Protein Kinase Cε Is Overexpressed in Primary Human Non–Small Cell Lung Cancers and Functionally Required for Proliferation of Non–Small Cell Lung Cancer Cells in a p21/Cip1-Dependent Manner

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Abstract

The protein kinase C (PKC) family of proteins plays important roles in growth regulation and is implicated in tumorigenesis. It has become clear that the role of PKC in tumorigenesis is cell context dependent and/or isoform specific. In this study, we showed for the first time by immunohistochemistry that overexpression of PKCε was detected in the vast majority (>90%) of primary human non–small cell lung cancers (NSCLC) compared with normal lung epithelium. Inhibition of the PKCε pathway using a kinase-inactive, dominant-negative PKCε, PKCε(KR), led to a significant inhibition of proliferation and anchorage-independent growth of human NSCLC cells in a p53-independent manner. This was accompanied by a specific induction of the cyclin-dependent kinase (cdk) inhibitor p21/Cip1 but not p27/Kip1. In response to serum stimulation, PKCε(KR)-expressing cells showed a prolonged G_1-S transition and delayed and reduced activation of cdk2 complexes, which was likely attributed to the increased binding of p21/Cip1 to cdk2. Furthermore, inhibition of PKCε function either by expressing PKCε(KR) or by small interfering RNA (siRNA)–mediated gene knockdown resulted in c-Myc down-regulation, which, in turn, regulated p21/Cip1 expression. Knockdown of PKCε or c-Myc expression using siRNA led to induction of p21/Cip1 and attenuation of G_1-S transition in NSCLC cells. Using *p21*$^{+/+}$ and *p21*$^{-/-}$ HCT116 isogenic cell lines, we further showed that growth inhibition by PKCε(KR) required the function of p21/Cip1. Collectively, these results reveal an important role for PKCε signaling in lung cancer and suggest that one potential mechanism by which PKCε exerts its oncogenic activity is through deregulation of the cell cycle via a p21/Cip1–dependent mechanism. [Cancer Res 2007;67(13):6053–63]

Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States, with an estimated 174,470 new cases and 162,460 deaths in 2006 (1). Tobacco smoking is the most prevalent cause of lung cancer with 80% to 90% of the disease arising in cigarette smokers. Lung tumorigenesis is a multistep process involving both genetic and epigenetic alterations in oncogenes and tumor-suppressor genes, and changes in activation of signal transduction pathways, resulting in progressive deregulation of cell proliferation and survival mechanisms (2, 3). Alterations in protein kinase C (PKC) expression and/or activity have been reported in human lung cancers (4, 5), and tobacco-related carcinogens have been shown to promote proliferation and survival of normal and neoplastic lung cells through PKC-dependent mechanisms (6–8). Thus, dysregulation of the PKC signaling is believed to contribute to lung tumorigenesis. The PKC family consists of 11 structurally related, phospholipid-dependent serine/threonine kinases that play important roles in proliferation, transformation, differentiation, and apoptosis (9–11). The heterogeneity of PKC isoform expression in human cancers and their distinct, sometimes paradoxical, roles in cellular functions highlights the need for understanding the role of each individual PKC isoform in the carcinogenic process.

PKCε is unique in its oncogenic potential. When overexpressed, PKCε acts as an oncogene that induces transformation in fibroblast and colonic epithelial cells (12, 13). Its transforming activity in these cells seems to be exerted by affecting the Ras-Raf-1 signaling pathway (12–14). PKCε has also been shown to mediate cyclin D1 induction and promote cell proliferation (15, 16). A growing body of evidence indicates that PKCε plays a critical role in tumor cell invasion and metastasis. PKCε is involved in the regulation of cell adhesion, cell spreading, and motility through integrin β1–dependent mechanisms (17, 18). Increased PKCε levels are associated with invasion and/or metastasis of human glioma and breast cancer (19, 20). Targeted disruption of PKCε inhibits cancer cell invasion and motility (21), and its overexpression results in developing highly malignant/metastatic skin carcinomas in mice (22). Emerging evidence indicates that PKCε also plays an important role in regulating tumor cell survival via its antiapoptotic function (23–25). These data suggest that alterations in the PKCε signaling could have profound effects in tumorigenesis.

In this report, we showed, for the first time, that significant increases in PKCε expression were detected in the vast majority of primary human non–small cell lung cancers (NSCLC) compared with normal lung epithelium. We found that genetic inhibition of PKCε by expressing a dominant-negative mutant led to significant inhibition of proliferation and transforming capacity of human NSCLC cells in a p53-independent manner, suggesting that the PKCε signaling is important for maintaining the transformed phenotype. Our results reveal that one potential mechanism by which PKCε exerts its oncogenic activity is through deregulation of the G_1-S transition of a cell cycle via a p21/Cip1–dependent mechanism.

Materials and Methods

Antibodies and plasmids. Antibody information can be found in Supplemental Information. A mammalian expression plasmid encoding a

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

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Table 1. Immunohistochemical analysis of PKCε expression

(A) Expression of PKCε in normal and malignant lung tissues

	n (%)	Expression of PKCε*			
		Neg (0)	Low (1+)	Med (2+)	High (3+)
Normal lung epithelium	16	2 (13)	14 (87)	0	0
NSCLC	37	1 (3)	1 (3)	16 (43)	19 (51)
Adenocarcinoma	19 (51)	1	1	4	13
Squamous cell carcinoma	18 (49)	0	0	12	6

(B) PKCε expression and patient characteristics

Variable	No. cases, *n* (%)	PKCε expression†	
		Mean ± SD	*P*‡
Total	37 (100)		
Age at time of diagnosis (y)§			
<64	17 (45.9)	135.8 ± 16.6	NS
≥64	20 (54.1)	142.0 ± 19.7	
Sex			
Male	21 (56.8)	ND	
Female	16 (43.2)	ND	
Histology			
Adenocarcinoma	19 (51.4)	146.0 ± 19.2	0.018
Squamous cell carcinoma	18 (48.6)	132.0 ± 14.7	
Stage			
I	19 (51.4)	140.1 ± 21.6	NS
II	7 (18.9)	133.1 ± 15.1	
III–IV	10 (27.0)	141.8 ± 15.0	
Unknown	1 (2.7)		
Tumor size			
T_1	16 (43.2)	146.4 ± 18.3	0.038
T_2–T_4	20 (54.1)	133.5 ± 17.2	
Unknown	1 (2.7)		
Lymph node involvement			
N_0	25 (67.6)	139.0 ± 19.4	NS
N_1–N_2	12 (32.4)	139.6 ± 16.8	

Abbreviations, ND, not defined; *n*, number of cases; NS, not significant.
*PKCε expression was assessed by manual scoring as described in Materials and Methods.
†PKCε expression was assessed by automated scanning and quantification in a scale of 0 to 255 using the Ariol SL-50 system. The values of PKCε staining were between 107 and 175.
‡ *P* values were calculated by unpaired Student's *t* test. *NS*, no statistical difference ($P > 0.05$).
§Age (mean ± SD), 63.6 ± 12.0 y.

FLAG-tagged PKCε(K437R) was described previously (26). The human p21/Cip1 promoter construct (WWP-LUC-1; ref. 27) was kindly provided by Dr. Vogelstein (Johns Hopkins University, Baltimore, MD).

Cell culture, growth curves, and soft agar assay. Human NSCLC lines (NCI-H23, NCI-H157, NCI-H358, and NCI-H460) were obtained from American Type Culture Collection and maintained as described previously (23). Human colon cancer cell lines, HCT116-$p21^{+/+}$ (wild-type), HCT116-$p21^{-/-}$ (clone 8054), HCT116-$p53^{+/+}$ (clone 40-16), and HCT116-$p53^{-/-}$ (clone 379.2), were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum. Growth studies and soft agar assays were done as described previously (28).

Stable PKCε(K437R)–expressing clones. Cells (1×10^6) were transfected with 5 μg of pcDNA3 (vector) or pcDNA3-FLAG-PKCε(K437R) using LipofectAMINE (20 μg/mL) in 3 mL of serum-free medium. Transfected cells were selected with 400 to 800 μg/mL G418 until resistant colonies formed. Individual PKCε(K437R)-expressing clones were expanded and tested for the expression of FLAG-PKCε(K437R) by immunoprecipitation with anti-FLAG followed by immunoblotting with anti-PKCε. A pool of G418-resistant clones from vector-transfected cells were used as controls and referred to as "vector" in all experiments.

