Protein Kinase Cζ Mediates Epidermal Growth Factor–Induced Growth of Head and Neck Tumor Cells by Regulating Mitogen-Activated Protein Kinase

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Abstract

Protein kinase C (PKC) ζ has been implicated as a mediator of epidermal growth factor (EGF) receptor (EGFR) signaling in certain cell types. Because EGFR is ubiquitously expressed in squamous cell carcinomas of the head and neck (SCCHN) and plays a key role in tumor progression, we determined whether PKCζ is required for tumor cell proliferation and viability. Examination of total and phosphorylated PKCζ expression in normal oral mucosa, dysplasia, and carcinoma as well as SCCHN tumor cell lines revealed a significant increase in activated PKCζ expression from normal to malignant tissue. PKCζ activity is required for EGF-induced extracellular signal-regulated kinase (ERK) activation in both normal human adult epidermal keratinocytes and five of seven SCCHN cell lines. SCCHN cells express constitutively activated EGFR family receptors, and inhibition of either EGFR or mitogen-activated protein kinase (MAPK) activity suppressed DNA synthesis. Consistent with this observation, inhibition of PKCζ using either kinase-dead PKCζ mutant or peptide inhibitor suppressed autocrine and EGF-induced DNA synthesis. Finally, PKCζ inhibition enhanced the effects of both MAPK/ERK kinase (U0126) and broad spectrum PKC inhibitor (chelerythrine chloride) and decreased cell proliferation in SCCHN cell lines. The results indicate that (*a*) PKCζ is associated with SCCHN progression, (*b*) PKCζ mediates EGF-stimulated MAPK activation in keratinocytes and SCCHN cell lines, (*c*) PKCζ mediates EGFR and MAPK-dependent proliferation in SCCHN cell lines; and (*d*) PKCζ inhibitors function additively with other inhibitors that target similar or complementary signaling pathways. (Cancer Res 2006; 66(12): 6296-303)

Introduction

The epidermal growth factor (EGF) receptor (EGFR), which is nearly universally expressed in squamous cell carcinomas of the head and neck (SCCHN), has been associated with cancer cell growth, survival, and metastasis. In nonmalignant tissues, the EGFR signaling pathway has been shown to require specific protein kinase C (PKC) isoforms for effective signaling (1–3). The PKC family of serine-threonine kinases consists of 10 members that are classified by their requirements for activation (4). Classic PKCs (α, β, and γ) require both Ca^{2+} and diacylglycerol (DAG); novel PKCs (δ, ε, η, and θ) are Ca^{2+} independent but still require DAG; and atypical PKCs (ζ and ι/λ) are Ca^{2+}, DAG, and phorbol ester independent.

Relatively, little is known about PKC expression, function, and effects of inhibition in SCCHN. Previously, we have shown, in a neuronal model, that PKCζ is necessary for EGF-induced mitogen-activated protein kinase (MAPK) activation, whereas PKCδ is required for basic fibroblast growth factor stimulation (2). However, the role of PKCζ or other isoforms in SCCHN signaling has not been determined.

Therefore, we undertook the current study to characterize PKCζ expression, activation, and function in normal and malignant head and neck tissues. We show that PKCζ is highly expressed in head and neck tumors, and inhibition of PKCζ reduces MAPK activation in normal human adult epidermal keratinocytes (NHEK) and five of seven head and neck tumor cell lines. Furthermore, SCCHN cell proliferation and viability is reduced by inhibition of PKCζ. Finally, PKCζ inhibition potentiates the action of other growth inhibitors in SCCHN. The findings of this study thus implicate PKCζ as a relevant target in SCCHN and suggest that PKCζ inhibition is a viable therapeutic strategy.

Materials and Methods

Cell lines and reagents. SQ20B, SCC61, SCC25, and JSQ3 cell lines were provided by Dr. Ralph Weichselbaum (University of Chicago, Chicago, IL). HN5 cells were provided by the Ludwig Institute for Cancer Research (London, United Kingdom). CCL 138 cells were purchased from the American Type Culture Collection (Manassas, VA). MSK 921 cells were provided by Dr. David Raben (University of Colorado Health Sciences Center, Aurora, CO). NHEK were purchased from Cambrex Corp. (East Rutherford, NJ). All cell lines were maintained as described previously (5–7). Myristoylated PKC pseudosubstrate (Myr-PS), bisindolylmaleimide 1 (BIM), chelerythrine chloride, and U0126 were purchased from EMD Biosciences (San Diego, CA). TAT-conjugated pseudosubstrate (TAT-PS; GRKKRRQ-RRRPPSIYRRGARRWRKL) and TAT-conjugated scrambled (TAT-Scr; GRKKRRQRRRPPRLYRKRIWRSAGR) peptides (8) were purchased from Tufts University Core Facility (Boston, MA). ZD1839 was provided by AstraZeneca Pharmaceuticals (Alderley Park, Cheshire, United Kingdom). Phosphorylated specific extracellular signal-regulated kinase (ERK) antibody (Thr^{202}/Tyr^{204} ERK 1/2), all phosphorylated specific PKC antibodies, and total ERK antibody were purchased from Cell Signaling (Beverly, MA). Total PKC isoform, α-tubulin, actin, and anti-mouse horseradish peroxidase (HRP)–conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human epidermal growth factor was obtained from Biomedical Technologies (Stoughton, MA). Recombinant PKC proteins were purchased from Invitrogen Corp. (Long Island, New York). Recombinant λ phosphatase and phosphatase

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

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reaction solution were obtained from New England Biolabs (Beverly, MA). The expression vector for kinase-dead PKCζ (KD-PKCζ) was generated as described previously (2).

