Protein Kinase Cê Negatively Regulates Hedgehog Signaling by Inhibition of Gli1 Activity*

Qingsong Ca†§1, Jing Li†§1, Tianyan Gao†§, Jingwu Xie†§, and B. Mark Evers†§2

From the †Department of Surgery, §Sealy Center for Cancer Cell Biology, and the ‡Department of Pharmacology and Toxicology, The University of Texas Medical Branch, Galveston, Texas 77555

Constitutive activation of the hedgehog pathway is implicated in the development of many human malignancies; hedgehog targets, PTCH1 and Gli1, are markers of hedgehog signaling activation and are expressed in most hedgehog-associated tumors. Protein kinase Cê (PKCê) generally slows proliferation and induces cell cycle arrest of various cell lines. In this study, we show that activated PKCê (wild-type PKCê stimulated by phorbol 12-myristate 13-acetate or constitutively active PKCê) decreased Gli1-luciferase reporter activity in NIH/3T3 cells, as well as the endogenous hedgehog-responsive gene PTCH1. In human hepatoma (i.e. Hep3B) cells, wild-type PKCê and constitutively active PKCê decreased the expression levels of endogenous Gli1 and PTCH1. In contrast, PKCê siRNA increased the expression levels of these target genes. Silencing of PKCê by siRNA rescued the inhibition of cell growth induced by KAAD-cyclopamine, an antagonist of hedgehog signaling element Smoothened, suggesting that PKCê acts downstream of Smoothened. The biological relevance of our study is shown in hepatocellular carcinoma where we found that hepatocellular carcinoma with detectable hedgehog signaling had weak or no detectable expression of PKCê, whereas PKCê highly expressing tumors had no detectable hedgehog signaling. Our results demonstrate that PKCê alters hedgehog signaling by inhibition of Gli protein transcriptional activity. Furthermore, our findings suggest that, in certain cancers, PKCê plays a role as a negative regulator of tumorigenesis by regulating hedgehog signaling.

The Hedgehog (Hh) signal pathway controls a variety of developmental processes such as pattern formation, differentiation, proliferation, and organogenesis (1, 2). Hh signaling is restricted in the adult organism where it is implicated in stem cell proliferation and tissue repair (3); persistent signaling or inappropriate reactivation results in cellular hyperproliferation and contributes to the formation and progression of human cancers, including basal cell carcinoma, lung, esophageal and biliary cancer, as well as breast, liver, pancreatic, and prostate cancers (4–8).

Hh signaling starts with association of the Hh ligand with its receptor Patched (PTCH), which releases PTCH inhibition of Smoothened (Smo), and allows Smo to transduce a signal for the activation and nuclear translocation of a family of transcription factors, cubitus interruptus in Drosophila and Glis (including Gli1, Gli2, and Gli3) in vertebrates, which, in turn, promotes expression of Hh signal target genes. In the absence of Hh ligand, phosphorylation of Gli2/3 targets latent Gli proteins to proteosome-dependent repressor formation (9, 10). Compared with Gli2/3, the mechanism of Gli1 regulation is poorly understood. The transcription factors Gli1, Gli2, and Gli3 are critical for the regulation of Hh signaling. Moreover, PTCH1 and Gli1 are transcriptional targets of the Hh signaling pathway expressed in most of these Hh-associated tumors and are used as markers of Hh signaling activation (2, 4).

The signaling proteins, protein kinase A, glycogen synthase kinase 3β, casein kinase-I, phosphoinositide 3-kinase/protein kinase B, and the mitogen-activated protein kinase kinase 1 (MEK1) affect mammalian Hh signal transduction by post-translational modifications (9, 11–16). Our laboratory is focused on signaling proteins, particularly phosphoinositide 3-kinase/protein kinase B and PKC, and their effects on tumorigenesis and interaction with other signaling pathways (17–23). The PKC family of proteins consists of three groups: the calcium-dependent conventional PKC isoforms (α, βI, βII, and γ), the calcium-independent novel PKC isoforms (δ, ε, η, and θ), and the calcium-independent atypical PKC isoforms (ζ, λ/τ) (24–26).

