PKCδ increases keratinocyte differentiation and suppresses keratinocyte proliferation and survival. However, the mechanism of proliferation suppression is not well understood. The present studies show that PKCδ overexpression increases p21<sup>Cip1</sup> mRNA and protein level and promoter activity and that treatment with dominant-negative PKCδ, PKCδ-siRNA, or rottlerin inhibits promoter activation. Analysis of the p21<sup>Cip1</sup> promoter upstream regulatory region reveals three DNA segments that mediate PKCδ-dependent promoter activation. The PKCδ response element most proximal to the transcription start site encodes six GC-rich DNA elements. Mutation of these sites results in a loss of PKCδ-dependent promoter activation. Gel mobility supershift and chromatin immunoprecipitation reveal that these DNA elements bind the Kruppel-like transcription factor KLF4. PKCδ increases KLF4 mRNA and protein level and KLF4 binding to the GC-rich elements in the p21<sup>Cip1</sup> proximal promoter. In addition, KLF4-siRNA inhibits PKCδ-dependent p21<sup>Cip1</sup> promoter activity. PKCδ increases KLF4 expression leading to enhanced KLF4 interaction with the GC-rich elements in the p21<sup>Cip1</sup> promoter to activate transcription.

PKC isoforms include three subfamilies of kinases that play a central role in the regulation of cell growth and differentiation (1). Classical PKCs (α, β, and γ) are calcium-, phospholipid-, and diacylglycerol-dependent; novel PKCs (nPKC δ, ε, η, and θ) are activated by diacylglycerol and phospholipids, but they do not respond directly to calcium; and atypical PKCs (ζ and λ) are calcium- and diacylglycerol-independent but undergo allosteric activation (2, 3). Epidermal keratinocytes express PKCa, PKCβIII, PKCδ, PKCε, PKCη, and PKCζ (4–10). These kinases have been studied in cultured keratinocytes and in animal models (11–19). A number of laboratories have shown that nPKC isoforms stimulate keratinocyte differentiation (15, 20–24, 26). Consistent with this role, studies from our group show that the novel PKC (nPKC) isoforms stimulate keratinocyte differentiation by activating MAPK signaling, which results in increased nuclear levels of AP1, CCAAT enhancer-binding protein, and Sp1 transcription factors and binding of these factors to target genes to increase transcription (27–29). Involucrin is a classical marker of differentiation, and our studies show that PKCδ is a potent activator of involucrin expression (21, 27, 30–32).

PKC isoforms have also been implicated in the regulation of keratinocyte proliferation (13, 20, 23, 33–35). This role is particularly important, because keratinocyte differentiation is associated with cessation of proliferation, and it would make mechanistic sense to have a common kinase activate both processes. A limited number of studies have examined the mechanism of nPKC regulation of keratinocyte proliferation. For example, the nPKC isoform PKCe binds to and activates Fyn, a Src kinase, and this is associated with reduced keratinocyte proliferation (36). PKCe forms a complex with cyclin E-ckd2-p21<sup>Cip1</sup> leading to phosphorylation of p21<sup>Cip1</sup> and cdk2 inhibition to reduce proliferation (37). In addition, PKCo suppresses proliferation in raft cultures of human keratinocytes by a mechanism that involves increased expression of p21<sup>Cip1</sup> (17).

This realization that PKC regulates cell proliferation points to the intriguing possibility that a single regulatory cascade may both increase differentiation and suppress proliferation, key processes that must proceed together during keratinocyte maturation. However, we have limited understanding regarding the mechanism of growth suppression. p21<sup>Cip1</sup> is an important regulator of cell cycle progression, and increased p21<sup>Cip1</sup> expression is associated with cessation of cell proliferation (38). Moreover, p21<sup>Cip1</sup> has been implicated as a key suppressor of proliferation progression in human keratinocytes (39–44). We now describe a novel mechanism whereby PKCδ suppresses keratinocyte proliferation. Our studies indicate that PKCδ increases expression of the Kruppel-like factor KLF4, which interacts at GC-rich DNA elements in the proximal p21<sup>Cip1</sup> promoter to activate p21<sup>Cip1</sup> gene expression leading to cessation of cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Keratinocyte serum-free medium (KSFM) and trypsin were purchased from Invitrogen. Rottlerin, G06796, actinomycin D, and sodium butyrate were from Calbiochem (La Jolla, CA). Me<sub>2</sub>SO was purchased from Sigma-Aldrich. Bromodeoxyuridine (BrdU) was from BD Biosciences (San Jose, CA). Mouse monoclonal antibodies for β-actin (A5441) and BrdU (B8434) were purchased from Sigma-Aldrich. Rabbit polyclonal antibodies for KLF4 (sc-20691), Sp1
(sc-59x), and Sp3 (sc-644x), and goat polyclonal antibodies for AP4 (sc-18593) and PKCδ (sc-957-G) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies for p21(1-6) (2947) and PKCδ-TyrP)-31 (2055S) were from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-p300 (05-257) was from Millipore (Billerica, MA). Peroxidase-conjugated anti-mouse IgG (NXA931) and peroxidase-conjugated anti-rabbit IgG (NA934V) were obtained from GE Healthcare. Peroxidase-conjugated anti-goat IgG (sc-2033) was obtained from Santa Cruz Biotechnology. 