Clonogenic growth assay. HCT116 isogenic cells were cotransfected with pcDNA3-FLAG-PKCε(K437R) or pcDNA3 along with a 1/10 amount of pSilencer4.1-puro (Ambion) using LipofectAMINE. At 48 h after transfection, cells were replated at a low density in 60-mm dishes and grown for 10 to 14 days in the presence of puromycin (1 μg/mL). Colonies were scored after crystal violet staining.

Cell cycle analysis. Cells were arrested in quiescence by serum starvation in serum-free medium supplemented with 1% bovine

serum albumin (BSA) for 48 h and subsequently stimulated with 10% serum to reenter the cell cycle. Cells were prepared for flow cytometry analysis as described previously (23).

Immunohistochemistry. Paraffin-embedded human lung tissue specimens were obtained from the archives of the Department of Pathology and the Molecular Tissue Bank at the University of Florida, including normal lung tissues and NSCLC specimens collected between 1997 and 2000. This study was approved by the University of Florida Institutional Review Board.

Immunohistochemical staining for PKCε was done on 4 μm sections of individual, paraffin-embedded lung specimens with the labeled avidin-biotin technique using DAKO LSAB2 system (DAKO Corp.). Expression of p21/Cip1 and c-Myc were evaluated by immunohistochemical analysis in a lung tissue array of IMH-358(CCA) (Imgenex, San Diego, CA), in which PKCε expression has been examined previously. Primary antibodies used were rabbit polyclonal anti-PKCε (C-15; 1–2 μg/mL), mouse monoclonal anti-p21/Cip1 (DCS60, 1:50), and anti–c-Myc (9E10; 1:50). Assay details and analysis of the results are given in Supplemental Information.

Immunoblotting, immunoprecipitation, and PKC activity assays. Immunoblotting and immunoprecipitation were done as described previously (26, 28). PKC activity was determined by immunocomplex kinase assays (26). To assess the activity of classic PKCs, 1 mmol/L EGTA was replaced with 100 μmol/L $CaCl_2$ in kinase buffer.

Cyclin-dependent kinase 2 and cyclin-dependent kinase 4 kinase assays. Cell lysates (100–500 μg) were incubated with 1 to 2 μg of anti–cyclin-dependent kinase 2 (cdk2) or anti-cdk4 for 2 h at 4°C, followed by incubation with protein A-agarose beads. The immunoprecipitates were washed thrice in lysis buffer and once in kinase buffer [50 mmol/L HEPES (pH 7.4), 10 mmol/L $MgCl_2$, 2.5 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L NaF, and 0.1 mmol/L $NaVO_3$]. Kinase reactions were carried out by incubating the beads at 30°C for 30 min in 30 μL of kinase buffer supplemented with 2 μg of histone H1 (Roche) for cdk2 or 1 μg of glutathione *S*-transferase-retinoblastoma (GST-Rb) for cdk4, 100 μmol/L ATP, and 5 μCi of [γ-^{32}P]ATP. The phosphorylated histone H1 or GST-Rb was resolved by SDS-PAGE followed by autoradiography and quantified by InstantImager (Packard Instruments).

Luciferase reporter assay. Cells (4×10^5 per well in six-well plates) were cotransfected with 1 μg of the human p21 promoter luciferase construct (WWP-LUC-1) and 0.2 μg of pSV-β-galactosidase (Promega) using LipofectAMINE in triplicate cultures. At 24-h posttransfection, cells were washed and cultured in serum-free medium supplemented with 1% BSA for an additional 24 h. Cell extracts were prepared in 1 × Reporter Lysis Buffer (Promega) and assayed for luciferase and β-galactosidase activities. The relative luciferase activity of each sample was normalized by the respective β-galactosidase activity.

Preparation of nuclear extracts. Cells were resuspended in a buffer of 10 mmol/L Tris-Cl (pH 7.4), 10 mmol/L NaCl, 0.03% NP40, 3 mmol/L $MgCl_2$, 1 mmol/L $NaVO_3$, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin by gentle vortexing. Nuclei were recovered by centrifugation at 700 × *g* for 5 min at 4°C. Nuclear extracts were obtained by incubating isolated nuclei in a buffer of PBS/0.5 mol/L NaCl containing phosphatase and protease inhibitors for 30 min at 4°C, followed by centrifugation at 10,000 × *g* for 10 min at 4°C.

RNA interference. The pools of small interfering RNA (siRNA) for human PKCε (Dharmacon), duplex c-Myc siRNA (Ambion), and the corresponding control siRNAs were used in gene knockdown experiments. siRNAs (100 nmol/L) were transfected into H157 cells using Oligofectamine. Significant down-regulation of c-Myc and PKCε protein was observed 48 h and 72 to 96 h after siRNA transfection, respectively.

Statistical analysis. Differences in anchorage-independent growth and treatment effects were evaluated by Student's *t* tests. Correlation between PKCε expression and p21/Cip1 localization was determined by χ^2 tests. *P* values <0.05 were considered to be statistically significant.

Results

PKCε expression in NSCLC specimens. NSCLCs represent ~80% of all lung cancers. It was reported that PKCε was highly expressed in NSCLC cells but was absent in normal human lung epithelial cells (23, 29). The lung has been estimated to consist of 40 or more different cell types (30). To reveal cell-specific distribution, we assessed PKCε expression by immunohistochemical analysis in 37 NSCLC specimens, including 19 cases of adenocarcinoma and 18 cases of squamous cell carcinoma (SCC), representing the two major subtypes of NSCLC. Intermediate (med) to high levels of PKCε staining were detected in 95% (35 of 37) of NSCLC specimens, 51% (19 of 37) of which showed high levels of PKCε staining (Table 1A). In contrast, negative or low levels of PKCε staining was observed in bronchiolar epithelium and alveolar cells of normal lung tissues (Fig. 1*A*, *first* versus *second panel*). We evaluated PKCε expression in relationship with various clinicopathologic variables. As indicated in Table 1B, PKCε expression was significantly higher in adenocarcinomas than in SCC ($P = 0.018$), and in patients with T_1 tumors than those with T_2 to T_4 tumors ($P = 0.038$). No significant correlations were observed between PKCε expression and age, pathologic stages, and lymph node involvement. The elevated expression of PKCε in NSCLC was further confirmed by immunohistochemical staining of NSCLC tissue microarrays containing an additional 51 specimens obtained from the NCI Tissue Array Research Program, in which 45% and 51% of the specimens showed intermediate and high levels of PKCε staining, respectively (data not shown). These results indicated that PKCε levels were elevated in the majority of human NSCLCs when compared with normal lung epithelium.

Dominant inhibition of PKCε suppressed the growth of human NSCLC cells. The elevated expression of PKCε in primary NSCLCs suggests that PKCε might play an important role in maintaining the transformed phenotype of lung cancer cells. To test this hypothesis, we introduced a kinase-deficient mutant of PKCε [PKCε(K437R)] into NSCLC cells and assessed its effects on cell growth and transforming potentials. Human NSCLC lines NCI-H23 (adenocarcinoma) and NCI-H157 (SCC), which express endogenous PKCε and mutant p53, were stably transfected to express FLAG-PKCε(K437R) [also called PKCε(KR)]. The empty vector–transfected cells (H23-vector and H157-vector) were used as controls. Clones expressing PKCε(KR) were confirmed by immunoprecipitation with anti-FLAG antibody, followed by immunoblotting using anti-PKCε antibody (Fig. 1*B*). Because PKCε(KR) acts in a dominant-negative fashion, we determined the effectiveness of this mutant in blocking PKCε activity by *in vitro* kinase assays. As shown in Fig. 1*B* (*bottom*), regardless of expression levels, cells expressing PKCε(KR) displayed significant decreases in PKCε activity compared with that in control cells. It is important to note that forced expression of PKCε(KR) did not alter levels of other PKC isoforms (Fig. 1*B, middle*), nor did it inhibit the activities of other PKC isoforms such as PKCα and PKCβII (data not shown). These data indicated that expression of PKCε(KR) in lung cancer cells was functionally effective and specific.