The effect of PKCê in cancer cells appears to be related to cell type and context. In some cancers, PKCê functions as a proapoptotic factor (27–29) but appears to have an anti-apoptotic effect in other cancers (30, 31). In this study, we found that PKCê antagonizes Gli protein transcriptional activity in NIH/3T3 cells, and endogenous PKCê has a negative effect on Gli activity in human hepatoma cells Hep3B. In addition, we found that PKCê expression is markedly decreased or undetectable in hepatocellular cancer (HCC) with active Hh signaling. These results are consistent with the general function of PKCê for attenuating proliferation and inducing cell cycle arrest (27–29, 32). Our findings provide the first evidence to suggest the negative regulation of the Hh pathway by PKCê.
**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit polyclonal anti-PKCδ specific antibody (ab47473) and rabbit polyclonal anti-Gli1 (ab7523) were purchased from Abcam, Inc. (Cambridge, MA). Mouse anti-HA.11 monoclonal antibody, and Alexa Fluor® Labeled (A488–101L) were purchased from CRP Inc. (Harrisburg, PA). Rat anti-HA monoclonal antibody (11867 and 423001) was purchased from Roche Applied Science. Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor® 568 goat anti-rabbit IgG (A-11011) was from Invitrogen Molecular Probes (Eugene, OR). Mouse anti-β-actin monoclonal antibody (A5441) was from Sigma-Aldrich. The enhanced chemiluminescence (ECL) system was from Amersham Biosciences. The concentrated protein assay dye reagent was from Bio-Rad (Richmond, CA). Tissue culture media and Lipofectamine 2000 transfection reagents were purchased from Invitrogen. Phorbol 12-myristate 13-acetate (PMA), rotterlin, and PD98059 were purchased from Sigma. KAAD-cyclopamine was from Calbiochem (Darmstadt, Germany). The Dual Luciferase Reporter Assay System was from Promega (Madison, WI); a High Capacity cDNA reverse transcription kit was from Applied Biosystems (Foster City, CA). All other reagents were of molecular biology grade and purchased from Sigma. pcDNA3 6× Myc human Gli1 was provided by Dr. Fritz Aberger (University of Salzburg, Salzburg, Austria) (16). 8× Gli-binding site luciferase reporter (8×GBS-luciferase) and 8× mutant Gli-binding site luciferase reporter (8×GBS-luciferase mutant) plasmids were kindly provided by Dr Hiroshi Sasaki (Riken Centre, Kobe, Japan) (33). Wild-type, kinase-dead, and constitutively active HA-tagged PKCδ were provided by Dr. Jae-Won Soh (Inha University, Korea) (34). pEF-neo, pEF-PKCα WT, and pEF-PKCα A25E were provided by Dr. Gottfried Baier (Medical University of Innsbruck, Austria) (35).

**Cell Culture and Transfection**—NIH/3T3 cells and Hep3B cells were purchased from the American Type Culture Collection (Manassas, VA). NIH/3T3 Cells (passages 2–15) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum at 37 °C in a humidified 5% CO2 atmosphere. Hep3B Cells (passages 10–25) were cultured in minimum essential medium (Eagle) with 2 mM l-glutamine, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and 10% fetal bovine serum FBS. NIH/3T3 and Hep3B cells were seeded in 24- or 12-well plates at 70% confluence and transfected with different vectors (0.5–0.8 μg) using Lipofectamine 2000 transfection reagent following the manufacturer’s directions; transfection efficiency was ~60–70% in NIH/3T3 and 80% in Hep3B cells. Hep3B cells were seeded in 12-well plates at 50% confluence and transfected with the non-targeting control siRNA, or SMARTPool PKCδ siRNA, or single Non-Targeting CONTROL siRNA (D-001210-01: UAGCGACUAAACACAUCCAA), or single PKCδ siRNA#1 (D-003524-04, AGAAGCCGCCAUGUAUU), or single PKCδ siRNA#2 (D-003524-05, GUUGAUGUCGUUCGUAUUU) (40 μM, Dharmaco, Inc., Lafayette, CO).

**mRNA Expression Analysis**—Total RNA was isolated from treated NIH/3T3 or/and Hep3B cells transfected with different vectors or siRNA with the RNeasy kit and RNase-Free DNase Set (Qiagen, Valencia, CA). An aliquot (1 μg) was subjected to reverse transcription with a High Capacity cDNA reverse transcription kit using random primers. One twentieth of the final cDNA was used in each PCR reaction. For mouse PTCH1, cDNA was amplified with CCGTTTCAGTCGCCAGACAG (forward primer) and CTCACTCGGGGTGTCACA (reverse primer), mouse β-actin GGTCTTTGCAAGCTCTTCGT (forward primer) and CTCCTTGCACCTTCCAC (reverse primer). For quantitative PCR analyses, we detected transcripts of Gli1 and PTCH1 using Applied Biosystem’s assays-by-demand assay mixtures (the sequences for human Gli1 and PTCH1 have been patented by Applied Biosystems) and pre-developed 18 S rRNA (VIC™-dye labeled probe) TaqMan® assay reagent (P/N 4319413E) was used as an internal control. The levels of Gli1 and PTCH1 were measured in the Real-time PCR Core Facility at the University of Texas Medical Branch.

