosine phosphorylation, or decrease SCC12 cell growth in the presence of BAPTA/AM (43), a chelator of intracellular calcium (Supplementary Fig. S3), suggesting that the classic Ca^{2+}-dependent pathway of PKC-α activation is triggered by GM3.

The formation of a complex of EGFR-GM3-CD82-caveolin-1-PKC-α further supports the role of distinct "glycosynaptic domains" that include ganglioside, tetraspanin, growth factor receptors, and integrins in regulating signaling. Todeschini et al. (44) have recently shown the association of GM2, but not GM3, with CD82, Met (the receptor for hepatocyte growth factor), and integrin $\alpha_3\beta_1$ in bladder epithelial cells as a regulator of cell adhesion and motility. In SCC12 cells, the association of EGFR, GM3, CD82, and PKC-α seems to be specific. Blotting with antibodies directed against the other four PKC isoforms in SCC12 cells (PKC-δ, PKC-η, PKC-ε, and PKC-ζ; ref. 25) revealed that no other SCC12 cell PKC isoform

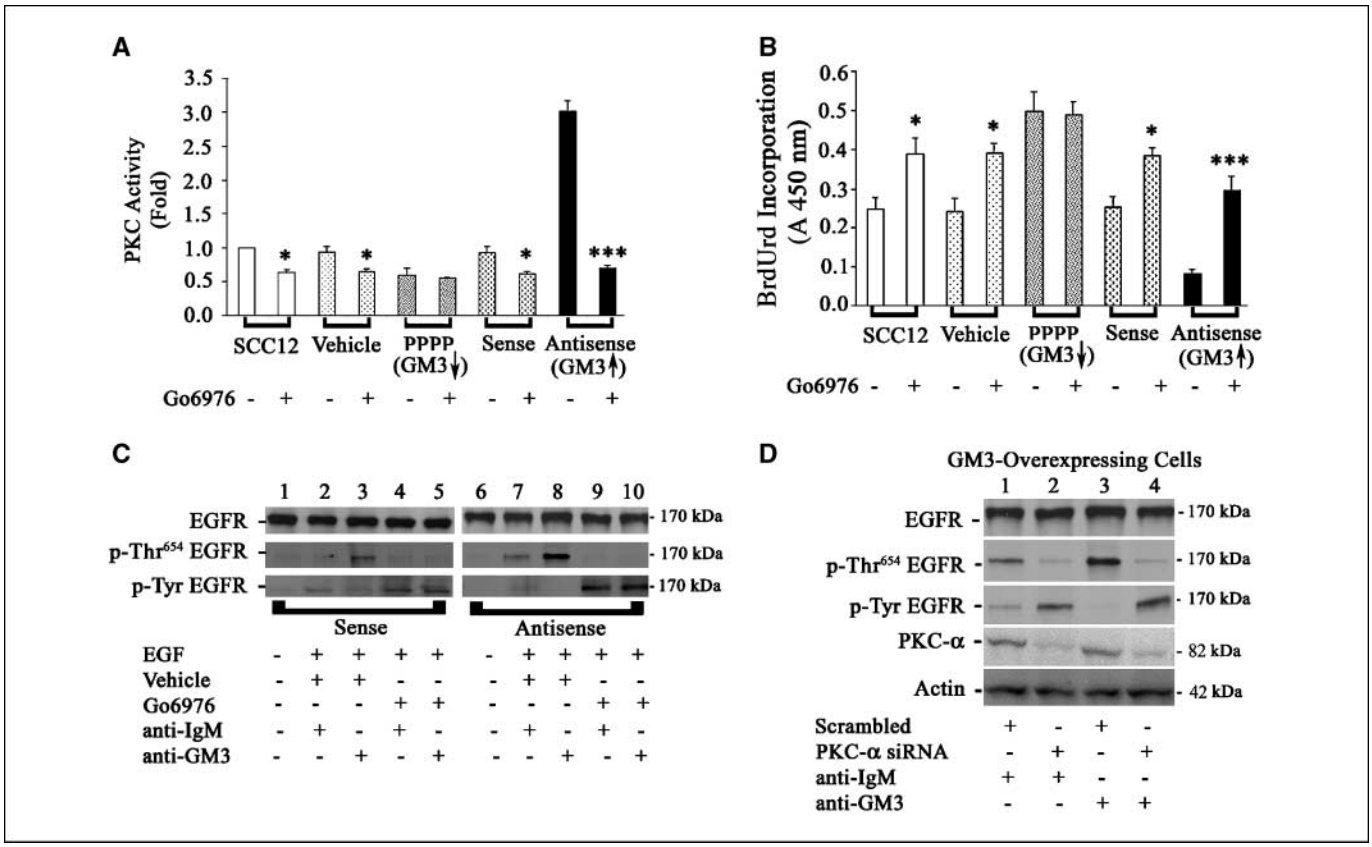

Figure 5. The expression and activation of PKC-α are required for GM3-induced inhibition of EGFR tyrosine phosphorylation and suppression of cell cycling. Cells prepared as indicated in Fig. 4 were treated with or without 500 nmol/L Go6976, which inhibits the activity of PKC-α, the only conventional PKC in SCC12 cells. *A,* PKC-α activity was