Immunohistochemistry. Immunohistochemistry for PKCζ and phosphorylated PKCζ/λ were done on custom human tissue microarrays. For PKCζ detection, deparaffinized sections were microwaved in citrate buffer, rabbit primary antibody (Santa Cruz Biotechnology) was applied at a dilution of 1:100 for 1 hour at room temperature, and the rabbit EnVision+ kit (DAKO, Carpinteria, CA) was used for detection. Phosphorylated PKCζ/λ was detected using a rabbit primary antibody (Cell Signaling) and HRP-conjugated secondary antibody to rabbit (Envision+, DAKO). All sections were counterstained with hematoxylin. All specimens were scored on a 1+ to 4+ scale.

Western blotting. Cells were treated and analyzed as described previously (2). All PKC isoform antibodies were used at 1:1,000 dilutions except the anti-PKCθ antibody (1:500). All phosphorylated specific PKC antibodies were used at dilutions of 1:500 except the phosphorylated PKCζ antibody (1:1,000). Recombinant PKC proteins were activated for use as positive controls as described previously (9).

Receptor tyrosine kinase immunoblotting. Proteome profiler human phosphorylated receptor tyrosine kinase (RTK) array that assays multiple phosphotyrosine receptors was purchased from the manufacturer (R&D Systems, Minneapolis, MN) and developed as instructed.

DNA plasmid electroporation. After trypsinization, SCC61 and SQ20B cells were counted and resuspended in cell line transfection buffer (Amaxa, Inc., Gaithersburg, MD) at 20 million/mL, and program U030 was utilized.

Construction of recombinant lentiviral vector containing the KD-PKCζ gene. DNA encoding COOH-terminal hemagglutinin (HA)–tagged PKCζ-K281R was obtained from Jae-Won Soh (Columbia University, New York, NY). pCDH expression lentivectors were purchased from System Biosciences (Mountain View, CA). pCDH-PKCζ-K281R was generated from the HA-tagged PKCζ-K281R as template by PCR using the 5′ forward primer (5′-AGCTCTAGAGCCACCATGCCCAGCAGGACCGGC-3′) and 3′ reverse primer (5′-GCGGAATTCCGCTCAGGCGTAGTCAGGCACGTC-3′). The resulting PCR product was digested with *Xba*I and *Eco*RI and cloned into pCDH-MCS1-EF1-copGFP.

Transduction with lentiviral vectors. Lentivirus was produced from 293T cells by transient cotransfection of either 8.5 μg pCDH-MCS1-EF1-copGFP empty vector or 8.5 μg pCDH-MCS1-EF1-copGFP-PKCζ-K281R with 4 μg pVSV-G and 6.4 μg pCMVΔR8.2 (from Naldini and Trono; Salk Institute, La Jolla, CA) to make pseudoviral particle. TransIT-LT1 reagent (Mirus Bio, Madison, WI) was used to transfect 293T cells. SCC61 cells were transduced according to the manufacturer's directions.

Bromodeoxyuridine proliferation assay. A bromodeoxyuridine (BrdUrd) proliferation assay kit (EMD Biosciences) was used for all experiments. Cells plated in quadruplicate in 96-well plates at 1×10^3 to 2×10^3 per well were starved and incubated with respective reagents for 24 hours. During the last 6 hours of treatment, 20 μL BrdUrd label was added to each well at a 1:2,000 dilution. After cell fixation and incubation with antibodies, HRP substrate plates were read using the Synergy HT Multidetection microplate reader (Bio-Tek, Winooski, VT) at dual wavelengths of 450 to 540 nm.

(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. A (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (Roche Diagnostics Corp., Indianapolis, IN) was used according to the manufacturer's instructions. Cells plated in quadruplicate in 96-well plates at 1 to 2×10^3 per well were starved and incubated with reagents. Plates were read using the Synergy HT Multidetection microplate reader (Bio-Tek) at dual wavelengths at 600 nm.

Results

Expression of PKCζ in normal, dysplastic, and malignant oral epithelium. Initially, we examined the expression of total PKCζ in normal human oral mucosa, dysplastic oral mucosa, and head and neck tumor biopsies by immunohistochemistry. The same samples were also stained with a phosphorylated specific antibody for the PKCζ activation loop at Thr^{410} (10, 11). Analysis of staining intensity reveals that the expression of both total and activated PKCζ increased significantly from normal to malignant tissue ($P < 0.0001$, Cuzick's trend test; Fig. 1*A*). In addition, only samples from malignant tumors showed membranous staining of phosphorylated PKCζ, implicating its involvement in receptor signaling (Fig. 1*B*). These results indicate that activated PKCζ is associated with malignant progression of SCCHN.

Expression of PKC isoforms in human keratinocytes and SCCHN cell lines. The high PKCζ expression in malignant tissue specimens prompted the characterization of PKCζ relative to other PKC isoforms in normal and malignant cells. A panel of PKC-specific antibodies was used to detect total and phosphorylated PKC isoform expression in NHEK. Expression of α, δ, ε, θ, and ζ (Fig. 1*C* and *D*) as well as phosphorylation of most of these isoforms (α, δ, θ, and ζ; Fig. 1*D* and *E, lane K*) was observed. NHEK cells expressed the phosphorylated forms of PKCθ and PKCδ but not PKCε. These results reveal that most of the expressed PKC isoforms, with the exception of PKCε, are phosphorylated and potentially activated in growing NHEK cells.

To determine the relative importance of PKC isoforms as mediators of head and neck tumor growth, we analyzed their expression in four radioresistant, EGFR-overexpressing SCCHN cell lines (SQ20B, SCC61, SCC25, and JSQ3; ref. 5) by immunoblotting with anti-PKC antibodies. Similar to NHEK cells, the PKC isoforms α, δ, ε, θ, and ζ are expressed at varying levels, whereas isoforms β1, β2, γ, and η do not seem to be expressed in these cell lines (Fig. 1*C* and *E, lanes SQ, 61, 25*, and *JS*). In addition, isoforms α, ε, and ζ are phosphorylated at activation sites to some degree in these lines (Fig. 1*D* and *E*). Interestingly, it seems that PKCζ is constitutively phosphorylated at Thr^{410} in the head and neck cell lines (Fig. 1*E, left*), as incubation with EGF did not alter phosphorylation. Treatment with phosphatase confirmed the phosphorylated specificity of the antibody, resulting in a loss of anti-phosphorylated PKCζ immunoreactivity (Fig. 1*E, right*). These results indicate that only a subset of PKC isoforms, including PKCζ, are expressed and potentially activated in both keratinocytes and head and neck tumor cells.