**Luciferase Assay**—NIH/3T3 cells and Hep3B cells were seeded in 24-well plates at 70% confluence and transfected with the Dual Luciferase Reporter Assay System (Promega) and Gli constructs and different PKC vectors (1–2 μg). Luciferase (firefly) and Renilla luciferase activities were determined in lysates of transfected cells with the Dual Luciferase Reporter Assay System as we have previously described (20, 23). The relative Gli-luciferase activity was normalized by TK-Renilla activity.

**Immunohistochemistry**—The status of Hh signaling of HCC tissues has been determined with in situ hybridization (4), and the sections were prepared as described previously (36). Tissue sections were deparaffinized, followed by rehydration with decreased concentrations of ethanol, and immersed in 3% H2O2 for 10 min. Following antigen retrieval in citrate buffer (pH 6.0), the tissue sections were incubated with normal goat serum to block nonspecific antibody binding (20 min at room temperature). The sections were then incubated with primary antibodies (at 1:200 dilution) at 37 °C in humid chambers for 2 h. After washing with phosphate-buffered saline three times, the sections were incubated with the biotinylated secondary antibody (goat anti-rabbit IgG) and streptavidin conjugated to horseradish peroxidase for 20 min at 37 °C, followed by phosphate-buffered saline wash. The sections were incubated with the diaminobenzidine substrate for <30 min. Hematoxylin was used for counterstaining. Negative controls were performed in all cases by omitting the first antibodies.

**Protein Preparation, Western Blotting, and Immunoprecipitation**—Protein preparation and Western blotting were performed as described previously (17, 37). In brief, cells were lysed with 1× cell lysis buffer from Cell Signaling Technology, Inc. (Danvers, MA). Equal amounts of protein were resolved on NuPAGE Bis-Tris gels (Invitrogen) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4 °C with primary antibodies in TBST buffer with 1% bovine serum albumin, followed with a horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. Membranes were developed using the ECL detection system. Immunoprecipitation was performed as
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described previously (17). In brief, protein samples (300 μg) were incubated with rat anti-HA monoclonal antibody (1 μl) and protein A/G beads (20 μl) on a shaker at 4 °C overnight. Beads were washed three times with lysis buffer, and 4 × Tris-glucine sample buffer was added. Samples were denatured and analyzed by Western blot.

Immunofluorescence Staining—Hep3B cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.2% Triton X-100 cells, incubated in blocking buffer (phosphate-buffered saline with 3% bovine serum albumin) for 15 min, and then with incubated primary antibody for 1 h in blocking solution at room temperature. Samples were then incubated with secondary antibodies for 30 min at room temperature in the blocking solution.

RNA Interference—The SMARTpool PKCδ siRNA and non-targeting siRNA were obtained from Dharmacon Research in the annealed and purified form. Transfection of siRNA duplexes (40 nM) was carried out using Lipofectamine 2000 following the instructions of the manufacturer. At 72 h after transfection, cells were lysed and subjected to either real-time PCR, immunoblot, or immunofluorescence microscopy analysis.

Cell Proliferation Assay—Hep3B cells were transfected with PKCδ siRNA or non-targeting siRNA, and 24 h later, replated into 24-wells plates with minimal essential medium containing 2% fetal bovine serum. Either KAAD-cyclosporine or vehicle (DMSO) was added, respectively. Cells were collected by trypsinization daily and counted by using a Coulter counter.

Statistical Analysis—All experiments were repeated at least three times, and data are reported as means ± S.E. For each experiment, real-time PCR values and relative luciferase activity were analyzed using a two-sample t test or analysis of variance for a one- or two-factor experiment according to the structure of factor(s). The factors were vector, drug, and/or dose. Main effects and interactions were assessed at the 0.05 level of significance. Multiple comparisons were conducted using a t statistic with the standard error computed from the residual mean square in the analysis of variance and the comparison-wise error rate with Bonferroni adjustment for the number of comparisons. Statistical computations were carried out using PROC TTEST and PROC GLM in SAS®, Release 9.1.