**Plasmids, Viruses, and Cell Culture**—The human wild-type p21(1-6) promoter-luciferase fusion plasmid, p21-2326, was a gift from Dr. Bert Vogelstein (45). Truncated p21(1-6) plasmids (p21-124, p21-101, and p21-60) were provided by Dr. Toshiyuki Sakai (46). Dominant-negative PKCδ(K376R), cloned in pLTR, was obtained from Dr. Weigun Li (47). pEGFP-N1 was from Clontech (Mountain View, CA). PKCδ-EGFP and PKCδ-Y311F-EGFP are described in our previous report (48). The p21(1-6) promoter truncation mutants were constructed using PCR, and the plasmids are named according to the 5’-most nucleotide from the p21(1-6) promoter sequence. p21-2326 was used as a template to construct Sp1 site mutants including p21-2326 Sp1(Δ1–6), p21-2326 Sp1(Δ1), p21-2326 Sp1(Δ2), p21-2326 Sp1(Δ3), and p21-2326 Sp1(Δ4) in pBluescript II KS(+). These plasmids lack the indicated Sp1 site. pMXs-hKLF4, a gift from Dr. Toshiro Kitamura (49), was used as a template to produce pcDNA3-hKLF4(1-470) (wild type), pcDNA3-hKLF4(1-388) (lacks zinc finger domain), and pcDNA3-hKLF4(335-470) (encodes only the zinc finger domain). PKCδ encoding adenovirus was obtained from Dr. T. Kuroki (29, 50). Ad5-EV and Ad5-PKCδ adenoviruses infect cells with greater than 90% efficiency (21). hKLF4 encoding cDNA was produced by PCR using pMXs-hKLF4 as template, and the EcoRI/Xbal product was cloned into pShuttle-Tet(ΔEcoRI) for production of virus using pAdeasy-1. The resulting virus is called tAd5-hKLF4. Expression of hKLF4 from this virus requires co-infection with Ad5-TA, which produces the tetracycline transactivator (51). For experiments, the keratinocytes were incubated with 10 μg/ml of plasmid and 0.5 μg of p21(1-6) promoter reporter plasmid and 0.5 μg of PKCδ expression plasmid were mixed, treated with FuGENE 6, and added to cells as indicated above. After 24 h, the cells were harvested, and the extracts were prepared for assay of luciferase activity.

**Promoter Activity**—For p21(1-6) promoter activity analysis, 0.5 μg of p21(1-6) promoter reporter plasmid was mixed with 1 μl of FuGENE 6 reagent diluted with 99 μl of KSFM. The mixture was incubated at 25 °C for 15 min and then added to a 50% confluent culture of primary human epidermal keratinocyte maintained in 2 ml of KSFM in a 9.6-cm² dish. For co-transfection experiments, with 0.5 μg of p21(1-6) promoter reporter plasmid and 0.5 μg of PKCδ expression plasmid were mixed, treated with FuGENE 6, and added to cells as indicated above. After 24 h, the cells were harvested, and the extracts were prepared for assay of luciferase activity.