Inhibition of PKCζ blocks ERK activation by EGF in NHEK and SCCHN cells. To determine whether PKCζ regulates EGF-stimulated MAPK (ERK1/2) activation in keratinocytes, NHEK cells were starved and exposed to EGF in the presence or absence of specific PKCζ inhibitors Myr-PS or TAT-PS. Incubation with either PKCζ inhibitor abrogated EGF-stimulated ERK1/2 phosphorylation (Fig. 2*A*). These results indicate that activation of ERK1/2 by EGF in normal human keratinocytes is dependent on PKCζ kinase activity.

Because the majority of SCCHN cells overexpress the EGF receptor, we determined whether inhibition of PKCζ influences MAPK signaling downstream of the EGF receptor in SCCHN cell lines. Specific PKCζ inhibitors, Myr-PS or TAT-PS, were incubated with starved or EGF-stimulated SCCHN cells. In five of the cell lines (SCC61, SQ20B, HN5, CCL-138, and MSK-921), the inhibition of PKCζ effectively reduced ERK activation as in NHEK cells (Fig. 2*B* and *C*). In contrast, two cell lines (SCC25 and JSQ3) were resistant to inhibition of MAPK by Myr-PS (Fig. 2*D*), suggesting that they have developed alternative pathways for MAPK activation by EGF. These results indicate that the majority of head and neck tumor cells retain their dependence on PKCζ for MAPK activation by EGF.

Previous studies have shown that regulation of the Raf/MAPK cascade by specific PKC isoforms is dependent on the cell type and

