Atypical protein kinase C (aPKC) isozymes play a critical role in human lung cancer cell growth and tumorigenicity. 

Lung cancer is the leading cause of cancer death in the United States, accounting for an estimated 160,440 deaths in 2004 (1). The vast majority (~90%) of lung cancer is related to tobacco use (2), the predominant risk factor for this disease. Over the past 30 years, the five-year survival rate of lung cancer patients has improved slightly from 7 to 14%, but there remains a dire need for more effective modalities for prevention, diagnosis, prognosis, and treatment. Lung cancer is divided into two major histopathologic classes, small cell lung cancer and non-small cell lung cancer (NSCLC), which are characterized by distinct patterns of genetic and epigenetic changes that likely dictate their distinct biologies and responses to therapy. The vast majority of lung cancer (~80%) is classified as NSCLC, with most remaining cases (~18%) being small cell lung cancer (3).

NSCLC results from progressive genetic and epigenetic changes often caused by exposure to cigarette smoke or other environmental carcinogens. Ten or more genetic and epigenetic changes are likely required to produce a malignant, invasive NSCLC (4). The most common molecular changes in NSCLC include oncogenic mutation of the K-ras gene (~50%), up-regulation of c-myc (~20–30%), mutation of the p53 gene (~50%), deletion of p16(ink4A)/Arf, and mutation of the fragile histidine triad gene (5, 6). NSCLCs frequently exhibit overexpression of the epidermal growth factor receptor, ErbB2 (Her2/Neu), and/or the hepatocyte growth factor/scatter factor receptor (Met). These receptors and their ligands (epidermal growth factor, transforming growth factor α, and hepatocyte growth factor) are often co-expressed in NSCLC tumors, generating autocrine and/or paracrine signaling loops that stimulate proliferation and survival of NSCLC cells. Signaling through epidermal growth factor receptor, ErbB2, and Met involves activation of cellular Ras, suggesting that Ras-mediated signaling, activated either through receptor-mediated paracrine/autocrine loops or oncogenic mutation, is central to NSCLC development.

The atypical PKC (aPKC) isozymes PKCγ and PKCζ have been linked to cellular Ras signaling (7–10). The Bcr-Abl oncoprotein induces PKCζ expression through a Ras/Mek-dependent pathway in chronic myelogenous leukemia cells (11), and Ras can activate aPKC (12, 13). Furthermore, two downstream effectors of aPKC, Rac1 and NFκB, are required for oncogenic Ras-mediated transformation (14, 15). aPKCs have also been implicated in oncogenic Ras signaling in fibroblasts (7–9, 16) and intestinal epithelial cells (17–20).

We recently demonstrated that PKCζ is important for Ras-mediated colon carcinogenesis in vivo in a murine model (17). However, essentially nothing is known about whether aPKCs play an important role in human cancers, and if so, which aPKC-mediated signaling pathway(s) are involved. Herein, we have investigated the involvement of atypical PKCζ and PKCγ in human NSCLC cell growth and transformation. We find that PKCζ is the major aPKC isozyme expressed in human lung cells and that PKCζ is overexpressed in NSCLC cell lines. Furthermore, we show that PKCζ is required for transformed growth of NSCLC cells in vitro and tumorigenicity in vivo by activating Rac1/Pak/Mek/Erk-dependent signaling pathway that regulates cell proliferation.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies were from the following sources and were used at the indicated concentrations: PKCζ, 1:100 (Santa Cruz Biotechnology, catalog number sc-17640); PKCγ, 1:4,000 (BD Transduction Laboratories, catalog number P20520); aPKC PB1 domain, 1:1,000 (Santa Cruz Biotechnology, catalog number sc-727); actin, 1:2,000 (Santa Cruz Biotechnology, catalog number sc-1616); the FLAG epitope, 1:2000.
Protein Kinase C is a Cancer Gene in Human Lung Cancer