RESULTS

PKCδ Increases Gli-luciferase Activity in NIH/3T3 Cells—Neill et al. (48) have shown that PKCδ is a potent regulator of Gli1 transcriptional activity in 293T cells. To test if the effect of PKCδ on the regulation of Gli1 is cell type-dependent, we first investigated the effects of PKCδ on Gli1-luciferase activity in NIH/3T3 cells. NIH/3T3 cells were co-transfected with a wild-type Gli1-luciferase reporter or a mutated Gli1-luciferase reporter (point mutation that abolishes the binding of Gli), Gli1, and constitutively active PKCδ AE. PKCδ AE significantly increased the wild-type Gli1-luciferase activity, but not the mutant, indicating the specific regulation of PKCδ on Gli activity (Fig. 1A, top panel). Western blot analysis was performed in cells overexpressing Gli1 and PKCδ AE to monitor the status of protein expression (Fig. 1A, bottom panel). Expression of Myc-tagged Gli1 was not altered by PKCδ AE compared with the control vector; phosphorylation of overexpressed PKCδ AE was detected using anti-phospho-PKCδ/βII (Thr-638/641) antibody to demonstrate the activation of PKCδ. It has been suggested that activation of MEK/ERK signaling is involved downstream of the PKC pathway (30, 31); therefore, we probed the membrane with anti-phospho-ERK1/2 (Thr-202/Tyr-204) antibody. ERK1/2 phosphorylation was not altered by PKCδ AE. To further determine if PKCδ regulation of Gli1 is mediated by the MEK/ERK pathway, cells were transfected with Gli1-luciferase reporter, Gli1 and wild-type PKCδ followed by combination treatment of PMA and PD98059, a specific inhibitor of MEK, for 6 h. The presence of PD98059 completely blocked Gli1 luciferase activity (Fig. 1B, top panel). Results from Western blot shows that PD98059 treatment effectively inhibited ERK1/2 phosphorylation; whereas, either PMA or PD98059 had no effect on Myc-tagged Gli1 protein expression (Fig. 1B, bottom panel). Our data suggest that PKCδ plays a positive role in the regulation of Gli1 activity; this effect is mediated by the MEK/ERK pathway.

PKCδ Down-regulates Gli-luciferase Activity in NIH/3T3 Cells—It has been demonstrated that PKCδ increased the activity of Gli1 in NIH/3T3 (13) and 293T cells (48). To examine the role of PKCδ in regulating Gli activity, we co-transfected NIH/3T3 cells with Gli luciferase reporter, Gli1, and either wild-type
increased Gli-luciferase activity in cells co-transfected with pcDNA3. In contrast, PMA treatment significantly decreased Gli-luciferase activity in cells transfected with PKC8 WT. In the cells transfected with PKC8ΔNPS, Gli-luciferase activity was further decreased either in the presence or absence of PMA, whereas Gli-luciferase activity was not altered in cells transfected with PKC8 KD. To further test the specificity of PKCδ-dependent Gli-luciferase activity by PMA treatment, NIH/3T3 cells were co-transfected with Gli luciferase reporter and PKC8 WT as well as pcDNA3 and treated with various doses of PMA (Fig. 2B, top panel). In the cells transfected with pcDNA3, PMA treatment enhanced Gli-luciferase activity in a dose-dependent fashion; however, in the presence of PKC8 WT, PMA treatment decreased Gli-luciferase activity in a dose-dependent manner. The decrease of Gli-luciferase activity in cells transfected with PKC8 WT, stimulated by PMA, was blocked by rottlerin (Fig. 2C, top panel), further suggesting the effect of PKCδ on Gli activity. Fig. 2D shows PD98059 decreased Gli-luciferase activity in the cells with PKC8 WT overexpression either in the absence or presence of PMA. In addition, Western blots were performed (Fig. 2, A–D, bottom panels) showing the effect of PKCδ on ERK signaling. PMA treatment deleted endogenous phosphorylated and total PKCδ expression (Fig. 2A), indicating that the endogenous PKCδ does not play an important role in PMA-stimulated Gli activity in NIH/3T3 cells. PMA treatment did not affect HA-tagged PKCδ expression in either total or phosphorylation level. Furthermore, ERK1/2 phosphorylation was not altered by overexpression of PKCδ. We also examined the effect of PKCδ on the expression of the endogenous Gli-regulated gene PTCH1. Reverse transcription-PCR (RT-PCR) assays showed that, in cells transfected with pcDNA3 as well as PKC8 KD, PTCH1 mRNA expression was not altered either with or without PMA treatment. In contrast, PMA treatment decreased PTCH1 mRNA levels in cells transfected with PKCδ.
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**FIGURE 3. PKCδ affects Hh pathway signaling and Gli1 protein expression in human hepatoma cells Hep3B.** A, NIH/3T3 and Hep3B cells were co-transfected with Gli-luciferase reporter, TK-RENilla, Gli1, and increased concentration of PKCδΔNPS plasmids as well as pcDNA3. Gli-luciferase activity was performed 24 h after transfection (*, p < 0.05 versus pcDNA3 alone; †, p < 0.05 versus Gli1 alone) (top panel); Western blot was performed to demonstrate the effects of PKCδΔNPS overexpression on Gli1 protein expression (bottom panels). B, Hep3B cells were transfected with PKCδ WT, PKCδΔNPS, PKCδ KD, and pcDNA3 as well. Total RNA was extracted 24 h after transfection, and real-time RT-PCR analysis was performed to detect mRNA expression of Gli1 and PTCH1 as well. Total RNA was extracted 24 h after transfection (*, p < 0.05 versus pcDNA3; baseline value = 1.0) (top panel); the expression of Myc-Gli1 and overexpression of PKCδ was monitored by Western blot (bottom panel). C, Hep3B cells were co-transfected with Myc-Gli1 and PKCδ WT, PKCδ KD, or pcDNA3. Co-transfection of PKCδ WT and Myc-PDK1 was used as a positive control. Immunoprecipitation and Western blot were performed as described under “Experimental Procedures.”