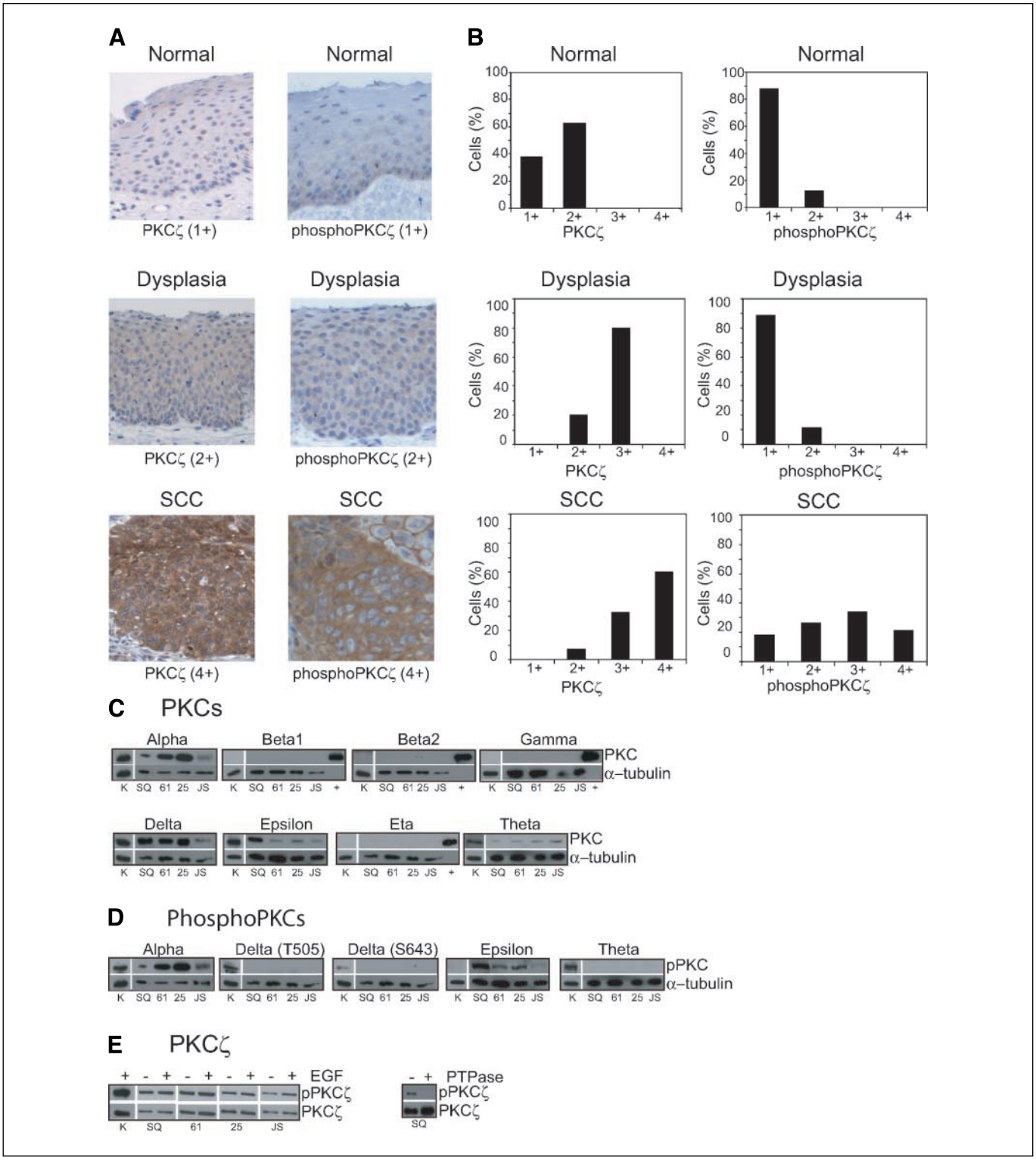

Figure 1. PKC expression in normal, dysplastic, and malignant SCCHN tissues and cell lines. *A,* tissue samples immunostained with either anti-PKCζ or anti-phosphorylated PKCζ antibodies. Staining intensity is expressed on a four-point scale from 1+ to 4+ (see Materials and Methods). Representative staining intensities (1+, 2+, and 4+) from the different tissues. *B,* relative distribution for PKCζ and phosphorylated PKCζ expression from a low of 1+ to a high of 4+ in normal, dysplastic, and malignant SCCHN tissues. $P < 0.0001$, Cuzick's trend test. *C,* expression of classic and novel PKC isoforms. Normal human epidermal keratinocytes (*K*), SQ20B (*SQ*), SCC61 (*61*), SCC25 (*25*), and JSQ3 (*JS*) tumor cells were analyzed for PKC expression. +, recombinant PKC proteins used as positive controls. Immunoblotting with PKC isoform-specific and anti-α-tubulin antibodies was done as described in Materials and Methods. *D,* expression of phosphorylated novel and classic PKC isoforms. Phosphorylated PKCδ was assessed using two phosphorylated specific antibodies directed against Thr^{505} or Ser^{643}. *E,* PKCζ expression in NHEK and SCCHN cell lines. *Left,* lysates from SCCHN cells in either serum-free conditions (−) or stimulated with 100 ng/mL EGF (+) were analyzed by immunoblotting with anti-PKCζ or anti-phosphorylated PKCζ antibodies; *right,* SQ20B cell lysates were incubated with or without phosphatase (*PTPase*) before immunoblotting with anti-phosphorylated PKCζ antibody. Representative of at least three independent experiments.

growth factor stimulus (2, 12). To determine whether other PKC isoforms mediate EGF-induced ERK1/2 phosphorylation, four of the cancer cell lines (SCC61, SQ20B, JSQ3, and SCC25) and NHEK cells were stimulated with EGF and incubated with an inhibitor of both classic and novel PKC isoforms, BIM (Fig. 3*B* and *D*; data not shown). Inhibition of either classic or novel PKC isoforms failed to block ERK activation in any of the cell lines following EGF stimulation, although BIM was effective at inhibiting phosphorylation of the classic and novel PKC substrate, myristolated alanine-rich C kinase substrate (data not shown). Taken together, these results show that inhibition of PKCζ, but not other PKC isoforms, blocks EGF-stimulated MAPK activation in normal keratinocytes, and the majority of head and neck cell lines were evaluated.

EGFR and MAPK are required for DNA synthesis in SCCHN cell lines. Because PKCζ regulates EGF signaling and EGF receptors are highly expressed in SCCHN, we investigated the role