measured as described in Fig. 4. *, $P < 0.05$; ***, $P < 0.001$. *B,* the effect of PKC-α activity inhibition on cell cycling was assessed by bromodeoxyuridine (*BrdUrd*) incorporation as per the manufacturer's instruction (Roche). *, $P < 0.05$; ***, $P < 0.001$. After being starved of serum and EGF overnight, cells were treated with or without 10 μg/mL anti-GM3 antibody in the presence or absence of PKC-α inhibitor (Go6976) as described in Materials and Methods. The whole-cell lysate was collected, and immunoblotting was done to detect EGFR expression (*C, top row*), phosphorylation at the Thr^{654} site (*second row*), and tyrosine sites (*bottom row*). *Vehicle,* DMSO-treated cells as a control for Go6976; *anti-IgM,* anti-IgM antibody-treated cells as a control for anti-GM3 antibody treatment. Cells with elevated GM3 were stably transfected with PKC-α siRNA or its scrambled control. *D, fourth row,* the expression of PKC-α was detected by immunoblotting with anti-PKC-α antibody; *bottom row,* equal loading was confirmed by immunoblotting with anti-actin antibody. The effect of PKC-α modulation on EGFR expression (*top row*) and EGFR phosphorylation at the Thr^{654} site (*second row*) and tyrosine sites (*third row*) was analyzed by immunoblotting with anti-EGFR, anti-phosphorylated Thr^{654} EGFR, and anti-phosphotyrosine EGFR antibodies.