WT in the presence of PMA; PTCH1 mRNA expression was also decreased in cells transfected with PKCδΔNPS either in the presence or absence of PMA (Fig. 2E). Taken together, our data demonstrate that PKCδ plays a negative role in the regulation of Gli1 activity stimulated by PMA. Similar results were also observed when Gli2 was used (data not shown).

PKCδ Affects Hh Pathway Signaling and Gli1 Protein Expression in Human Hepatoma Hep3B Cells—Our findings imply an antagonistic role for PKCδ on the activity of Gli, suggesting a role of PKCδ in regulating tumor cell progression via Hedgehog signaling. Therefore, we next focused on PKCδ to test whether PKCδ plays a similar role in Hep3B hepatoma cells, which possess constitutive Hh activity (4). For this purpose, Hep3B cells were co-transfected with Gli luciferase reporter, Gli, and various concentrations of the PKCδ ΔNPS; NIH/3T3 cells were used as a positive control (Fig. 3A, top panel). Similar to NIH/3T3 cells, overexpression of PKCδ ΔNPS significantly decreased Gli-luciferase activity in Hep3B cells in a dose-dependent pattern. To understand how PKCδ down-regulates Gli1 activity, we tested if overexpression of PKCδ ΔNPS affected Myc-tagged Gli1 protein expression by Western blot analysis. The Myc-tagged Gli1 was decreased by the overexpression of PKCδ ΔNPS in a dose-dependent fashion in either NIH/3T3 cells (Fig. 3A, bottom panel, left) or Hep3B cells (Fig. 3A, bottom panel, right). Next, we determined whether PKCδ affects endogenous Hh target genes, PTCH1 and Gli1, in Hep3B cells by real-time RT-PCR. mRNA expression of PTCH1 and Gli1 was decreased in cells transfected with PKCδ WT and PKCδ ΔNPS, whereas PKCδ KD did not affect the expression of these genes (Fig. 3B, top panel). Furthermore, endogenous Gli1 protein levels in Hep3B cells were decreased by PKCδ WT and PKCδ ΔNPS but not PKCδ KD as shown by Western blot in Fig. 3B, bottom panel.