**Electroporation and siRNA-mediated Knockdown**—Keratinocytes were electroporated with siRNA or plasmids using the Amaxa electroporator and the VPD-1002 nucleofection kit (Germany). For electroporation, keratinocytes were harvested with trypsin and replated 1 day prior to use. On the day of electroporation, 1 × 10⁵ of the replated cells were harvested with trypsin and resuspended in KSFM. The cells are collected at 2000 rpm, washed with 1 ml of sterile phosphate-buffered saline (pH 7.5), and suspended in 100 μl of keratinocyte nucleofection solution. The cell suspension, which included 3 μg of gene-specific siRNA, was mixed by gentle pipetting and electroporated using the T-018 settings. Warm KSFM (500 μl) was added, and the suspension was transferred to a 21.3-cm² cell culture dish containing 3.5 ml of KSFM. When required, the cells were electroporated a second time with luciferase reporter or expression plasmid. This was accomplished by harvesting the cells with trypsin and resuspension in KSFM. The cells were collected, washed with PBS, and resuspended in nucleofection solution as above. The nucleofection suspension, which included 2 μg of plasmid, was electroporated using the T-018 settings. The cells were plated and maintained for various times before the extracts were prepared for assay. Our electroporation method delivers nucleic acid reagents with greater than 90% efficiency.

**Immunological Analysis**—Equivalent amounts of protein were electrophoresed on a 4–15% denaturing polyacrylamide gradient gel and transferred to nitrocellulose. The membranes were blocked, incubated with a specific primary antibody, washed, and exposed to an appropriate horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection was used to visualize secondary antibody binding. To assess intracellular p21(1-6) distribution, total and nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagent (Pierce). To monitor DNA synthesis, the cells were treated for 24 h with stimulus, and during the final 6 h prior to harvest, the cells were incubated with 10 μM BrdU. BrdU uptake was monitored in fixed cells by indirect immunofluorescence.

**Real Time PCR**—Total RNA was isolated (Illustra RNAspin Mini kit; GE Healthcare) and reverse transcribed. Quantification was using the LightCycler 480 system (Roche Applied Science). PCR primers were designed to quantify the abundance of p21Cip1 mRNA Half-life—To analyze p21(1-6) mRNA decay kinetics, keratinocytes were infected with 15 MOI of Ad5-EV or Ad5-PKCδ for 24 h prior to the addition of 5 μg/ml actinomycin D. At 0, 0.5, 1, 2, 3, and 4 h after actinomycin D addition, RNA was isolated and analyzed for p21(1-6) and cyclophilin A.
mRNA content by quantitative RT-PCR using primers described in the previous section. The values for each mRNA level at each time point are the means ± S.D. derived from triplicate quantitative RT-PCRs of independent samples. First order decay constants ($k$) were determined by nonlinear regression analysis (PRISM v3.03; GraphPad) of plots measuring the percent p21Cip1 mRNA remaining versus time of actinomycin D treatment. p21Cip1 mRNA decay constants were calculated based on the means ± S.D. of $n$ of independent time course experiments where $n = 3$, permitting pair-wise statistical assessment using the Student’s $t$ test. Differences were considered significant if $p < 0.05$.

**Gel Mobility Shift and Supershift**—Human keratinocytes were infected Ad5-EV or Ad5-PKÇδ adenovirus (15 MOI), and at 24 or 48 h, the cells were washed with phosphate-buffered saline, and nuclear extract was prepared using NE-PER nuclear and cytoplasmic extraction reagent (Pierce). Binding of transcription factors to p21Cip1 promoter sites was monitored by gel mobility shift assay. Five micrograms of nuclear extract was incubated for 40 min at room temperature in a volume of 20 μl containing 20 mM HEPEs (pH 7.5), 10% glycerol, 50 mM KCl, 2 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 1 μg/ml poly(di-l-c), 0.1 mg/ml bovine serum albumin, and 50,000 cpm radioactive, double-stranded, 32P-end labeled Sp1(1/2) site oligonucleotide (5’-GGAGGGGGGTCCCGGGGCGC-3’, which encodes the Sp1 and Sp2 binding sites. The Sp1-1 and Sp1-2 binding sites. The Sp1 and Sp2 binding sites. The Sp1 and Sp2 binding sites. For gel mobility supershift assay, 2 μg of Sp1, Sp3, or KLF4 factor-specific antibody was added to the reaction mixture and incubated at 4 °C overnight with rotation. The 32P-labeled probe was then added, and the incubation was continued for an additional 40 min at 25 °C. Protein–DNA complexes were resolved in 4% polyacrylamide gels under non-denaturing conditions.