**NFκB Signaling and Transcriptional Activity Assays—**NFκB transcriptional activity was assayed using a dual luciferase reporter system (Promega), as described previously (22). In brief, A549 cells stably expressing PKCz or pBabe vector control were transiently transfected with 500 ng of 3xMHC-Luc, a plasmid containing three NFκB response elements from the major histocompatibility complex promoter linked to a luciferase reporter gene, and 25 ng of phRL-SV40 using the FuGENE 6 lipofection reagent (Roche Applied Science), as described by the manufacturer. Twenty four hours after transfection, NFκB activity was stimulated with 50 ng/ml TNFα (R & D Systems) for 2 h. Total cell extracts were prepared for the dual luciferase assay according to the manufacturer’s (Promega) instructions. Firefly and Renilla luciferase activity were measured using a Veritas microplate luminometer (Turner BioSystems). The activity of Renilla luciferase was used as an internal control for transfection efficiency.

The response of A549/pBabe and A549/kdPKCα cells to TNFα was assessed by treating the cells with 50 ng/ml TNFα for the time indicated in the figure legend. The cells were lysed into lysis buffer containing 20 mM MOPS, pH 7.4, 150 mM NaCl, 5 mM EGTA, 1% Nonidet P-40, 10 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Total cell lysates (10 μg/lane) were subjected to SDS-PAGE and immunoblot analysis for IκBα, Erk, phospho-(Ser51)Erk, phospho-(Thr202/Tyr204)-Erk, and PKCz protein were from Upstate Biotechnology, catalog numbers 4697 and 16-200, respectively. The myristoylated atypical PKC pseudosubstrate inhibitor peptide was from BIOSOURCE (catalog number 77-749).

**Cell Culture, Plasmids, Transfections, and Drug Treatments—**Human NSCLC cell lines (A549, H1299, H292, H520, 3110, and SK-MES-1) were maintained in Ham’s F12 supplemented with 10% or 2% or no fetal bovine serum. A549 cell transfectants were harvested and resuspended in serum-containing medium. 4–6-week-old female nude mice were injected subcutaneously into the flank with 5 × 106 cells in 100 μl of growth medium. Once palpable tumors were established, tumor size was measured once a week. Tumor growth was quantified by measuring the tumors in three dimensions with calipers. Tumor volume (mm3) was calculated using the formula 0.5236(L × W × H), where L represents the length of the tumor, W represents the width of the tumor, and H represents the height. Each group of mice was comprised of 4–6 mice. Drugs were administered by intraperitoneal injection. At the conclusion of the study, mice were injected intraperitoneally with 100 μg/g 5-bromo-2-deoxyuridine (BrdUrd) 1 h prior to sacrificing the mice by CO2 asphyxiation. Tumors were excised and divided into sections for protein extraction and tumor fixation. Total tumor extracts were prepared in SDS buffer (2% w/v) SDS, 4 mM urea, 62.5 mM Tris-HCl, pH 6.8, 1 mM EDTA, 5% (v/v) β-mercaptoethanol), and equal amounts of protein were subjected to immunoblot analysis. A section of tumor was also fixed in 10% buffered formalin, embedded in paraffin, sectioned (5 μm thickness), and stained for appropriate antibodies.無

**Immunoblot Analysis—**Cells were harvested by washing with phosphate-buffered saline and scraping off the plate. The cell pellet was lysed in SDS sample buffer. Protein lysates were quantitated using the Bradford assay method. Equal amounts of proteins (2–20 μg) were loaded for each sample, resolved in 12 or 4–20% SDS-polyacrylamide gels (Invitrogen), and transferred to polyvinylidene difluoride membrane (Millipore Immobilon-P). A solution of 5% milk and phosphate-buffered saline-Tween 20 was used for blocking. A solution of 5% milk and phosphate-buffered saline-Tween 20 was used for blocking. Blots were incubated with antibodies to the appropriate antigens.