To address whether PKCδ regulates Gli1 through direct protein-protein interaction, we performed co-immunoprecipitation experiments. The results show that both PKCδ WT and KD associated with Gli1 in cells (Fig. 3C). As a positive control, the interaction between PKCδ and its upstream kinase PDK1 was readily detected as well. In addition, the time course for Gli1 protein degradation was monitored using pulse-chase experiments. Hep3B cells expressing Gli1 and co-transfected with a control vector or PKCδ ΔNPS were treated with cycloheximide, a protein synthesis inhibitor. The rate of Gli1 degradation was similar in the control and PKCδ ΔNPS-expressing cells (data not shown), suggesting that PKCδ is not involved in regulating the protein stability of Gli1 in Hep3B cells. Taken together, our results indicate that overexpression of an active form (either WT or ΔNPS) but not a kinase-dead mutant of PKCδ negatively regulates Gli1 expression likely through transcriptional down-regulation of Gli1 mRNA.

Wild-type and Constitutively Active PKCδ Decreases Nuclear Gli1 Expression—We have shown that PKCδ down-regulates exogenous and endogenous Gli activity. Modulation of Gli accumulation in the nucleus is a major mechanism for controlling its activity (39). To further understand how PKCδ down-regulates Gli1 activity, we examined the effects of overex-
pressed PKCδ on the distribution of endogenous Gli1 in Hep3B cells by immunofluorescent staining. As shown in Fig. 4, HA-tagged PKCδ WT (green) localized in the cytosol as well as the nucleus, whereas PKCδ ΔNPS predominantly localized in the cytosol in an accumulated pattern. Endogenous Gli1 (red) is mainly localized in the nucleus and is only faintly detected in the cytoplasm in non-transfected cells. Cells transfected with PKCδ WT or PKCδ ΔNPS display a significant decrease in the expression levels of endogenous Gli1 (76–86% and 93–100%, respectively), whereas Gli1 expression levels are not altered in cells transfected with PKCδ KD. The PKCδ WT and the PKCδ ΔNPS have different localization patterns to nucleus but similar effects in the regulation of Gli1 expression, suggesting the cytosolic PKCδ plays a more important role in the regulation of nuclear Gli1 expression. PKCδ may affect the shuttle of Gli1 between the nucleus and cytosol. This result further suggests that PKCδ affects Hh signaling by regulating Gli1 protein expression.

PKCδ Suppression Increases Hh Pathway Signaling—To further confirm that PKCδ plays a negative role in the regulation of Gli1 activity in Hep3B cells, endogenous PKCδ was knocked down by PKCδ siRNA. mRNA levels of PTCH1 and Gli1 were increased in cells transfected with SMARTpool PKCδ siRNA (Fig. 5A, top panel). Furthermore, PKCδ siRNA increased Gli1 protein levels compared with NTC siRNA (Fig. 5A, bottom panel). We also determined if the regulation of Hh signaling by PKCδ affects cell function, such as cellular proliferation. Treatment with the Smo antagonist KAAD-cyclopamine (2 μM) alone decreased Hep3B cell proliferation. In contrast, PKCδ knockdown with siRNA enhanced the proliferation, and transfection with PKCδ siRNA significantly blocked the inhibitory effects of KAAD-cyclopamine (Fig. 5B, left panel). The PKCδ knockdown was monitored by Western blot as shown in Fig. 5B, right panel. The results were confirmed with two individual PKCδ siRNA (Fig. 5C). Taken together, the loss of PKCδ increases Hh signaling and Gli1 protein expression and rescues the inhibitory effect of KAAD-cyclopamine on cellular proliferation, demonstrating that PKCδ negatively regulates Hh signaling downstream of Smo. However, we cannot exclude the possibility that PKCδ indirectly regulates Gli1 by inhibiting Smo.

Reduced PKCδ Expression Is Associated with Activation of Hh Signaling in HCC Tissue—To determine whether PKCδ plays a role in regulating Hh signaling in human cancer, we screened a set of HCC clinical specimens for PKCδ expression. The activation status of Hh signaling in this set of samples has previously been determined by in situ hybridization using probes against Gli1 and PTCH1 (4). The samples with positive staining of these Hh target genes are considered to have activated Hh pathway. The level of PKCδ expression in this set of HCC specimens was detected using immunohistochemical staining. Among the 11 total cancer samples screened, 6 samples are known to have activated Hh signaling. Interestingly, the expression of PKCδ was not detected in any of these specimens with activated Hh signaling (three representative cases are shown in Fig. 6, A–C). Nuclear staining with PKCδ antibody was only observed in two samples screened, and both of these had no detectable Hh signaling (Fig. 6D and Table 1). The remaining three specimens with undetectable Hh signaling did not demonstrate expression of PKCδ (data not shown). These results suggest that decreased expression of PKCδ may account for activation of Hh signaling in certain HCC specimens, underscoring the importance of PKCδ mediated negative regulation in suppressing the oncogenic Hh signaling.