**ChIP Assay**—ChIP assays were conducted as described (52) with minor modifications. Briefly, human keratinocytes were cross-linked with 1.42% formaldehyde at room temperature for 15 min followed by quenching with 125 mM glycine. The cells were collected by centrifugation, and cell pellets were washed twice with ice-cold PBS containing histone deacetylase inhibitors. Cross-linked cells were collected by centrifugation and lysed in 150 μl of lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS, 1 mM PMSF, 20 mM sodium butyrate, and protease inhibitors). The samples were chilled on ice, and DNA was sheared using a Branson Sonifier (three 30-s pulses on ice at 25 °C) to produce fragments of 1,000 bp. Radioimmune precipitation assay buffer (400 μl) containing protease inhibitors and histone deacetylase inhibitors was added, and the chromatin was centrifuged at 12,000 × g for 10 min. Aliquots were used for generating input DNA and for antibody immunoprecipitation. ChIP grade antibodies were added to Dynabeads protein A and incubated for 2 h at 4 °C with rotation at 40 rpm. Sheared chromatin was added, and mixture was incubated at 4 °C overnight with rotation. The chromatin-antibody complex was washed twice with radioimmunoprecipitation assay buffer, and 40 μl of Chelex 100 slurry (10% w/v) was added to the washed beads, and samples were boiled for 10 min. The samples were then treated with proteinase K for 30 min at 55 °C, and the samples were boiled for 10 min. Enrichment of KLF4-associated DNA sequences in immunoprecipitated samples and input samples was detected by quantitative RT-PCR using sequence specific primers and LightCycler 480 SYBR Green I Master mix. ChIP primers were as follows: p21Cip1 proximal promoter located at nucleotides −150/−4 (5‘-GCTGGGCGACGAGCCTG-3’, 5‘-CTGTCACACCTCAGCTGCC-3’) and p21Cip1 distal promoter located at nucleotides −827/−673 (5‘-CTGCTGCAACCAGGGATTTCTT-3’, 5‘-TGTTGATTTGCACATGCTTTTCCG-3’).

**RESULTS**

**PKÇδ Increases p21Cip1 mRNA Level and Promoter Activity**—Although overexpression of PKÇδ in epidermis in vivo suppresses cell division (33), few studies have assessed the underlying mechanism. The present studies focus on p21Cip1 as a PKÇδ target and potential mediator of growth suppression. Fig. 1A shows that PKÇδ expression produces a concentration-dependent increase in p21Cip1 promoter activity. Rottlerin, a PKÇδ inhibitor, inhibits this increase, but Go6976, an inhibitor of PKCa and β, does not (Fig. 1B). We next tested the impact of dominant-negative PKÇδ and PKÇδ-siRNA on p21Cip1 promoter activity. As shown in Fig. 1 (C and D), expression of dominant-negative PKÇδ or treatment with PKÇδ-siRNA reduces p21Cip1 promoter basal activity. The immunoblot in Fig. 1E confirms a PKÇδ-siRNA-dependent partial knockdown of PKÇδ. We have previously shown that PKÇδ-Tyr-311 phosphorylation is required for optimal PKÇδ activity (31). We therefore tested the ability of the PKÇδ-Y311F mutant to increase p21Cip1 promoter activity. As shown in Fig. 1F, PKÇδ-Y311F produces a reduced increase in promoter activity as compared with wild-type PKÇδ. Taken together, these findings suggest that PKÇδ regulates p21Cip1 expression.

To assure that this regulation is biologically meaningful, we monitored the impact of PKÇδ expression and knockdown on endogenous p21Cip1 mRNA and protein level. PKÇδ expression increases (Fig. 2A) and PKÇδ knockdown reduces p21Cip1 mRNA and protein level (Fig. 2B). To further make the case that this is transcriptional regulation and is not due to changes in mRNA stability, we monitored the impact of increased PKÇδ expression on p21Cip1 mRNA turnover. Although PKÇδ expression substantially increases p21Cip1 mRNA (Fig. 2A), p21Cip1 mRNA half-life is not changed in cells expressing basal versus elevated levels of PKÇδ (τ½ = 2.2 in Ad5-EV cells versus 1.94 in Ad5-PKÇδ cells) (Fig. 2C). Taken together, these studies strongly argue that PKÇδ controls p21Cip1 level via transcriptional mechanisms. It is interesting that PKÇδ-siRNA reduces p21Cip1 protein level by more than 80%, indicating that tonic PKÇδ activity is required to maintain endogenous p21Cip1 expression.