We did a series of studies to characterize the proliferative and oncogenic potentials of NSCLC cells expressing PKCε(KR). As shown in Fig. 1*C*, ectopic expression of PKCε(KR) resulted in a significant inhibition of anchorage-independent growth. Compared with the controls (vector), PKCε(KR)-expressing cells formed fewer colonies in soft agar. Anchorage-independent colony formation was reduced in a range of 61% to 74% in H157-PKCε(KR) cells and 70% to 93% in H23-PKCε(KR) cells. Growth kinetic studies over a time course of four to five doublings showed that PKCε(KR)-expressing cells displayed significant defects in proliferation (Fig. 1*D*). Decreases in proliferation and colony formation in soft agar in response to expressing PKCε(KR) were also observed in NCI-H460 (wild-type p53) and NCI-H358 (p53

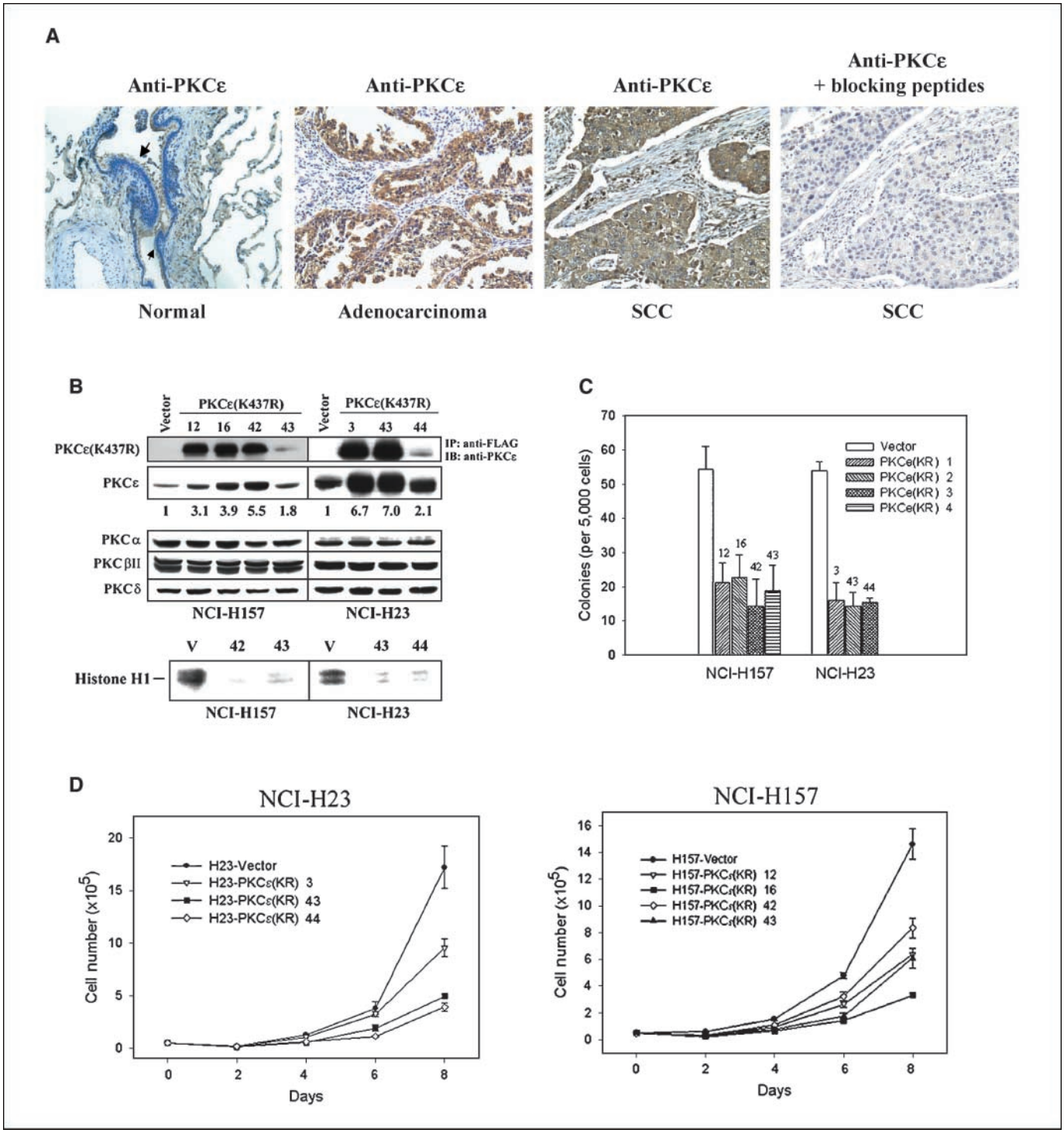


Figure 1. PKCε function is important for proliferation and transforming activities. *A,* immunohistochemical analysis of PKCε expression in human lung tissues. Tissue sections were stained by the labeled avidin-biotin technique using an anti-PKCε rabbit polyclonal antibody in the absence (*first* to *third panels*) or the presence (*fourth panel*) of the corresponding blocking peptide. *First* and *second panels,* matched normal lung tissue (*filled arrows*, bronchiolar epithelium) and adenocarcinoma, respectively; *third* and *fourth panels,* squamous cell carcinoma (*SCC*). *B* to *D,* dominant inhibition of PKCε results in growth suppression in lung NSCLC cells. *B,* biochemical characterization of clonal cell lines stably expressing a FLAG epitope–tagged PKCε(KR). *Top two panels,* expression of PKCε(KR) was detected by immunoprecipitation (*IP*) of whole-cell lysates from controls (vector) and individual PKCε(KR) clones with anti-FLAG antibody, followed by immunoblotting (*IB*) with anti-PKCε antibody. The total level of PKCε in cells [endogenous plus FLAG-PKCε(KR)] was shown by immunoblotting of whole-cell lysates with anti-PKCε antibody (*second row from the top*). The number under each lane indicates fold increases in total PKCε levels in PKCε(KR) clones relative to that in vector controls based on densitometric analyses. *Middle three panels,* Western blot analysis of PKC isoform expression. *Bottom, in vitro* kinase assays. Whole-cell lysates were subjected to immunoprecipitation with anti-PKCε antibody. Kinase activities of PKCε immunoprecipitates were assessed using histone H1 as substrates in the presence of [γ-^{32}P]ATP and cofactors. *C,* anchorage-independent growth. Cells were seeded in six-well plates in growth medium containing 0.3% agarose over a base layer of 0.6% agarose. Colonies were scored 14 to 21 d after seeding. *Columns*, mean of two independent experiments done in triplicate; *bars,* SD. *D,* growth curves in complete medium. Cells were plated at 5×10^4 per well in six-well plates. Cell numbers were determined by trypan blue exclusion every 48 h. *Lines*, mean of triplicate cultures of one representative experiment; *bars,* SD. Three independent experiments were done with similar results.