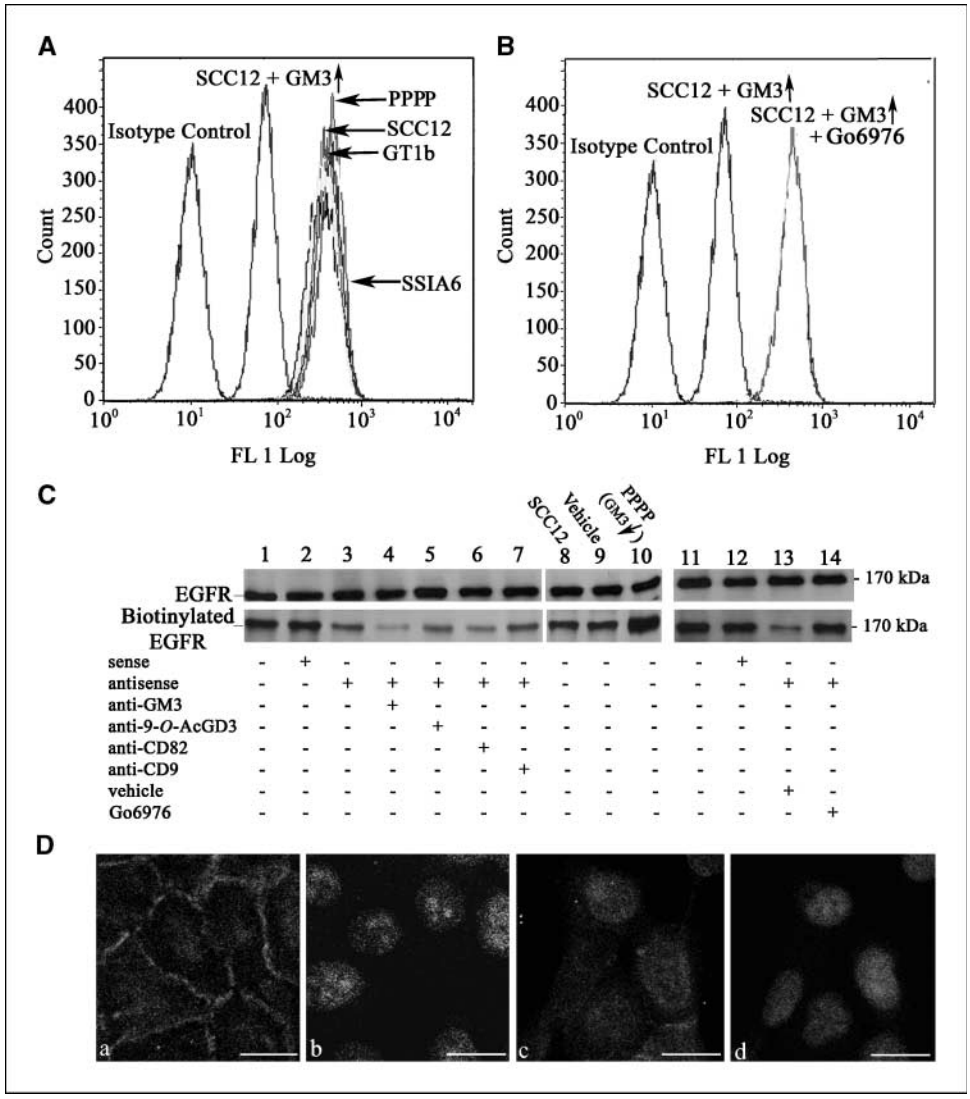

Figure 6. Increases in GM3 trigger EGFR internalization but not degradation. *A,* SCC12 cells were stably transfected with a human plasma membrane ganglioside-specific sialidase (*SSIA*) to functionally deplete gangliosides or treated with or without 50 μmol/L purified GM3 (to increase GM3), 1 μmol/L purified GT1b (to increase GT1b, as a control; ref. 27), or 2 μmol/L PPPP (to inhibit the GM3 synthesis). After starvation of serum and EGF overnight, flow cytometry assay was done to detect the cell surface EGFR as described in Materials and Methods. *B,* SCC12 cells with increased GM3 were also treated with or without 500 nmol/L Go6976 as described in Materials and Methods to block PKC-α activation. The content of cell surface EGFR was detected by flow cytometry using FITC-EGF as described in Materials and Methods. *C,* surface protein in cells prepared as indicated in Fig. 4 was biotinylated and the biotinylated EGFR at the cell surface was detected as described in Materials and Methods. *D,* untreated control cells (*a* and *b*) and cells with increased GM3 (*c* and *d*) were plated onto glass slides and allowed to grow for 2 d. The distribution of EGFR (*a* and *c*) was detected by immunofluorescence staining and confocal microscopy as described in Materials and Methods. *b* and *d,* nuclei are outlined by propidium iodide. *Bar*, 35 μm.

coimmunoprecipitates with either EGFR or CD82 (data not shown). Similarly, there is no evidence that other tetraspanin molecules participate. Tetraspanin CD9 has previously been shown to associate with both GM3 (14, 23) and PKC-α (11), and interactions between CD9 and CD82 have been described. However, previous coimmunoprecipitation studies have linked GM3 with CD9 in association with $\alpha_3\beta_1$ and tetraspanin CD81 (45). We found no CD9 in the EGFR-PKC-α complex by immunoblotting with anti-CD9 antibody (Supplementary Fig. S4), and treatment of SCC12 cells with anti-CD9 antibody or CD9 siRNA had no effect on GM3-induced PKC-α activation or suppression of EGFR signaling. EGFR Tyr^{845}, a site phosphorylated by depletion of GM3 (21), is a target for Src, and Src is regulated by PKC; however, we have not found Src in the EGFR-PKC complex, eliminating its role as an intermediary.

Despite these molecular specificities, the ganglioside in the complex seems to be cell specific. CD82 overexpression in HB2 cells suppresses EGFR phosphorylation, but these cells express no GM3 (9). Instead, the HB2/CD82 cells show increased ganglioside GD1a in comparison with parental HB2 cells, and depletion of GD1a in HB2/CD2 cells weakens (but does not eliminate) the association of EGFR with CD82 (38).