**PKÇδ Stimulates Nuclear Accumulation of p21Cip1**—The above studies indicate that PKÇδ drives p21Cip1 expression;
however, it is important to assess the impact on activity. Nuclear accumulation is an index of p21Cip1 activity (54). Fig. 3A, shows that the total p21Cip1 level increases, and this is associated with increased nuclear levels. Although the increase in nuclear content is largely a response to the overall increase in p21Cip1 level, there is also an impact on subcellular distribution. This is apparent when cells are stained to assess p21Cip1 location. We detect cytoplasmic p21Cip1 in 96% of Ad5-EV-infected cells. This cytoplasmic staining is evident in the upper panels of Fig. 3B. In contrast, in Ad5-PKCδ-infected cells, cytoplasmic staining is observed in only 1% of cells (lower panels). This consistent observation suggests that in addition to increasing p21Cip1 level, PKCδ promotes movement into the nucleus. As shown in Fig. 3C, these changes are associated with a reduction in BrdU incorporation from 45.2% in empty virus-infected to 1.3% in PKCδ-expressing cells, indicative of reduced cell proliferation in the presence of increased PKCδ.

PKCδ Regulation of p21Cip1 Promoter Activity—To study the mechanism of the PKCδ-dependent increase in p21Cip1 expression, we sought to identify PKCδ response elements in the p21Cip1 promoter. The p21Cip1 promoter upstream regulatory region encodes six GC-rich sites in the proximal promoter that are reported to bind Sp1 transcription factors (Sp1, Sp2, etc.), and two p53 binding sites in the distal promoter (Fig. 4A) (55). We constructed a p21Cip1 promoter truncation series and monitored the ability of PKCδ to activate expression of each construct. As shown in Fig. 4B, PKCδ produces a three-peak pattern of promoter activation. To our knowledge, this is a novel pattern of activation. PKCδ response regions are located at nucleotides −251/−60, −2001, and −2326. This complicated
pattern suggests that the p21Cip1 promoter encodes multiple PKC- responsive elements. The fact that these regions are separated by nonresponsive regions suggests the presence of inter- spersed enhancer and suppressor elements. This arrangement is typical of complex promoters (56). In the present manuscript, we focus on the element located within nucleotides 11002–11006.

**Role of the GC-rich DNA Elements**—As noted in Fig. 4A, the p21Cip1 promoter encodes six GC-rich DNA elements, previously characterized as Sp1 factor binding sites (Sp1-1, Sp1-2 etc.), clustered in the proximal promoter between nucleotides 11002–11006 (46). We assessed whether these sites are required for PKC- dependent regulation. We challenged full-length p21Cip1 promoter constructs encoding GC-rich site mutations with PKC. Fig. 4C shows that mutation of the Sp1-1, Sp1-2, Sp1-3, or Sp1-4 sites partially reduces PKC- stimulated activity but that elimination of all six Sp1 sites, Sp1(Δ1–6), is required to eliminate the response. This suggests that GC-rich element interacting proteins may drive PKC- dependent p21Cip1 transcription. A number of transcription factors and co-factors have been described as interacting at the proximal p21Cip1 promoter to regulate transcription, including Sp1 and Sp3 (55), the β-helix-loop-helix factor, AP4 (57), and the histone deacetylase, p300 (55). Kruppel-like transcription factors also interact at these sites (58). Fig. 5A shows that PKC- expression increases p21Cip1 mRNA level, and the level of klf4. In contrast, there is no change in Sp1, Sp3, AP4, or p300 level. The klf4 increase is associated with a parallel increase in KLF4 mRNA (Fig. 5B).

**Transcription Factor Interaction with GC-rich Elements**—These findings suggest that KLF4 may mediate the PKC- dependent increase in p21Cip1 promoter activity. We therefore
PKCδ and KLF4 Regulate p21Cip1 Expression  

A, keratinocytes were infected with 1S MOI of Ad5-EV or Ad5-PKCδ, and after 24 h, extracts were prepared for detection of the indicated transcription factors/co-factors and KLF4 mRNA. B, the KLF4 mRNA level was assessed by quantitative RT-PCR. The values are the means ± S.D. We observed similar results in three separate experiments.