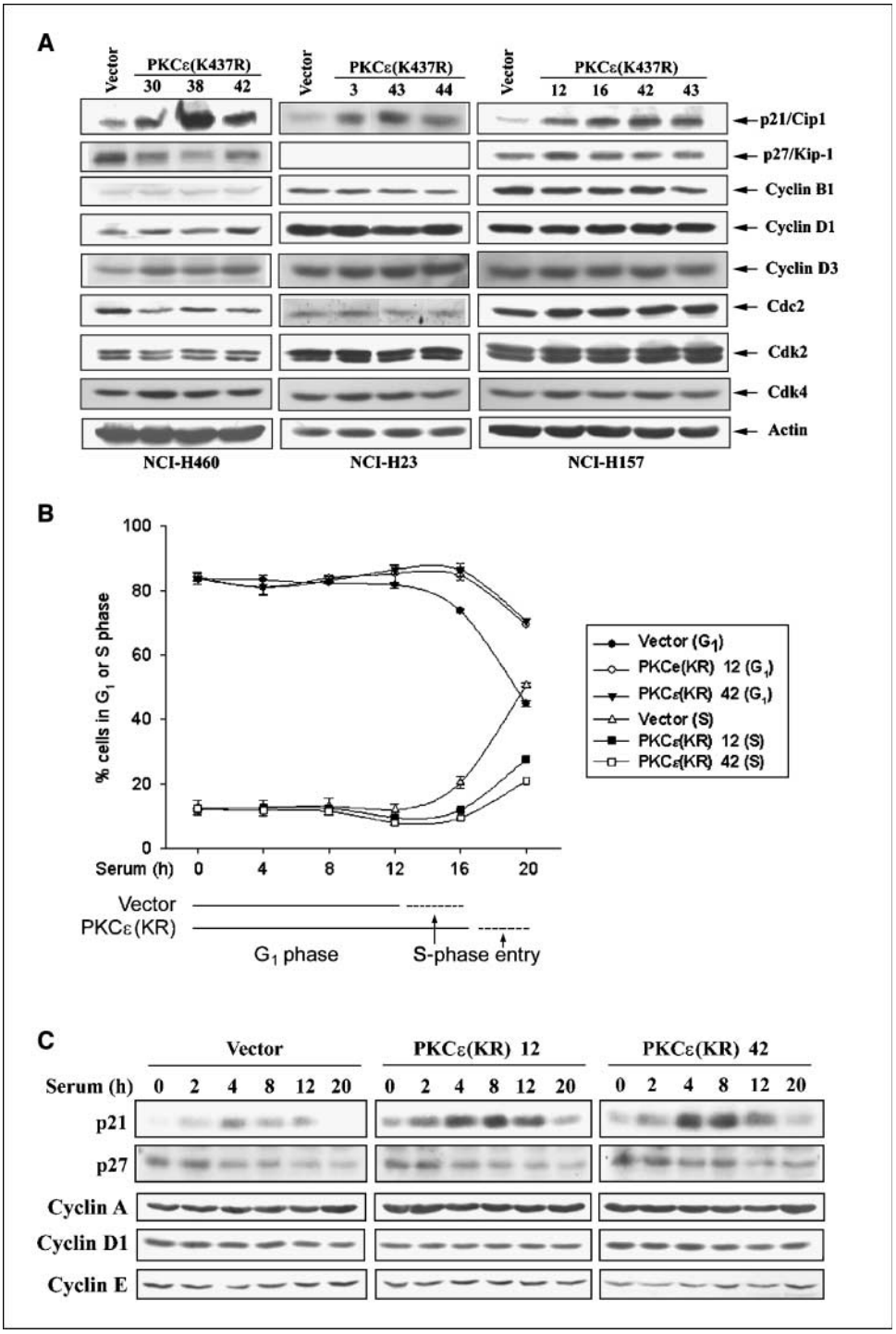


Figure 2. Dominant inhibition of PKCε results in up-regulation of p21/Cip1 and prolonging G_1-S transition. *A,* equal amounts of whole-cell lysates from exponential growing cells were separated by SDS-PAGE and transferred to membrane. The expression of cell cycle regulatory proteins was evaluated by immunoblotting with antibodies as indicated. Actin expression was detected for loading controls. *B,* flow cytometry analysis of cell cycle profiles. H157-vector and H157-PKCε(KR) clones (clones 12 and 42) were cultured in serum-free medium for 48 h and subsequently stimulated with serum to reenter the cell cycle. Cells collected at different times after stimulation were fixed, labeled with propidium iodide, and assessed for DNA contents by fluorescence-activated cell sorting. Histograms illustrate the distribution of cells in the G_0-G_1 and S phases over a time period of 20 h. Data are the average of two independent experiments. *Dashed lines at the bottom,* time points of the onset of the S phase. *C,* temporal expression of p21/Cip1 in response to serum stimulation. H157-vector and PKCε(KR) clones (clones 12 and 42) were synchronized in quiescence by serum starvation and induced to reenter the cell cycle by the addition of serum. Expression of p21/Cip1, p27/Kip1, cyclin A, cyclin D1, and cyclin E was assessed by immunoblotting of whole-cell lysates from cells harvested at indicated times after stimulation.

null) cells (Supplemental Table S1). Together, these results reveal an important role for PKCε in the regulation of proliferation and transforming activity of lung cancer cells.

Dominant inhibition of PKCε resulted in up-regulation of p21/Cip1 expression and delayed S phase entry. Activation of PKCε has been shown to induce cyclin D1 expression and enhance cell cycle progression (15). To determine whether the decline in proliferation in PKCε(KR)-expressing cells is accompanied with alterations in expression of cell cycle regulatory proteins, we did Western blot analysis. Results showed that the level of the CDK-inhibitory protein p21/Cip1 was selectively increased in PKCε(KR)-expressing cells (Fig. 2*A*; Supplementary Table S1), whereas p27/Kip1 expression was not detected in H23 cells and was not significantly altered in H157 and H460 cells. There were no appreciable differences in levels of cdc2 (cdk1), cdk2, cdk4, cyclin D1, cyclin D3, cyclin B1, cyclin A, and cyclin E (Fig. 2*A* and *C*).

p21/Cip1 has both positive and negative effects on cell cycle progression. p21/Cip1 is required for assembling and activation of cyclin D-cdk4/6 complexes in early G_1; however, high levels of p21/Cip1 potently inhibit cdk activity, resulting in G_1 or G_2 cell cycle