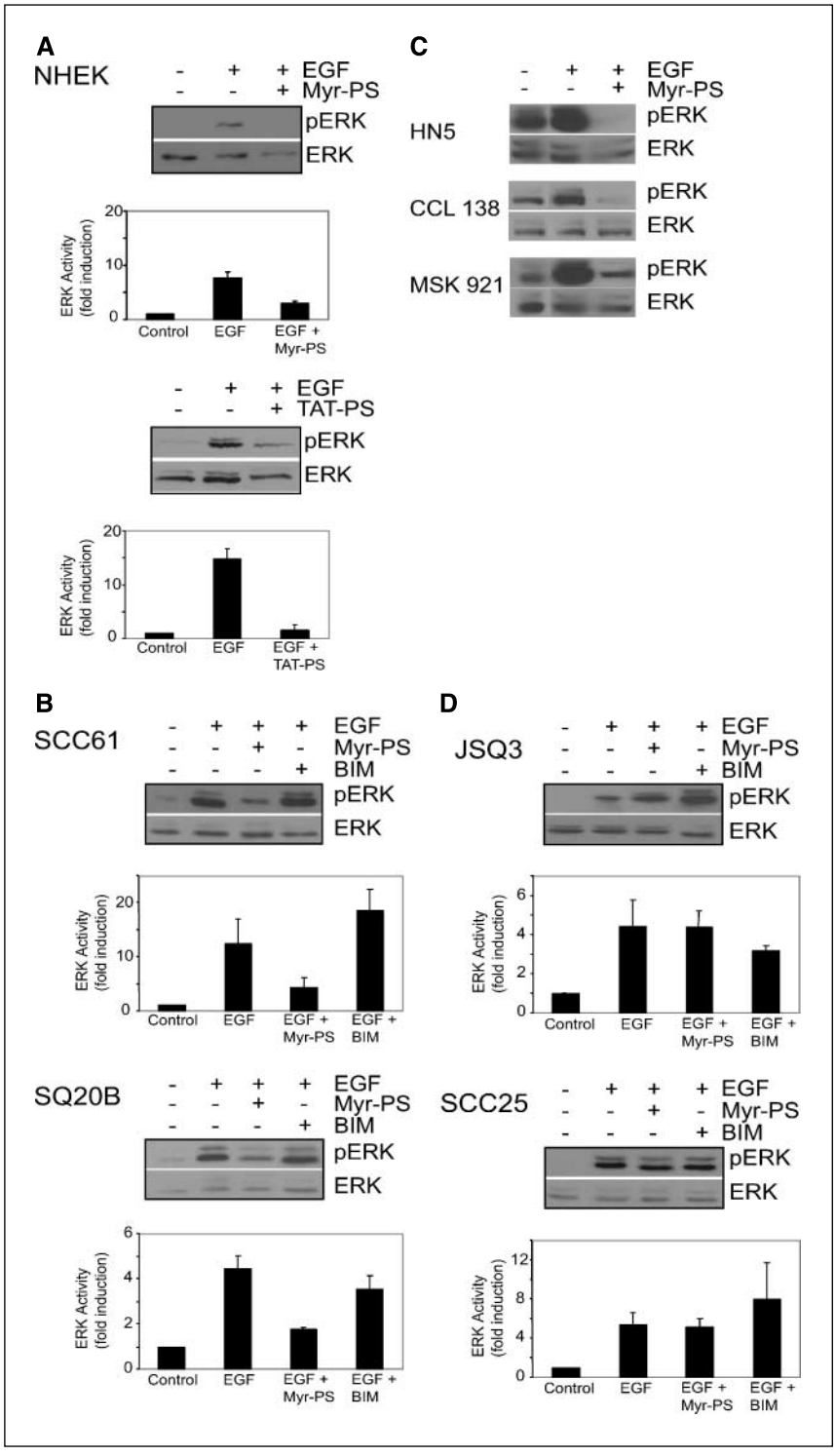

Figure 2. Inhibition of PKCζ decreases ERK activation in keratinocytes and five of seven squamous cell carcinoma cell lines. *A,* suppression of ERK activation by PKCζ inhibitors in NHEK cells. NHEK cells were preincubated with 15 μmol/L Myr-PS or TAT-PS before preincubation in either serum-free (control) medium or 100 ng/mL EGF as described in Materials and Methods. Lysates were analyzed by immunoblotting with anti-phosphorylated ERK and anti-ERK antibodies. *Columns,* mean of three samples; *bars,* SE. ERK activity represents the ratio of pERK/ERK intensity on immunoblots. Representative of three independent experiments. *B,* suppression of ERK activation by PKCζ inhibitors in SCC61 and SQ20B cells. Cells were pretreated with either Myr-PS (15 μmol/L) or BIM (200 nmol/L) and then incubated in serum-free (control) or 100 ng/mL EGF. *Columns,* mean of three samples; *bars,* SE. ERK activity represents the ratio of pERK/ERK intensity on immunoblots. Representative of three independent experiments. *C,* suppression of ERK activation by PKCζ inhibitors in HN5, CCL 138, and MSK 921 cells. Cells were pretreated with Myr-PS (15 μmol/L) and then incubated in serum-free (control) or 100 ng/mL EGF. Representative of two independent experiments. *D,* lack of suppression of ERK activation by PKCζ inhibitors in SCC25 and JSQ3 cells. Cells were pretreated with either Myr-PS (15 μmol/L) or BIM (200 nmol/L) and then incubated in serum-free (control) or 100 ng/mL EGF. Representative of three independent experiments. *Columns,* mean of three samples; *bars,* SE. ERK activity represents the ratio of pERK/ERK intensity on immunoblots.