Impact of KLF4 on p21Cip1 Promoter Activity—We next assessed the functional impact of KLF4 on p21Cip1 promoter activity. Cells were transfected with the p21Cip1 promoter reporter and full-length hKLF4, a hKLF4 mutant lacking the zinc finger domain, hKLF4(1–388), and a mutant encoding only the zinc finger domain, hKLF4(335–470) (58). Treatment with hKLF4(1–470) (wild type) increases transcription, but the inactive mutants, hKLF4(1–388) and hKLF4(335–470), do not (Fig. 7A). hKLF4(1–470) and hKLF4(1–388) were confirmed to be expressed at similar levels by immunoblot, thereby confirming that the difference p21Cip1 promoter activation is not due to a difference in KLF4 expression (Fig. 7A). Because anti-KLF4 binds to KLF4 within amino acids 1–180, expression of hKLF4 that we delivered to the cells in these experiments is comparable with that observed following stimulation with PKCδ (Fig. 7A). In addition, simultaneous expression of hKLF4(1–470) with PKCδ augments the PKCδ-dependent increase (Fig. 7B). Consistent with these findings, reducing KLF4 level with siRNA reduces p21Cip1 protein level and p21Cip1 promoter activity (Fig. 7C). In contrast, we could not demonstrate interaction of Sp1 or Sp3 by this method. We next assessed whether these proteins interact with the proximal promoter region (−150/−4) using chromatin immunoprecipitation. This method is extremely useful, because it can demonstrate interaction in situ in intact cells (59). Fig. 6D shows low KLF4 interaction in empty virus-infected cells and a 2.5-fold increase in this interaction in PKCδ-expressing cells. In contrast, KLF4 does not bind to the −827/−673 region, which lacks GC-rich binding sites.

Fig. 4A reveals a shifted band when \(^{32}\)P-3P1(1/2) probe is incubated with nuclear extract. An important finding is that the mobility of this band increases in extracts prepared from PKCδ-expressing cells, suggesting an altered composition or structure of the binding complex. Accumulation of this faster migrating band is time-dependent; it is variably present in cells treated for 24 h with PKCδ but is always present when cells are exposed to PKCδ for 48 h (Fig. 6A). Fig. 6B shows appropriate self-competition of radioinert probe against \(^{32}\)P-3P1(1/2), which indicates that the binding is specific. We next monitored Sp1, Sp3, and KLF4 interaction with \(^{32}\)P-3P1(1/2) by gel mobility supershift. Incubation of the extract with anti-KLF4 results in a loss of the supershifted band. This suggests that KLF4 interacts at this transcription site (Fig. 6C). In contrast, we could not demonstrate interaction of Sp1 or Sp3 by this method. We next assessed whether these proteins interact with the proximal promoter region (−150/−4) using chromatim immunoprecipitation. This method is extremely useful, because it can demonstrate interaction in situ in intact cells (59). Fig. 6D shows low KLF4 interaction in empty virus-infected cells and a 2.5-fold increase in this interaction in PKCδ-expressing cells. In contrast, KLF4 does not bind to the −827/−673 region, which lacks GC-rich binding sites.

Impact of KLF4 on p21Cip1 Promoter Activity—We next assessed the functional impact of KLF4 on p21Cip1 promoter activity. Cells were transfected with the p21Cip1 promoter reporter and full-length hKLF4, a hKLF4 mutant lacking the zinc finger domain, hKLF4(1–388), and a mutant encoding only the zinc finger domain, hKLF4(335–470) (58). Treatment with hKLF4(1–470) (wild type) increases transcription, but the inactive mutants, hKLF4(1–388) and hKLF4(335–470), do not (Fig. 7A). hKLF4(1–470) and hKLF4(1–388) were confirmed to be expressed at similar levels by immunoblot, thereby confirming that the difference p21Cip1 promoter activation is not due to a difference in KLF4 expression (Fig. 7A). Because anti-KLF4 binds to KLF4 within amino acids 1–180, expression of hKLF4 that we delivered to the cells in these experiments is comparable with that observed following stimulation with PKCδ (Fig. 7A). In addition, simultaneous expression of hKLF4(1–470) with PKCδ augments the PKCδ-dependent increase (Fig. 7B). Consistent with these findings, reducing KLF4 level with siRNA reduces p21Cip1 protein level and p21Cip1 promoter activity (Fig. 7C). Moreover, KLF4 knockdown is not associated with changed PKCδ level, suggesting that the reduced p21Cip1 promoter activity is not due to feedback reduction of PKCδ level.