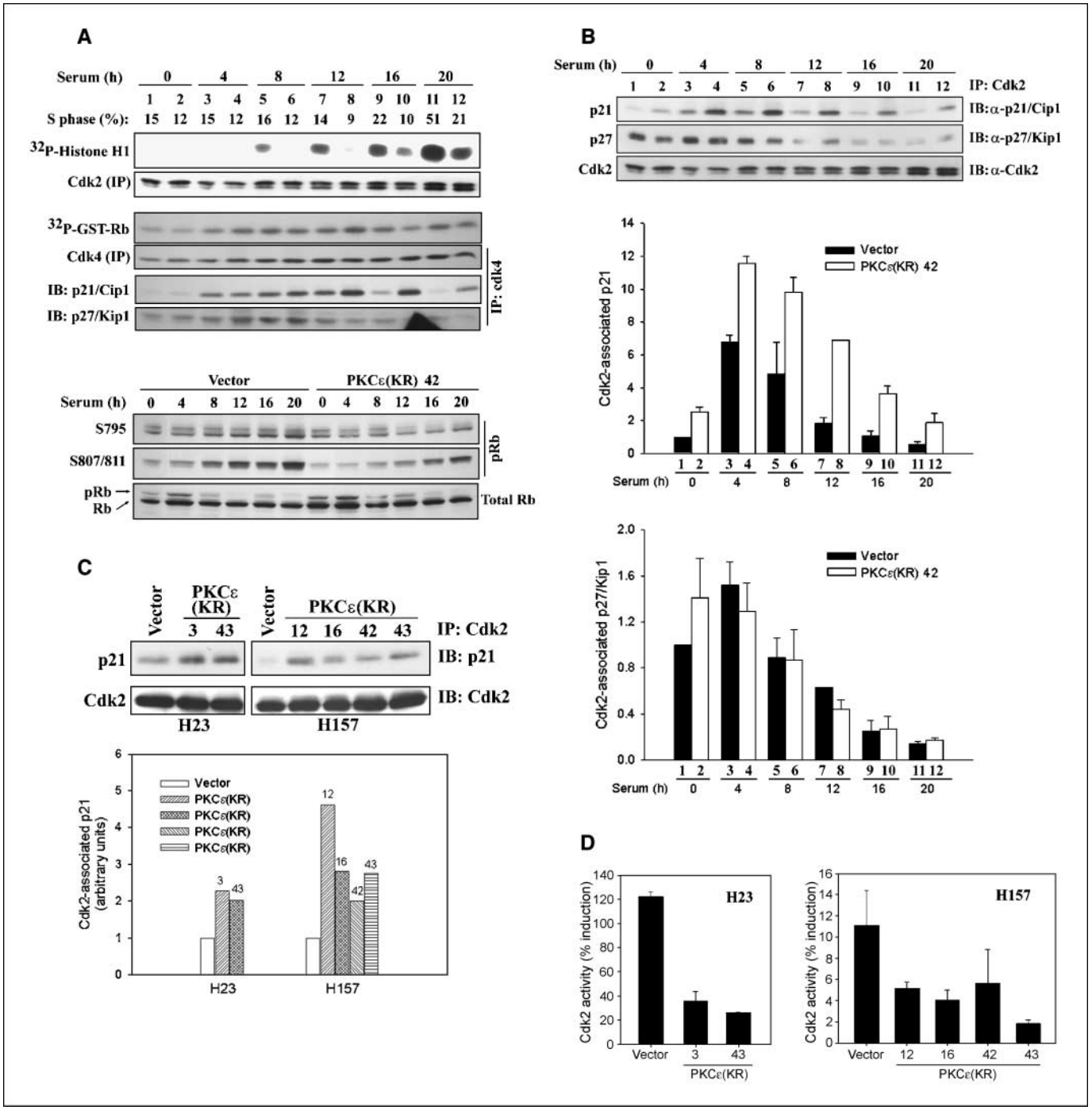


Figure 3. PKCε(KR) impairs cdk2 activation. *A* and *B,* H157 cells [vector and PKCε(KR) clone 42] were synchronized in quiescence by serum starvation and induced to reenter the cell cycle by the addition of serum. At indicated times after serum stimulation, whole-cell lysates were prepared and subjected to immunoprecipitation with anti-cdk2 or anti-cdk4. *Lanes 1, 3, 5, 7, 9,* and *11,* H157-vector; *lanes 2, 4, 6, 8, 10,* and *12,* H157-PKCε(KR) clone 42. Representative of three independent experiments with similar results. *A,* cdk2 and cdk4 activities were determined by *in vitro* kinase assay using histone H1 (*top section, two panels*) and GST-Rb (*middle section, top two panels*) as substrates, respectively. Levels of cdk4-associated p21/Cip1 and p27/Kip1 (*middle section, bottom two panels*) were assessed by immunoblotting of cdk4 immunoprecipitates with anti-p21/Cip1 and anti-p27/Kip1, respectively. The proportion of cells in the S phase at each time point is indicated. *Bottom section (three panels),* the status of Rb phosphorylation was determined by immunoblotting with Rb phosphorylation site-specific antibodies. Total Rb was determined using an antibody recognized as both hypophosphorylated and hyperphosphorylated Rb. *B,* levels of cdk2 associated p21/Cip1 and p27/Kip1 in H157 cells [vector and PKCε(KR) clone 42] were assessed by immunoblotting of cdk2 immunoprecipitates with anti-p21/Cip1 and anti-p27/Kip1, respectively (*top section*), analyzed by densitometry, normalized to respective cdk2 levels, and presented as columns. *Columns,* mean of three experiments; *bars,* SE. *C* and *D,* H23 and H157 cells were synchronized in quiescence by serum starvation and then stimulated with serum for 12 h. Whole-cell lysates were subjected to immunoprecipitation with anti-cdk2 antibody. *C,* cdk2-associated p21/Cip1 was detected by immunoblotting of cdk2 immunoprecipitates with an anti-p21/Cip1 antibody. Levels of cdk2 in immunoprecipitates were detected by immunoblotting with anti-cdk2 antibody. Cdk2-associated p21/Cip1 was analyzed by densitometry, normalized to respective cdk2 levels, and presented as fold increase relative to that in controls (vector; *columns*). Data are the averages of two independent experiments reaching almost identical results (<5% variations). *D,* Cdk2 activities were determined by *in vitro* kinase assay of cdk2 immunoprecipitates using histone H1 as substrate and quantified by InstantImager. Data are presented as percent induction (H23, *left*) or fold induction (H157, *right*) relative to that of unstimulated controls (0 time point). *Columns,* mean of three independent experiments; *bars,* SE.

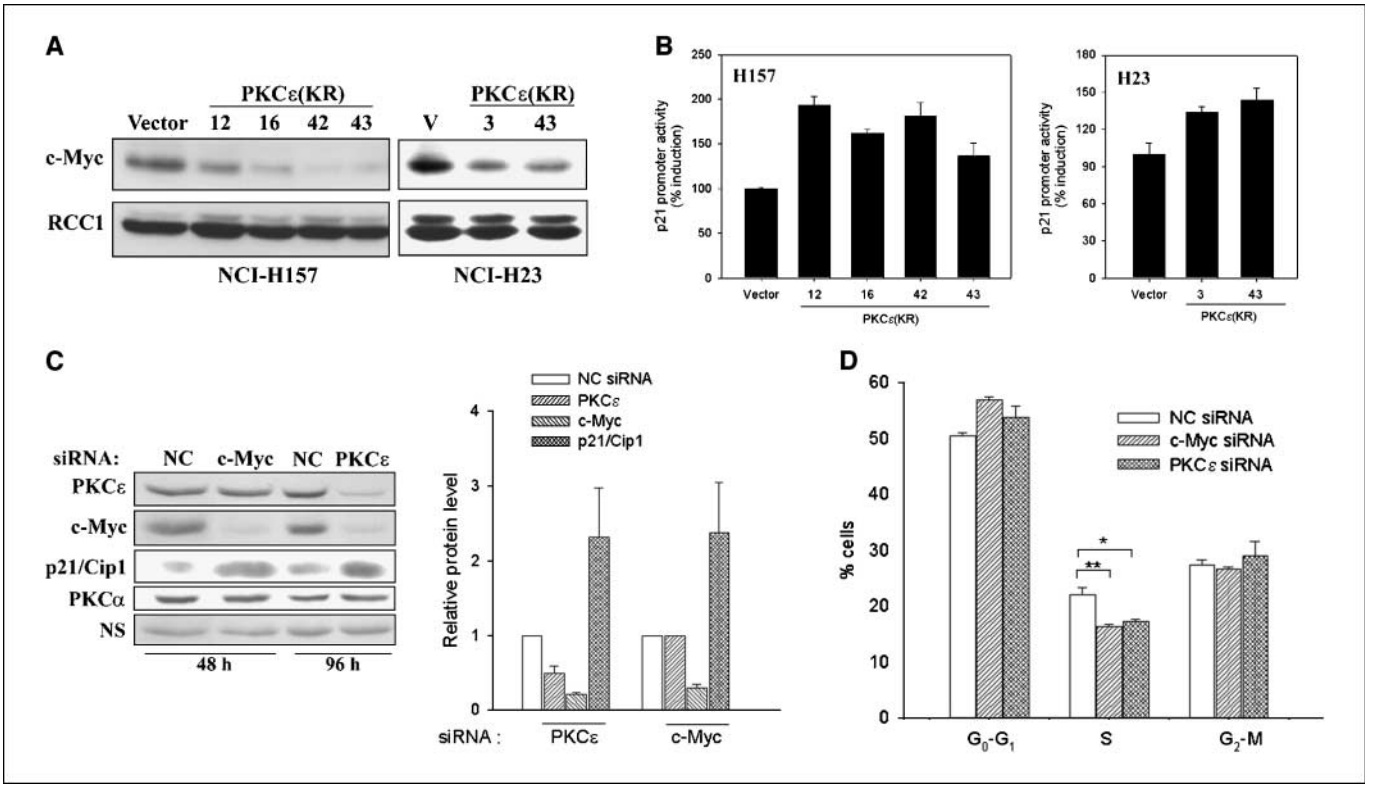


Figure 4. c-Myc is a downstream effector of the PKCε proliferating signaling pathway. *A,* c-Myc expression was determined by immunoblotting of nuclear extracts of H23 and H157 cells after subcellular fractionation. Expression of RCC1 (regulator of chromosome condensation), a chromatin-associated nuclear protein, was detected for loading controls. *B,* effects of PKCε(KR) on the p21/Cip1 promoter activity. Vector and PKCε(KR)-expressing clones were transiently transfected with the human p21/Cip1 promoter-luciferase construct (WWP-LUC-1) along with pSV-β-gal plasmid. Luciferase activity was measured after transfected cells were serum starved for 48 h and normalized with respective β-galactosidase activities. Results presented are relative luciferase activities compared with that in vector cells, which was designated as 100. *Columns,* mean of one representative experiment preformed in triplicate; *bars,* SE. *C,* knockdown of PKCε and c-Myc by RNA interference led to p21/Cip1 induction. H157 cells were transfected with 100 nmol/L siRNA for PKCε, c-Myc, or nontargeting control siRNA (*NC*). Levels of c-Myc and PKCε were assessed by Western blot analysis at 48 and 96 h after transfection, respectively (*left*). No significant changes in PKCα levels were observed. *NS,* nonspecific. Quantitation of protein expression was analyzed by densitometry, normalized to NS, and presented as fold changes relative to controls (*NC*; *right*). *Columns,* mean of three independent experiments; *bars,* SE. *D,* effects of siRNA-mediated gene knockdown on cell cycle progression. siRNA-transfected cells were serum starved for 24 h, and then stimulated with serum for 16 h, followed by fluorescence-activated cell sorting. The distribution of cells in G_0-G_1, S, and G_2-M phases was plotted as columns. *Columns,* mean of three independent experiments; *bars,* SE. *P* values were assessed by the Student's *t* test. *, $P = 0.03$ (PKCε siRNA versus NC siRNA); **, $P = 0.01$ (c-Myc siRNA versus NC siRNA).