DISCUSSION

The Hh signaling pathway, acting through transcriptional factors such as Gli1, has been identified as critical for the initiation and growth of a number of cancers (4–8). PKCα generally stimulates cell growth, but PKCδ slows proliferation and induces cell cycle arrest of various cell lines (32, 40, 41). The data from reporter assay reveal that PKCα and PKCδ have different effects on Gli activity. Activation of PKCα (by PMA or the constitutively active mutant) increased Gli-luciferase activity in NIH/3T3 cells. Activation of PKCδ decreased Gli-luciferase activity and down-regulated the mRNA levels of the Hh target gene. In Hep3B cells, wild-type PKCδ and constitutively active PKCδ decreased the expression levels of the endogenous genes Gli1 and PTCH1, whereas PKCδ siRNA increased the expression of these Hh target genes.

Using a different PKCδ plasmid (pCO2-PKCδ-cat) and Gli reporter plasmid (pGL3P-6Gb), Neill et al. (48) reported that constitutively active PKCδ appeared to increase Gli1 transcriptional activity in HEK293 cells. We found PMA increased the activity of overexpressed Gli1 in NIH/3T3 cells as previously reported by Riobo et al. (13). Based on the finding that rottlerin prevented the stimulation of endogenous Hh signal by PMA in LIGHT2 cells, these investigators concluded that PKCδ plays a
positive role in Hh signaling. We found that rottlerin decreased overexpressed Gli activity, but in combination with overexpression of wild-type PKCδ, rottlerin partially rescued the decrease of Gli-luciferase activity by PMA. Although rottlerin was initially described as a selective PKCδ inhibitor (32), subsequent studies showed that it is an inappropriate and ineffective inhibitor of PKCδ, and can uncouple mitochondrial respiration from oxidative phosphorylation and exert an inhibitory effect in PKCδ−/− cells (32, 49–51). The nonspecific effect of rottlerin may play a role in blocking Hh signaling stimulated by PMA. In addition, long term PMA treatment will deplete all of the conventional and novel PKC isoforms (13, 52). Thus, it is difficult to form conclusions regarding the function of PKCδ in the regulation of Gli activity by either rottlerin or PMA.

Phosphorylation of Gli2/3 by protein kinase A, GSK3 and casein kinase-I targets latent Gli proteins to proteasome-dependent repressor formation (2). As an Hh signaling target gene, Gli1 is regulated mainly at the transcriptional level (2). Recent reports identify protein kinase A regulation of Gli1 localization (14), and that β-Trcp and Numb regulate Gli1 degradation (39, 53). We found that PKCδ significantly decreased the activity of overexpressed Gli1, indicating that the function of PKCδ is rate limiting for Gli1 function. In an attempt to understand the mechanism by which Gli1 is down-regulated by active forms of PKCδ, we performed immunoprecipitation studies and found that Gli1 interacts with PKCδ in Hep3B cells. However, the binding does not depend on the kinase activity of PKCδ, because both the WT and KD PKCδ co-immunoprecipi-
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We found that all tumors with active Hh signaling demonstrated no or weak known Hh signaling status (4) were used in this study (see Table 1 for details). Antibodies from Abcam (cat# ab47473). A total of 11 HCC specimens with increase Gli transactivation activity of possibly other regulatory proteins resulting in a decrease of the Hh target genes. Moreover, the effects of PMA depend on the specific intracellular isoforms of PKC (32). Recently, Lasfer et al. (45) reported that PKCδ is required for apoptosis induction in Hep3B cells, and Huang et al. (4) found that constitutive Hh activity is important in these cells. PMA treatment induced a marked translocation of PKCα, PKCδ, and PKCε in Hep3B cells (46). Our finding, that the negative regulation of Hh signaling by PKCδ, is in agreement with a role for PKCδ in apoptosis induction of certain cancer cells.