We next examined whether KLF4 activation of p21Cip1 promoter activity requires the proximal GC-rich elements. KLF4...
**PKCδ and KLF4 Regulate p21<sup>Cip1</sup> Expression**

**A**

![Graph A](image1)

**B**

![Graph B](image2)

**C**

![Graph C](image3)

**D**

![Graph D](image4)

PKCδ and hKLF4 in regulating p21<sup>Cip1</sup>, we show that treatment with PKCδ-siRNA reduces KLF4 and p21<sup>Cip1</sup> protein level and that p21<sup>Cip1</sup> expression can be restored by virus-mediated expression of hKLF4 (Fig. 10).

**DISCUSSION**

**PKCδ Regulates Keratinocyte Differentiation and Proliferation**—Epidermal keratinocytes express the PKCδ, β1, 8, ε, η, and ζ isoforms (4–10). Among these, PKCδ is present in all epidermal layers (12, 20). A major role has been proposed for PKCδ in driving keratinocyte differentiation (15, 22, 23, 24, 31, 48). We have extensively studied the mechanism of PKCδ regulation of differentiation using involucrin as a target (28). Involucrin is exclusively present in the suprabasal epidermal layers (60). PKCδ enhances keratinocyte differentiation as measured by increased involucrin expression (21, 27, 30–32).

PKCδ activates a p38/ERK signaling complex that triggers changes in transcription factor function leading to increased involucrin gene expression (27, 28, 32, 61–66). The transcription factors include AP1, CCAAT enhancer-binding protein, and Sp1, which move to the nucleus and bind to specific sites on the involucrin promoter to activate expression (48, 67–71). This molecular mechanism has been confirmed using transgenic mouse models (72–76).

Various studies indicate that PKCδ also regulates keratinocyte proliferation. PKCδ expression in HaCaT keratinocytes reduces cell proliferation (23), and PKCδ also increases keratinocyte susceptibility to apoptosis (23, 32, 61). However, little information is available regarding the mechanism whereby PKCδ suppresses proliferation. In the present report, we identify that p21<sup>Cip1</sup> is a PKCδ target. BrdU uptake is an index of cell progression through the S phase and therefore cell proliferation. We show that PKCδ expression reduces the percentage of BrdU-positive cells from 45 to 1.3%. This change is associated with a substantial increase in p21<sup>Cip1</sup> mRNA and p21<sup>Cip1</sup> protein. Rottlerin, which inhibits the PKCδ isoform, suppresses this increase. Because rottlerin inhibits other targets in addition to PKCδ (77), we also show that treatment with PKCδ-siRNA and expression of dominant-negative PKCδ suppresses the response. In contrast, treating with PKCα/β inhibitor, Go6976, does not reduce the increase, suggesting that PKCα and β do not regulate p21<sup>Cip1</sup> expression. Phosphorylation of PKCδ at tyrosine 311 is associated with increased activity (31). We show that the phosphorylation-defective PKCδ mutant, PKCδ-Y311F, has reduced ability to increase p21<sup>Cip1</sup> promoter activity, further suggesting it has a key role. We further show that p21<sup>Cip1</sup> accumulates in the nucleus of PKCδ-expressing cells, suggesting that PKCδ may stimulate nuclear translocation.

**PKCδ Mediates the PKCδ Stimulus**—Sp1 factors and p53 are important regulators of p21<sup>Cip1</sup> expression (45, 55). Two p53 response elements are located in the distal p21<sup>Cip1</sup> promoter, and six GC-rich elements are present in the proximal promoter (nucleotides −140/−60) (46, 55). Although p53 induces p21<sup>Cip1</sup> expression in some systems (45), initial studies indicated that the increase we observe is not mediated by p53 (not shown). However, promoter truncation and mutagenesis experiments indicate that the GC-rich elements are required for regulation. These elements interact with Sp1 and Kruppel-

expression increases activity of the full-length wild-type p21<sup>Cip1</sup> promoter (p21-2326) (Fig. 8B). Basal and KLF4-stimulated promoter activity is increased for some (Δ2) and decreased for other (Δ1, Δ3, and Δ4) Sp1 site deletion mutants. However, deletion of all six sites (Δ1–6) results in a complete loss of KLF4-dependent regulation.