arrest (31–33). To determine whether the PKCε(KR)-mediated p21/Cip1 up-regulation is associated with alterations in cell cycle progression, we compared cell cycle profiles of H157-vector and H157-PKCε(KR) (clones 12 and 42) by flow cytometry analysis. Cells were first cultured in serum-free medium for 48 h and then stimulated with 10% serum. After serum starvation, both control and PKCε(KR)-expressing cells were arrested in the G_0-G_1 phase [vector, 83% G_1, 15% S, 2% G_2-M; PKCε(KR), ~85% G_1, 12% S, 3% G_2-M]. However, H157-vector cells started entering into S phase at 12 h after serum stimulation, whereas the S phase entry in PKCε(KR)-expressing cells did not occur until 16 h after serum stimulation (Fig. 2*B*). At 16 h after serum stimulation, ~22% of control cells (H157-vector) were in S phase, whereas <12% of PKCε(KR)-expressing cells were in S phase, which was comparable with that at the 0 time point. At 20 h poststimulation, cells in S phase were 51% and 21% to 28% for vector and PKCε(KR)-expressing cells, respectively. These data indicate that the inhibition of proliferation by PKCε(KR) is mediated, at least in part, by prolonging the G_1-S transition.

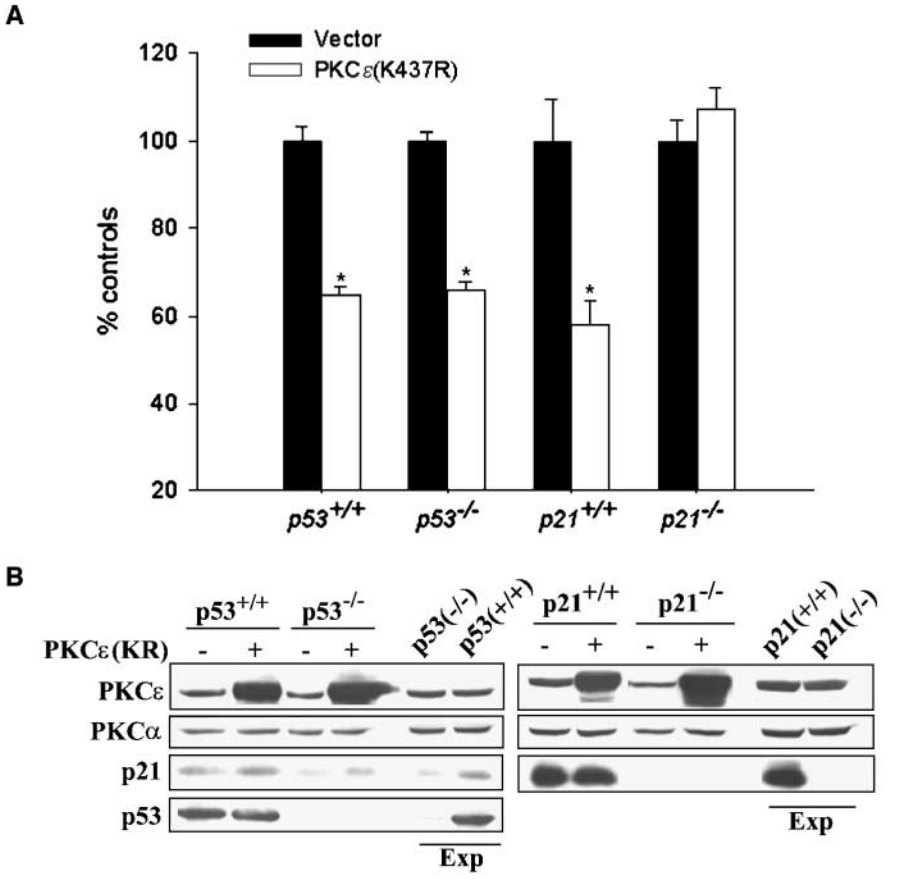
To understand the potential role of PKCε(KR)-induced up-regulation of p21/Cip1 in cell cycle progression, we investigated the temporal expression of p21/Cip1 in response to serum stimulation. As shown in Fig. 2*C*, H157-vector cells showed elevated p21/Cip1 between 2 and 12 h, and the level subsequently reduced to a very low level between 12 and 20 h after serum stimulation. In contrast, PKCε(KR)-expressing cells showed much higher basal levels of p21/Cip1 (0 time point), which were further induced by serum. The elevated p21/Cip1 levels were maintained for over 20 h after serum stimulation. In contrast, p27/Kip1 expression was down-regulated after serum stimulation. There was no significant difference in the temporal expression of p27/Kip1, cyclin A, cyclin D1, and cyclin E between H157-vector and H157-PKCε(KR) cells (Fig. 2*C*). Thus, the sustained p21/Cip1 up-regulation might be responsible for the delayed S-phase entry in PKCε(KR)-expressing cells.

Expression of PKCε(KR) was associated with increased binding of p21/Cip1 to cdk2 and inhibition of cdk2 kinase activity. Progression through late G_1 into S phase requires activation of cdk2. p21/Cip1 is a potent inhibitor of cdk2-containing complexes, which directly binds to cyclin-cdk2 complexes, thereby inhibiting its kinase activity (31, 32). As PKCε(KR) affects the G_1-S transition in association with up-regulation of p21/Cip1, we sought to investigate whether cdk2 is targeted for inactivation in PKCε(KR)-expressing cells. H157 cells were

synchronized at G_0-G_1 by serum deprivation and subsequently released from cell cycle arrest by serum stimulation. Cdk2 immunoprecipitates were examined for cdk2 activities by kinase assay (Fig. 3*A*) and for expression of cdk2-associated p21/Cip1 and p27/Kip1 by immunoblotting (Fig. 3*B*). Compared with that in H157-vector cells, a significant delay in activation of cdk2 as well as reduction in its activation magnitude were observed in H157-PKCε(KR) cells (clone 42) after serum stimulation (Fig. 3*A*). Accompanied with the impaired activation of cdk2 complexes, it was found that over a 20-h time course of serum stimulation, much higher levels of p21/Cip1 were detected in cdk2 immunoprecipitates in PKCε(KR)-expressing cells than that in vector controls, whereas little differences in levels of cdk2-associated p27/Kip1 were observed (Fig. 3*B*). Thus, the sustained elevation of p21/Cip1 and its association with cdk2 is responsible for the delay in activation of cdk2 in PKCε(KR)-expressing cells.

To further confirm the importance of p21/Cip1 in inactivating cdk2 complexes, we investigated the relationship between p21/Cip1 binding to cdk2 and cdk2 activation in additional PKCε(KR) clones at 12 h after serum stimulation, at which control cells (vector) started entering the S phase. Compared with controls, cdk2-associated p21/Cip1 was significantly increased in all PKCε(KR)-expressing cells (Fig. 3*C*). Densitometric analyses indicate that levels of cdk2-associated p21/Cip1 were 2- and 2- to 5-fold higher in PKCε(KR)-expressing H23 and H157 cells, respectively. Importantly, kinase assays showed that an increased binding of p21/Cip1 to cdk2 was directly associated with decreased cdk2 kinase activities (Fig. 3*D*).