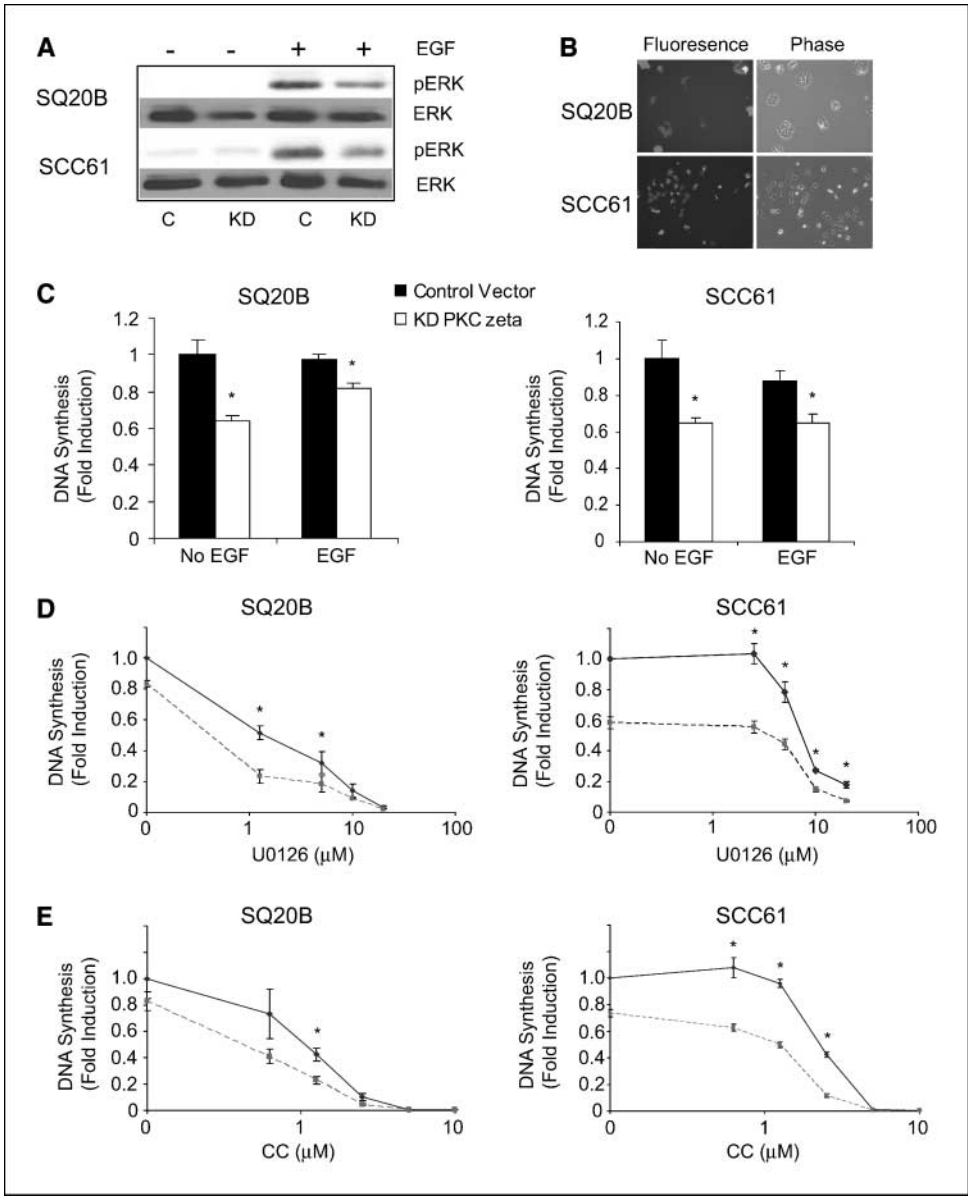

Figure 3. Transfection of SCCHN cells with KD-PKCζ inhibits ERK activation and DNA synthesis and potentiates suppression of DNA synthesis by MEK and PKC inhibitors. *A,* suppression of EGF-induced ERK activation by KD-PKCζ in SQ20B and SCC61 cells. Cells were transfected with KD-PKCζ and then incubated in serum-free (control) or 100 ng/mL EGF. Samples were resolved by SDS-PAGE and immunoblotting with anti-phosphorylated ERK and anti-ERK antibodies. *B,* transfection efficiency of KD-PKCζ expression in SQ20B (62%) and SCC61 (54%) cells. Cells were cotransfected with expression vectors for GFP and KD-PKCζ and examined 4 days later for GFP expression by fluorescence microscopy and cell number by phase microscopy. *C,* suppression of DNA synthesis by KD-PKCζ in SQ20B and SCC61 cells. Cells were transfected with KD-PKCζ or control vector and then incubated in serum-free medium (*No EGF*) or 100 ng/mL EGF. BrdUrd incorporation assays were done as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SE. *D,* inhibition of DNA synthesis by U0126 in SQ20B or SCC61 cells transfected with either KD-PKCζ (*dashed line*) or control (*solid line*) vector. Cells were transfected with KD-PKCζ or control vector and then incubated in 100 ng/mL EGF. BrdUrd incorporation assays were done as described in Materials and Methods. Representative of three independent experiments. *Columns,* mean of quadruplicate samples; *bars,* SE. *E,* inhibition of DNA synthesis by chelerythrine chloride (*CC*) in SQ20B or SCC61 cells transfected with either KD-PKCζ (*dashed line*) or control (*solid line*) vector. Cells were transfected with KD-PKCζ or control vector and then incubated in 100 ng/mL EGF. BrdUrd incorporation assays were done as described in Materials and Methods. Representative of three independent experiments. *Columns,* mean of quadruplicate samples; *bars,* SE. *, $P < 0.05$.

of activated EGF receptors in tumor cell DNA synthesis. Analysis of 80 RTKs by immunoblotting for tyrosine phosphorylation revealed that the EGFR (ErbB1) is constitutively activated in the absence of serum in all cells tested (Supplementary Fig. S1A; data not shown). We also observed that other members of the EGFR family were activated, such as ErbB3, in some of the tumor cell lines. To determine whether inhibition of the EGF-RTK activity is sufficient to block DNA synthesis of the cells in serum, the SQ20B and SCC61 cell lines were exposed to ZD1839, an EGFR tyrosine kinase inhibitor (13). Phosphorylation of the EGFR and the downstream ERKs in these cells was inhibited in a dose-dependent manner by ZD1839 (Supplementary Fig. S1B). ZD1839 also reduces both autocrine- and serum-stimulated DNA synthesis in these cells as measured by BrdUrd incorporation (Supplementary Fig. S1C). These results indicate that EGFR is selectively activated and regulates DNA synthesis in SCCHN cells.

MAPK activation has been found to correlate with EGF receptor expression in SCCHN and to regulate cell proliferation in EGFR-overexpressing tumor cell lines (14). To determine whether activation of the Ras-Raf-MAPK pathway is critical to DNA synthesis in SCCHN, the SQ20B and SCC61 cell lines were exposed to an inhibitor of MAPK/ERK kinase (MEK; U0126). The MEK inhibitor blocked both EGF-stimulated ERK (Supplementary Fig. S1D) and DNA synthesis (Supplementary Fig. S1E). These results indicate that MAPK, a downstream effector of the EGFR, is required for DNA synthesis by the radiation-resistant, EGFR-dependent SCCHN cells.

Inhibition of PKCζ reduces DNA synthesis. Because PKCζ is required for MAPK activation by EGF, we examined the role of PKCζ in autocrine SCCHN cell DNA replication. The Myr-PS peptide was nonspecifically toxic during prolonged incubation, and RNA interference approaches were ineffective at PKCζ depletion in these cell lines (data not shown). Therefore, a KD-PKCζ construct was transfected into SQ20B and SCC61 cells and was able to inhibit ERK activation (Fig. 3*A*). Cotransfection with green fluorescent protein (GFP) revealed that the transfection efficiency of the

electroporated KD-PKCζ expression vector is at least 50% in SQ20B and SCC61 cells (Fig. 3*B*). When the KD-PKCζ-transfected cells were assayed for BrdUrd incorporation, DNA synthesis in both SCC61 and SQ20 cells was significantly reduced (Fig. 3*C*). The rate of DNA synthesis was similar in the presence or absence of exogenous EGF; however, in SQ20B cells, KD-PKCζ was less effective in the presence of exogenous EGF. Consistent with the suppressive effects of EGFR and MEK inhibitors, these results indicate that KD-PKCζ reduces both autocrine and exogenous EGF-stimulated EGFR signaling in SCCHN cell lines.