The spatial configuration and precise sites of potential interaction of these molecules within the membrane deserve further investigation. Caveolin-1 seems to play a central role in the EGFR-PKC-α interaction. The scaffolding region of caveolin-1 has a motif that binds *in vitro* with EGFR (3), suggesting that caveolin-1 can bind directly to EGFR. However, caveolin-1 does not coimmunoprecipitate with EGFR when GM3 is eliminated by either PPPP treatment or stable transfection with ganglioside-specific membrane sialidase (Neu3; ref. 20). Similarly, knockdown of CD82 prevents coimmunoprecipitation of caveolin-1 with EGFR, although this is partially overcome by increasing GM3 content. These observations suggest that GM3 and CD82 are both required for the caveolin-1/EGFR association and that EGFR is not always able to bind to caveolin-1 *in vivo*. In addition to the GM3-induced membrane shift in caveolin-1 that apposes caveolin-1 and EGFR, it is possible that GM3 and CD82 may stabilize the association of caveolin-1 with EGFR or induce a conformational change in EGFR so that its binding region is accessible to the scaffolding domain of caveolin-1. Suppression of caveolin-1 expression not only eliminates the association of CD82/PKC-α with EGFR but also markedly reduces the association of CD82 with PKC-α itself even when GM3 is present. Consistently, overexpression of caveolin-1 increases both

the association of EGFR with CD82 and the amount of PKC-α that coimmunoprecipitates with CD82. Treatment with caveolin-1 antisense oligomers not only stimulates EGFR autophosphorylation but also reduces Thr^{638} phosphorylation/activation of PKC-α. It is possible that CD82 does not bind directly to PKC-α but rather that caveolin-1 serves as a linker molecule through its known ability to bind PKC-α via the scaffolding domain of caveolin-1 (46).

More PKC-α than EGFR coimmunoprecipitates with CD82 in parental and control SCC12 cells (Fig. 1). In contrast to the effects of caveolin-1 manipulation, the extent of coimmunoprecipitation of PKC-α with CD82 does not change when cells are depleted of GM3 by PPPP treatment or when cells accumulate GM3 by antisense treatment. Thus, the association of CD82 with PKC-α is independent of GM3 and likely regulates effects in addition to suppression of EGFR signaling, such as integrin signaling. Surprisingly, anti-CD82 antibody is able to increase PKC-α activation in PPPP-treated cells, although this activation does not phosphorylate EGFR Thr^{654} or PKC-α Thr^{638} or decrease EGFR tyrosine phosphorylation in these GM3-depleted cells.[4] We hypothesize that antibody may stabilize activated PKC-α without PKC-α Thr^{638} phosphorylation, although without functional consequence on EGFR (i.e., EGFR remains activated) because EGFR and PKC-α are dissociated.

Raft disruption by MβCD has previously been shown to increase EGFR tyrosine phosphorylation in a variety of cells other than SCC12 cells, among them COS-1 cells, normal keratinocytes, and HaCaT cells. Given that GM3 mobilizes caveolin-1 from caveolar domains to EGFR-containing noncaveolar domains (20), we disrupted the cholesterol-enriched lipid raft domains from which most caveolin-1 is mobilized. Our studies suggest that cholesterol-enriched membrane domains are critical to the association of EGFR with PKC-α. Recent studies of tetraspanin-integrin and tetraspanin-phosphatidylinositol-4-kinase associations support the presence of a "tetraspanin-enriched membrane" (TEM) domain as a site of molecular interactions (8). These TEM domains are usually resistant to cholesterol depletion (47, 48) and soluble in Triton X-100 (49). The cholesterol requirement for the association of CD82 with EGFR and PKC-α thus differs from the shown resistance to cholesterol depletion of tetraspanin interactions with other molecules, such as integrins. Previous biochemical investigations with sucrose density gradients have suggested that both the EGFR/caveolin-1/GM3 interaction (20) and CD82 (50) localize outside of caveolar domains in lipid-enriched areas. Future studies should address the membrane site of interactions of these molecules with activated PKC-α.

In summary, we propose that GM3 and CD82 act with caveolin-1 as membrane organizers that control the interactions between the EGFR and PKC-α in normal and neoplastic epithelial cells. GM3 enables the CD82/EGFR interaction, whereas caveolin-1 plays a role in both stabilizing the CD82/PKC-α association and mobilizing PKC-α to interact with EGFR. Given the participation of these membrane molecules in suppressing EGFR signaling, up-regulation of GM3, caveolin-1, and/or CD82 function may prove to be an effective adjunctive therapy for treating epithelial cell malignancies.

[4] X. Wang and A.S. Paller, unpublished data.

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