Recent studies indicate that function of cdk2 and cdk4 is important for timing S-phase entry and in the control of embryonic cell proliferation (34, 35), and inhibition of Rb by phosphorylation represents a critical checkpoint of the G_1-S transition (36). We investigated the effects of PKCε(KR) on cdk4 activation and Rb phosphorylation. As shown in Fig. 3*A* (*middle section, top two panels*), there was little difference in cdk4 activation after serum stimulation in H157-vector and H157-PKCε(KR) cells (clone 42).



C

PKCε level	Total (n = 38)	p21/Cip1, n (%) (P < 0.05)			c-Myc, n (%)	
		Negative (n = 13)	Nuclear (n = 6)	Cyto (n = 19)	Negative (n = 14)	Positive (n = 24)
Low (1+)	7	0 (0%)	2 (29%)	5 (71%)	4 (57%)	3 (43%)
Med (2+)	18	5 (28%)	4 (22%)	9 (50%)	6 (33%)	12 (67%)
High (3+)	13	8 (62%)	0 (0%)	5 (38%)	4 (31%)	9 (69%)

Figure 5. p21/Cip1 is required for PKCε(KR)-induced growth suppression. *A,* clonogenic assay. HCT116 isogenic colon cancer cell lines ($p21^{+/+}$, $p21^{-/-}$, $p53^{+/+}$, and $p53^{-/-}$) were transfected with a PKCε(KR) expression construct or the empty vector along with a puromycin expression plasmid. At 48 h posttransfection, cells were replated in 60-mm dishes at a low density and grown in the presence of puromycin for 10 to 14 d. Colonies were scored after crystal violet staining. *Columns,* mean of two independent experiments done in triplicate; *bars,* SD. *, P < 0.01 compared with the vector-transfected cells. *B,* expression of PKCε(KR) was detected by Western blot analysis of whole-cell lysates from transfected cells. Membranes were striped and reblotted with anti-PKCα antibody to control loading. Status of p21/Cip1 and p53 in each isogenic cell line was assessed by immunoblotting. *Exp,* untransfected, asynchronously growing cells as controls. *C,* inverse correlation between PKCε expression and subcellular localization of p21/Cip1 in NSCLC specimens. Expression of p21/Cip1 and c-Myc were evaluated by immunohistochemical analysis using NSCLC tissue arrays. The association between the levels of PKCε and the cellular localization of p21/Cip1 is statistically significant (P < 0.05, by χ^2 tests). *Cyto,* cytoplasmic.

Although increased binding of p21/Cip1 to cdk4 was observed at 12 h, this had no effect on cdk4 activity but was associated with the onset of cdk2 activation in PKCε(KR)-expressing cells (Fig. 3*A*, *top four panels*, compare *lane 7* versus *8* and *9* versus *10*), suggesting a release of the inhibitory effect of p21/Cip1 on cdk2 complexes. Consistently, Rb phosphorylation at Ser^{795} (targeted for cdk4 phosphorylation) was not significantly altered, whereas Rb phosphorylation at $Ser^{807/811}$ (preferred for cdk2 phosphorylation) was significantly reduced in PKCε(KR) cells (clone 42; Fig. 3*A*, *bottom section*). Similar effects of PKCε(KR) on cdk4 activation and Rb phosphorylation were also observed in other PKCε(KR) clones (Supplementary Fig. S1). Collectively, these data indicate that a loss of cdk2 activity in PKCε(KR)-expressing cells is responsible for delaying the G_1-S progression.

c-Myc is a downstream effector of the PKCε proliferating signaling pathway. It has been shown that ectopic expression of c-Myc promotes cell cycle progression and shortens the G_1 phase in cycling cells (37). Several lines of evidence suggest that PKCε is involved in the regulation of c-Myc expression (38, 39). The prolonged G_1-S transition observed in PKCε(KR)-expressing cells prompted us to investigate the effect of PKCε(KR) on c-Myc expression. As shown in Fig. 4*A*, c-Myc levels were significantly reduced in PKCε(KR)-expressing cells compared with that in vector cells. As transcriptional repression of p21/Cip1 is thought to be one of the mechanisms by which c-Myc regulates cell cycle (31, 40, 41), we assessed the p21/Cip1 promoter activities using a p21/Cip1 reporter consisting of a 2.3-kb fragment of the human p21/Cip1 promoter linked to the luciferase reporter gene (27). As shown in Fig. 4*B*, inversely correlated with c-Myc down-regulation, PKCε(KR)-expressing cells displayed increases in p21/Cip1 promoter activity compared with that in vector cells, with the mean induction of 34% to 44% ($P < 0.05$) and 40% to 90% ($P < 0.05$) in H23 and H157 cells, respectively. Importantly, knockdown of PKCε expression (~50% reduction) by siRNA resulted in significant down-regulation (~80% reduction) of c-Myc and up-regulation (2- to 3-fold) of p21/Cip1 (Fig. 4*C*, *columns*). Consistent with its role in the repression of p21/Cip1 expression, siRNA-mediated knockdown of c-Myc (~70% reduction) led to a 2- to 3-fold induction of p21/Cip1 (Fig. 4*C*), and ectopic expression of c-Myc repressed the p21/Cip1 promoter activity (data not shown). Furthermore, siRNA-mediated knockdown of PKCε or c-Myc resulted in attenuation of G_1-S transition in response to serum stimulation (Fig. 4*D*). At 16 h after serum stimulation, significantly fewer cells were in the S phase in PKCε siRNA–transfected cells ($P = 0.03$) and c-Myc siRNA–transfected cells ($P = 0.01$) compared with that in control siRNA–transfected cells. These data suggest that c-Myc may function downstream of PKCε, which could directly repress p21/Cip1 expression, thereby leading to cell cycle progression.

Growth suppression by PKCε(KR) required the function of p21/Cip1. Because enforced expression of PKCε(KR) was associated with the induction of p21/Cip1 expression in p53 mutant (H23 and H157), p53 null (H358), and p53 wild-type cells (H460), we investigated the functional significance of p53 and p21/Cip1 in mediating PKCε(KR) growth suppression using HCT116 isogenic human colon cancer cell lines, including $p21^{+/+}$, $p21^{-/-}$, $p53^{+/+}$, and $p53^{-/-}$ cells. Forced expression of PKCε(KR) caused a significant inhibition (a 42% reduction) of colony growth in $p21^{+/+}$ cells, but not in p21-deficient ($p21^{-/-}$) cells (Fig. 5*A*). In contrast, PKCε(KR) inhibited colony growth at the comparable level (~35% reduction) in both $p53^{-/-}$ and $p53^{+/+}$ cells (Fig. 5*A*, compare *columns 2* and *4* from left). Western blot analysis indicated that expression of PKCε(KR) resulted in a modest induction of p21/Cip1 independent of p53 status (Fig. 5*B*, compare *lane 2* versus *1* and *lane 4* versus *3* in *left*). However, no clear induction of p21/Cip1 was observed in $p21^{+/+}$ cells, which may be attributed to the high levels of basal p21/Cip1 in these cells. Collectively, results obtained using HCT116 isogenic cell lines are consistent with the findings in human lung cancer cells, suggesting that PKCε(KR)-induced growth suppression requires p21/Cip1 function, but is independent of p53.

To further determine the significance of p21/Cip1 in the PKCε proliferating signaling, we evaluated p21/Cip1 and c-Myc expression in relationship with PKCε levels in human NSCLC specimens. As p21/Cip1 nuclear localization is critical for its growth-inhibiting activity (33), we assessed p21/Cip1 based on its subcellular localization. Immunohistochemical analysis (Fig. 5*C*) indicates that increased PKCε levels were accompanied with significant decreases in the expression/distribution of p21/Cip1 ($P < 0.05$). Particularly, high levels of PKCε expression ($n = 13$) were associated with the absence of nuclear p21/Cip1 staining and increased expression of c-Myc. Although not statistically significant, c-Myc expression seems associated with PKCε expression in NSCLCs. Therefore, inhibition of p21/Cip1 is an important mechanism underlying PKCε growth-promoting function in human lung cancer.