KD-PKCζ potentiates the inhibitory effects of U0126 and chelerythrine chloride. We determined whether suppression of PKCζ can potentiate the action of other cell proliferation inhibitors by investigating the effect of KD-PKCζ on the potency of U0126, a more general inhibitor of the MAPK cascade. As shown in Fig. 3*D*, electroporation of either SQ20B or SCC61 cells with KD-PKCζ reduces the concentration of the MEK inhibitor required to achieve a specific reduction in DNA synthesis. Because other PKC isoforms (α, δ, and ε) are also expressed and phosphorylated in these SCCHN cells and a broad PKC inhibitor, chelerythrine chloride, can inhibit tumor growth in mouse xenograft models (15), we tested the effect of electroporating KD-PKCζ on the inhibition of EGF-induced DNA synthesis by chelerythrine chloride. As shown in Fig. 3*E*, an additive reduction in DNA synthesis was observed compared with control vector. These results indicate that PKCζ suppression enhances the action of other inhibitors that target similar or complementary signaling pathways.

PKCζ inactivation reduces SCCHN proliferation. Lentivirus expressing KD-PKCζ or TAT-PS peptide also inhibited PKCζ activity in SQ20B or SCC61 cells. Immunoblotting for PKCζ confirmed expression of the lentiviral KD-PKCζ protein in the SCCHN cells (Fig. 4*A*). As shown using SCC61 cells, introduction of