In summary, we have shown that PKCδ was weakly expressed in all of the HCC sections with detectable Hh signaling, and high expression of PKCδ was found in only two HCCs, both of them had no detectable Hh signaling. It has been reported that nuclear retention of full-length PKCδ can be cleaved to form the catalytic fragment (47). In this study, we found nuclear staining of PKCδ in HCCs without detectable Hh signaling, suggesting PKCδ may directly regulate Gli in the nucleus and play a role in regulating Hh signaling in hepatocarcinogenesis. However, this does not appear to be the only mechanism by which Hh signaling activation is regulated in HCC. Three HCC specimens with no detectable Hh signaling also had no detectable expression of PKCδ, suggesting down-regulation of PKCδ is not sufficient to activate Hh signaling. A large cohort study on the correlation between Hh signaling and PKCδ will be useful to show the significance contribution of PKCδ to Hh signaling activation in HCCs. Nevertheless, our data from limited tumor specimens are in favor of our hypothesis that PKCδ negatively regulates Hh signaling in HCC. Thus, elevated expression of PKCδ may cause reduced Hh signaling, whereas loss of PKCδ may contribute to Hh signaling activation.

In summary, we have shown that PKCδ down-regulates Hh signaling by inhibiting the activity of the transcription factor expression or knockdown of PKCδ did not affect MEK/ERK signaling in NIH/3T3 and Hep3B cells. Therefore, our findings, using complementary approaches, suggest that PKCδ plays a negative role in the regulation of Gli activity, which is independent of the MEK/ERK pathway. Consistent with a previous report (13), our data indicate that activation of PKC by PMA increased Gli-luciferase activity through MEK/ERK signaling. Individual PKC isoforms have specific functions (42–44). For example, PKCα and PKCβ stimulate growth, whereas PKCδ generally slows proliferation and induces cell cycle arrest (32, 40, 41). Our data are consistent with the anti-apoptotic function of PKCα and the inhibitory function of PKCδ in cell proliferation. The effects of PMA depend on the specific intracellular isoforms of PKC (32).

### TABLE 1

| Number | Age | Sex | HBV | Size | Type of tumor                      | Hh signaling | PKCδ expression   |
|--------|-----|-----|-----|------|------------------------------------|--------------|-------------------|
| 1      | 50  | F   | NA* | 3 x 2 | HCC                               | Negative     | Negative/weak     |
| 2      | 67  | M   |      | 5 x 5 | HCC                               | Positive     | Negative/weak     |
| 3      | 66  | M   | +   | 3.5 x 2 | HCC                               | Positive     | Negative/weak     |
| 4      | 32  | M   | NA  | 3.5 x 3 | HCC and hepatocirrhosis           | Negative     | Positive          |
| 5      | 61  | M   | +   | 4.5 x 4.5 | HCC                               | Negative     | Negative/weak     |
| 6      | 28  | M   |      | NA   | HCC                               | Negative     | Positive          |
| 7      | 58  | M   | +   | Large | HCC and cholecytitis              | Positive     | Negative/weak     |
| 8      | 55  | M   | +   | 3.5 x 3 | HCC and hepatocirrhosis           | Positive     | Negative/weak     |
| 9      | 73  | M   | +   | NA   | HCC                               | Positive     | Negative/weak     |
| 10     | 46  | M   | +   | 5 x 4  | HCC                               | Positive     | Negative/weak     |
| 11     | 42  | M   | +   | 8 x 8 | HCC                               | Positive     | Negative/weak     |

* NA, not available.

**FIGURE 6. Immunohistochemical analysis of PKCδ expression in HCC sections.** Immunohistochemical detection of PKCδ was performed with specific antibodies from Abcam (cat# ab47473). A total of 11 HCC specimens with known Hh signaling status (4) were used in this study (see Table 1 for details). We found that all tumors with active Hh signaling demonstrated no or weak PKCδ expression (arrow in A and B; A is from specimen #2, B is from specimen #11). Positive staining of PKCδ was noted in the stromal compartment in one specimen (arrowhead in C), although the tumor was negative for PKCδ expression (C is from specimen #7). Two specimens with positive PKCδ staining (arrow in D) were negative for Hh signaling activation (see Table 1 for details; D is from specimen #6).
Hedgehog Pathway Regulation by PKCδ

Gli in NIH/3T3 cells and Hep3B cells. In fact, the balance between PKCα and PKCδ is important in the regulation of Gli activity by PMA. When PKCα is dominant, the negative effect of PKCδ is small, and PMA increases Gli activity through the PKCα/MEK/ERK pathway. However, when PKCδ is dominant, PMA treatment decreases Gli activity through the activation of PKCδ. Our study provides a better understanding of the complex cross-talk between the Hh and the PKC pathways. Studies detailing the molecular mechanism for PKCδ regulation of Gli activity will provide a basis for the design of more efficient targeted therapies for Hh responsive cancers.

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