Discussion

PKC has been implicated in both positive and negative regulation of cell cycle progression at two critical sites: the G_1-S and the G_2-M transitions. Numerous studies indicate that PKC-mediated control of these transitions is highly dependent on the timing of PKC activation during a cell cycle, the specific PKC isoforms involved, and/or the cell types being examined (42). The complexity of PKC signaling in cell cycle control may be attributed to the fact that multiple PKC isoforms are present in a given cell type, and commonly used PKC activators (e.g., phorbol esters) and PKC inhibitors can simultaneously affect several, if not all, PKC isoforms. PKC isoforms that are likely to play a negative role in control of the G_1-S transition include PKCδ and PKCη (43, 44), whereas PKCα seems to have either positive or negative roles in promoting G_1-phase progression in a cell-specific manner (45–47). In contrast, increasing evidence has pointed to a general, positive role for PKCε in control of G_1-S transition. Activation of PKCε is required for activation of the mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase/ERK signaling during phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate–induced cell cycle progression in the G_1 phase in C3H 10T1/2 cells (48). Expression of constitutively active PKCε induces cyclin D1 expression and enhances cell proliferation in NIH 3T3 cells (15). Furthermore, PKCε-dependent ERK1/2 activation facilitates *Mycobacterium leprae*–induced nuclear accumulation of cyclin D1 and G_1-S phase progression in human Schwann cells (49). These observations suggest that one mechanism by which PKCε enhances the G_1-S progression is through up-regulation of cyclin D1 expression via activation of the ERK signaling. Consistent with its positive role in regulation of G_1-phase progression, we found that the persistent inhibition of PKCε by PKCε(KR) resulted in prolonging G_1-S transition in human lung cancer cells. However, we did not observe any changes in the overall expression of D-type cyclins (D1 and D3) and nuclear accumulation of cyclin D1 in

PKCε(KR)-expressing cells in response to serum-induced cell cycle reentry (data not shown). This is consistent with no alterations in ERK1/2 activation in response to mitogens in these cells.[4] Furthermore, analysis of the effects of PKCε(KR) on other G_1 regulatory molecules shows no changes in expression of cdk2, cdk4, cdk6, and cyclin E. This suggests that modulation of G_1 cyclin/cdk expression could not account for the PKCε(KR)-induced delay in S-phase entry in lung cancer cells.

Cell cycle progression through G_1 phase into S phase is a major checkpoint for proliferating cells, which is controlled by the assembly and activation of cyclin D–cdk4/6 and cyclin E–cdk2 complexes. Cell cycle arrest occurs when cyclin-cdk complexes cannot form or when the catalytic activity of these complexes is suppressed through binding of cdk inhibitory molecules, such as p21/Cip1 (31, 32). In this report, we showed that PKCε(KR)-induced growth suppression of lung cancer cells was associated with the specific induction of p21/Cip1 and inactivation of cyclin-cdk2 complexes but not cyclin D-cdk4 complexes. Time course studies showed that a 4-h delay in S-phase entry in PKCε(KR)-expressing cells was associated concomitantly with a delay in activation of cdk2 (Fig. 3*A*). This indicates that PKCε(KR)-induced cell cycle arrest at the G_1-S boundary occurs as a consequence of the specific loss of cdk2 activity. This observation seems somewhat contradictory to the recent finding that cdk2 is dispensable for cancer cell proliferation and cell cycle progression (35, 50), which challenged the importance of cdk2 in control of the G_1-S transition. Although deletion of cdk2 did not cause profound effects on cell proliferation, *cdk2*$^{-/-}$ mouse embryonic fibroblasts did show a delay in S-phase entry upon release from quiescence (35), indicating that cdk2 affects the timing of S phase. Importantly, the recent study by Berthet et al. (34) indicates that both cdk2 and cdk4 are important for proliferation in a way that they can compensate for each other's function in promoting cell cycle progression. Furthermore, given the heterogeneity in cellular background, it is likely that cancer cells may respond differently to different growth-inhibitory signals. Perhaps, in lung cancer cells, owing to the induction of p21/Cip1 by PKCε(KR), cdk2 activity was primarily affected as the result of increased association between cdk2 and p21/Cip1. Although increased binding of p21/Cip1 was observed in some but not all PKCε(KR) clones, it has no significant effects on cdk4 activity (Supplementary Fig. S1). This may be due to the fact that cyclin-cdk2 complexes are much more susceptible to inhibition by p21/Cip1 than cyclin D-cdk4/6 complexes, and p21/Cip1 is required for assembling active cyclin D-cdk4/6 complexes (32, 33). Consistent with a delayed activation of cdk2 during cell cycle reentry, phosphorylation of Rb at the cdk2-targeted sites (S807/811) was significantly reduced in PKCε(KR)-expressing cells. Moreover, compared with the corresponding vector cells, proliferating PKCε(KR)-expressing cells showed reduced phosphorylation of Rb at S807/811, whereas phosphorylation of Rb at the cdk4-targeted site (S795) seems not significantly altered (Supplementary Fig. S1). Altogether, our data strongly support the notion that a loss of cdk2 activity is responsible for prolonging the G_1-S progression in response to PKCε inhibition in lung cancer cells.

Cell cycle regulation by PKC-dependent signaling has been linked to the alteration in p21/Cip1 expression (44–47). The essential role for p21/Cip1 in negative regulation of the PKCε signaling is supported by the observation that the HCT116 isogenic line lacking p21/Cip1 (*p21*$^{-/-}$) was refractory to the PKCε(KR)-induced growth inhibition. The significance of p21/Cip1 as a negative effector of the PKCε proliferating signal is further underscored by an inverse correlation between the levels of PKCε and expression/function of p21/Cip1 in primary NSCLCs (Fig. 5*C*). Furthermore, PKCε(KR)-mediated p21/Cip1 up-regulation occurred in a p53-independent manner in both lung cancer cells and in HCT116 isogenic cells. Interestingly, concurrent inhibition of PKCα and PKCθ induced G_1 cell cycle arrest was also associated with p53-independent induction of p21/Cip1 in fibroblasts (46). It seems that p53-independent induction of p21/Cip1 may be a common mechanism underlying negative regulation of G_1 progression via PKC inhibition.

One important observation from this work is that expression of PKCε(KR) led to the down-regulation of c-Myc expression. c-Myc elicits its transforming activity mainly through overexpression, which occurs in ~50% of lung cancers (3). A primary function of c-Myc is to activate transcription of a large number of target genes encoding proteins important for cell growth. However, recent studies suggest that transcriptional repression of negative regulators of cell cycle such as p21/Cip1 represents an additional mechanism by which c-Myc promotes cell cycle progression (40, 41). c-Myc was shown to repress p21/Cip1 transcription independent of p53, possibly by sequestering transcription factors Sp1/Sp3 (31, 40). We found that PKCε(KR)-mediated down-regulation of c-Myc expression was associated with transcriptional induction of *p21/Cip1*. Importantly, knockdown of c-Myc expression by siRNA led to p21/Cip1 induction and attenuation of the G_1-S transition, and c-Myc expression seems associated with PKCε expression in NSCLCs (Fig. 5*C*). These results suggest that c-Myc is a negative regulator of p21/Cip1 expression downstream of PKCε and its down-regulation provides an intriguing mechanism by which PKCε(KR) suppress oncogenic activity in lung cancer cells. Consistent with our findings, it has been reported that PKCε activation or overexpression can lead to c-Myc induction and enhanced cell proliferation (38, 39). Collectively, it suggests that c-Myc might act as an important mediator in the propagation of growth/survival signals downstream of PKCε.

In summary, this study presents the first evidence of overexpression of PKCε in human NSCLCs. We present mechanistic insights as to how alterations in PKCε signaling could contribute to abnormal proliferation in lung cancer cells. Our data suggest that one mechanism by which PKCε exerts its oncogenic function is through the dysregulation of cell cycle control at the G_1-S boundary in a p21/Cip1–dependent manner. Given that genetic deletion of *PKCε* results in only minor phenotypes in mice (51), targeted inhibition of PKCε expression via antisense or RNA interference strategies may represent novel therapeutics against human lung cancer.

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[4] Xiao et al., unpublished observations.

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Protein Kinase Cε Is Overexpressed in Primary Human Non–Small Cell Lung Cancers and Functionally Required for Proliferation of Non–Small Cell Lung Cancer Cells in a p21/Cip1-Dependent Manner

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