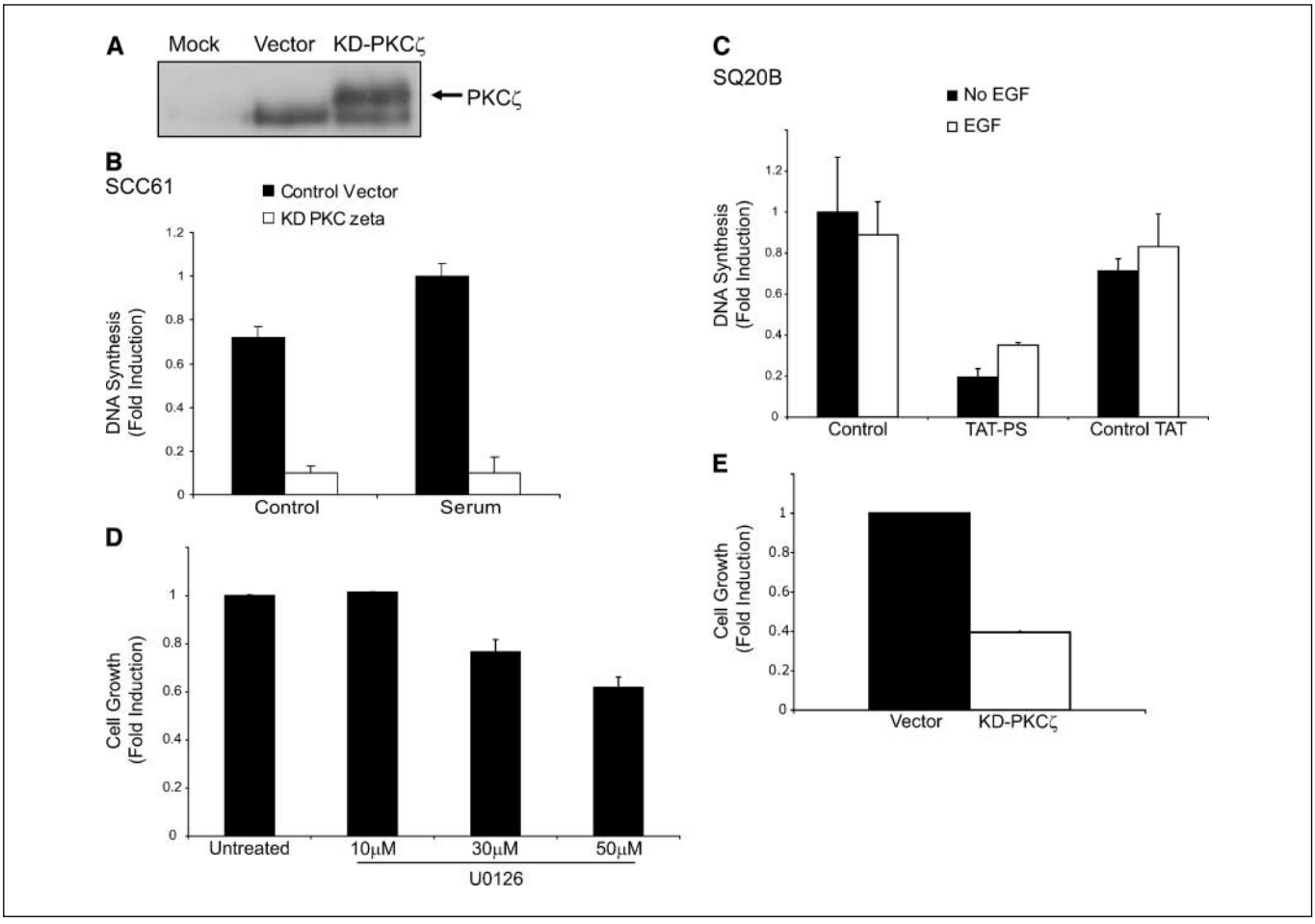

Figure 4. PKCζ inactivation reduces SCCHN proliferation. *A,* expression of the KD-PKCζ protein in lentivirus-infected cells. SCC61 cells were mock infected (*mock*) or infected with pCDH-EV expression lentivirus (vector) or with pCDH-KD-PKCζ-expressing lentivirus (KD-PKCζ) and harvested at 48 hours after infection. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting using anti-PKCζ antibody as described in Materials and Methods. *B,* suppression of DNA synthesis by KD-PKCζ in SCC61 cells infected with pCDH-EV-expressing lentivirus (control vector) or pCDH-KD-PKCζ-expressing lentivirus (KD-PKCζ). Cells were infected for 48 hours with KD-PKCζ or control vector and then incubated in serum-free medium (control) or 20% serum-containing medium for 24 hours. BrdUrd incorporation assays were done as described in Materials and Methods. Representative of two independent experiments. *Columns,* mean of quadruplicate samples; *bars,* SE. *C,* suppression of DNA synthesis by TAT-PS inhibitory peptide in SQ20B cells. Cells were grown in serum-free or 100 ng/mL EGF-containing medium (*EGF*) for 24 hours. TAT-PS or Tat-Scr (2 μmol/L; control TAT) was then added every 8 hours for 24 hours. BrdUrd incorporation assays were done as described in Materials and Methods. Representative of two independent experiments. *Columns,* mean of quadruplicate samples; *bars,* SE. *D,* reduction of cell proliferation by MEK inhibitor. SCC61 cells were grown in 20% serum-containing medium for 72 hours and incubated with increasing concentrations of U0126. MTT assays were done as described in Materials and Methods. Representative of at least three independent experiments. *Columns,* mean of quadruplicate samples; *bars,* SE. *E,* reduction of cell proliferation by KD-PKCζ in SCC61 cells infected with pCDH-EV-expressing lentivirus (control vector) or pCDH-KD-PKCζ-expressing lentivirus (KD-PKCζ). Cells were grown in 20% serum-containing medium for 72 hours. MTT assays were done as described in Materials and Methods. Representative of two independent experiments. *Columns,* mean of quadruplicate samples; *bars,* SE.

KD-PKCζ almost completely abrogated serum-stimulated DNA synthesis (Fig. 4*B*). Similarly, exposure of SQ20B and SCC61 cells to low but repeated doses of the TAT-PS peptide also significantly inhibited DNA synthesis compared with a scrambled TAT-fusion control peptide (Fig. 4*C*; data not shown). Taken together, these results indicate that PKCζ inhibition by multiple approaches is sufficient to completely inhibit DNA synthesis in aggressive head and neck tumor cell lines.

We also examined whether inhibition of PKCζ was able to reduce the number of metabolically active SCCHN cells using the MTT assay. Paralleling its effects on DNA synthesis, but to a lesser degree, the MEK inhibitor, U0126, reduced proliferating SCC61 cells in a dose-dependent manner (Fig. 4*D*). Lentiviral transfection of SCC61 cells with KD-PKCζ similarly reduced cell metabolism (Fig. 4*E*) consistent with its role in MAPK activation. Overall, these results show that PKCζ is a critical mediator of SCCHN proliferation.

Discussion

In this study, we explored the role of one specific isoform, PKCζ, in normal oral mucosa and keratinocytes as well as SCCHN tumors and cell lines. The expression of activated PKCζ increases dramatically with malignant progression in normal and SCCHN tissue. PKCζ inhibition can reduce EGFR-mediated MAPK signaling, DNA synthesis, and cell viability and can act in an additive fashion with other more general inhibitors in radioresistant SCCHN cell lines. These results identify a key role for PKCζ in the development of aggressive SCCHN tumors and highlight an alternative strategy for selective suppression of EGF-mediated SCCHN growth.

Our work extends previous studies of PKC expression in NHEK and SCCHN cells and tissues. Although similar expression of PKCs (α, δ, ε, η, and ζ) in normal human keratinocytes was observed (16), different expression patterns have also been reported (17). There is limited data available about PKC expression in SCCHN, and the results are conflicting (18, 19). Our study would suggest that the novel isoforms (δ, ε, and θ) are highly expressed in SCCHN cell lines. Furthermore, we report a successive increase in total and phosphorylated PKCζ expression in normal, dysplastic, and malignant SCCHN tissue. PKC isoform phosphorylation, necessary but not sufficient for activity, is currently the best measure of activation available in archival tissue specimens. The increase in total PKCζ along with phosphorylation in the malignant specimens underscores a significant function for this isoform in SCCHN.

In addition to mediating EGFR-dependent cell proliferation, it is likely that PKCζ plays other critical roles in SCCHN tumor progression. In breast cancer cell lines, inhibition of PKCζ reduced EGF-induced chemotaxis, whereas inhibition of other PKC isoforms had minimal effects (20). In a renal cancer model, PKCζ activated hypoxia-inducible factor (HIF)-1α and HIF-2α and promoted their association with p300 (21). Therefore, PKCζ mediates several key steps in tumor progression, including cell proliferation, survival, cell migration, and angiogenesis.

Our studies indicate that the requirement for PKCζ to activate MAPK can be overridden, as two of the SCCHN cell lines were not sensitive to inhibition of PKCζ. The dysregulation of PKCζ-dependent MAPK activation has not been described previously and raises the possibility that loss of regulation by this enzyme through alternative mechanisms of MAPK activation plays an important role in the maintenance of the malignant state in specific SCCHN cell lines.

Inhibition of PKCζ in SCCHN cells potentiates the action of MEK and PKC inhibitors. Because U0126 blocks MEK activation of MAPK, whereas PKCζ inhibits Raf kinase activation (2), the two inhibitors act at different steps along the pathway but seem to be additive. Similar results were obtained when combining KD-PKCζ with chelerythrine chloride, a broad spectrum PKC inhibitor that is most potent against classic and novel isoforms. Previous studies by Chmura et al. reported that chelerythrine chloride was effective at suppressing the growth of SQ20B xenografts in mice (15). The use of chelerythrine chloride does not allow identification of the specific isoform responsible for the additivity, although it is noteworthy that only PKCα, among the classic and novel isoforms, is consistently expressed and phosphorylated in NHEK and SCCHN cells. Thus, it seems that targeting the same pathway or complementary pathways by different mechanisms lowers the amount of active enzyme and the threshold necessary to reduce DNA synthesis and cell proliferation.

The current study would suggest that PKCζ is a valid target in SCCHN based on its expression pattern in malignant disease, its critical role in EGF-induced MAPK activation, and its ability to inhibit SCCHN proliferation. Suppression of the proliferative stimulus from the EGFR signaling cascade by PKCζ should significantly lower the threshold for effective repression by other less selective and perhaps dose-limited inhibitors.

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