**PKCδ Is Required for PKCδ-dependent Growth Suppression and p21<sup>Cip1</sup> Induction**—We also examined the impact of KLF4 knockdown on the PKCδ-dependent increase in p21<sup>Cip1</sup> and reduction in cell number. Fig. 9A shows that PKCδ overexpression increases hKLF4 level and that this increase is suppressed by KLF4-siRNA. Consistent with our previous studies, keratinocyte cell number increases 2.5-fold during a 3-day growth time (Fig. 9B). This increase is enhanced by treatment with KLF4-siRNA and suppressed by PKCδ overexpression. In addition, knockdown of KLF4 partially reverses the PKCδ-dependent reduction in cell number. The PKCδ-dependent growth suppression is associated with a PKCδ-dependent increase in p21<sup>Cip1</sup> mRNA. Treatment with hKLF4-siRNA partially attenuates this increase (Fig. 9C). To further confirm the role of
PKCδ and KLF4 Regulate p21^{cip1} Expression

We observe increased Kruppel-like factor 4 expression and binding to the GC-rich elements in PKCδ/H9254-expressing cells. Moreover, wild-type KLF4 increases p21Cip1 promoter activity, but activity is not regulated by inactive KLF4 mutants. We had anticipated that Sp1 factors may be important in this regulation, but Sp1 and Sp3 did not interact with the p21Cip1 promoter GC-rich elements.

KLF4 is an interesting member of the Kruppel-like factor family of regulators (78). KLF4 is unusual compared with other Kruppel-like factors in that it can activate or suppress transcription in a context-dependent manner (78). KLF4 has a role in epidermis where it is expressed in suprabasal cells (79). Its presence is essential for epidermal barrier formation (79), because KLF4 knock-out mice do not form a competent barrier and do not survive (80), and mice that overexpress KLF4 in epidermis display enhanced barrier formation (81). KLF4 also has a role in regulating proliferation in tissues such as the oral epithelium (79) and colon (82). However, only limited information is available regarding the mechanism of this regulation. In
the colonic epithelium, KLF4 reduces proliferation by suppressing cyclin D1 level (82) via a mechanism that involves Sp1 binding to the cyclin D1 gene promoter (83). Our studies are novel in that the link PKC\(\alpha\)/H9254 activation to increased KLF4 binding to the p21Cip1 promoter and increased p21Cip1 expression. It is important to note that multiple regulatory mechanisms control p21Cip1 level in cells (45, 45, 55). Our present studies suggest an important role for KLF4 in mediating the PKC\(\alpha\)/H9254-dependent increase in p21Cip1. However, it would be naïve to suggest that KLF4 is the only transcription factor that mediates this regulation, and it is very likely that other transcription factors have a context-dependent role.

Coordinate Regulation of Keratinocyte Proliferation and Differentiation—Previous studies indicate that a PKC\(\alpha\)/H9254, Ras, MEKK1, MEK3, and p38\(\delta\)/ERK cascade increases AP1 and Sp1 factor expression and nuclear accumulation and that these factors bind to elements in the involucrin promoter to drive gene expression (Fig. 11) (27, 28, 32, 63, 66). Our present studies suggest that this same cascade increases KLF4 expression and binding to the p21Cip1 promoter to increase p21Cip1 expression and thereby suppress cell proliferation (Fig. 11). This regulation is particularly interesting, because it indicates a mechanism whereby PKC\(\alpha\) can coordinately control keratinocytes differentiation and proliferation. This appears to make mechanistic sense because cessation of proliferation and initiation of differentiation must occur simultaneously during epidermal development. These findings are also interesting because a limited number of studies have examined the impact of PKC isoforms on keratinocyte proliferation. PKC\(\delta\) binds to and activates Fyn, a Src kinase, and this is associated with reduced keratinocyte proliferation (36). PKC\(\alpha\) also forms a complex with cyclin E-cdk2-p21Cip1 leading to phosphorylation of p21Cip1 and cdk2 inhibition to reduce proliferation (37). In addition, PKC\(\delta\) suppresses proliferation in raft cultures of human keratinocytes via a mechanism that involves increased expression of p21Cip1 (17). The present studies identify a new mechanism whereby PKC\(\delta\) regulates both proliferation and differentiation. Additional studies will be necessary to gain insight regarding the molecular details of this regulation.

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