**RESULTS**

PKCα Is Required for Human NSCLC Cell Transformation in Vitro—Little is known about the expression or function of atypical PKC isoforms in human cancers. Because NSCLC is one of the most prevalent and deadliest of human cancers, we assessed the expression of the two atypical PKC isoforms, PKCα, and PKCζ, in a panel of human NSCLC cell lines. Each of six human NSCLC cell lines tested (A549, H1299, H292, ChaGoK1, Sk-Mes1, and H520) expressed elevated PKCα and PKCζ, as determined by immunoblot analysis in whole cell lysates using specific antibodies. We also investigated the role of PKCα, PKCζ, and PKCα and PKCζ in human NSCLC cell transformation, as determined by immunoblot analysis in whole cell lysates using specific antibodies. We also investigated the role of PKCα, PKCζ, and PKCα and PKCζ in human NSCLC cell transformation, as determined by immunoblot analysis in whole cell lysates using specific antibodies.
Atypical protein kinase C is critical for A549 human NSCLC transformation. A, immunoblot analysis of human NSCLC cell lines for PKCβ, PKCδ, and actin. PKCβ is overexpressed in all NSCLC cell lines, whereas PKCδ is undetectable. Purified recombinant human PKCβ and PKCδ were included as controls to confirm antibody specificity. B, immunoblot analysis of A549 cell transfectants expressing either pBabe, wild-type human PKCβ (wtPKCβ), or kinase-deficient human PKCβ (kdPKCβ) for FLAG-tagged PKCβ, total PKCβ, and actin. C, growth of A549 transfectants in adherent culture in growth medium supplemented with 10%, 2%, or no serum. Data represent means ± S.E. of three independent determinations. FCS, fetal calf serum. D, anchorage-independent growth of A549/pBabe, A549/wtPKCβ, and A549/kdPKCβ cells in soft agar. Data represent the mean ± S.E. of three independent determinations.

Fig. 1. Atypical protein kinase Cβ is critical for A549 human NSCLC transformation. A, immunoblot analysis of human NSCLC cell lines for PKCβ, PKCδ, and actin. PKCβ is overexpressed in all NSCLC cell lines, whereas PKCδ is undetectable. Purified recombinant human PKCβ and PKCδ were included as controls to confirm antibody specificity. B, immunoblot analysis of A549 cell transfectants expressing either pBabe, wild-type human PKCβ (wtPKCβ), or kinase-deficient human PKCβ (kdPKCβ) for FLAG-tagged PKCβ, total PKCβ, and actin. C, growth of A549 transfectants in adherent culture in growth medium supplemented with 10%, 2%, or no serum. Data represent means ± S.E. of three independent determinations. FCS, fetal calf serum. D, anchorage-independent growth of A549/pBabe, A549/wtPKCβ, and A549/kdPKCβ cells in soft agar. Data represent the mean ± S.E. of three independent determinations.

R. P. Regala and A. P. Fields, unpublished data.
The PB1 Domain of PKC\textsubscript{i} Is Important for A549 Cell Transformation—PKC\textsubscript{i} couples to Rac1 through interactions involving the PB1 domain of PKC\textsubscript{i} (29). Therefore, we reasoned that the PKC\textsubscript{i} PB1 domain may act as a competitive inhibitor of Rac1 activity in NSCLC cells. Transfection of A549 cells with an LZRS retrovirus expressing the PKC\textsubscript{i} PB1 domain (PKC\textsubscript{i}-(1–113)) inhibits Rac1 activity to levels comparable with those observed after treatment with pseudosubstrate peptide inhibitor (Fig. 3B).

**FIG. 2.** PKC\textsubscript{i} does not regulate NF\textsubscript{kB} signaling in A549 cells. A, immunoblot analysis of A549 cell transfectants for cIAP2, Bcl-XL, and actin. B, analysis of A549 cell transfectants for PARP and cleaved PARP. HeLa cells treated with taxol for either 24 or 48 h served as a positive control for cleaved PARP. C, A549/pBabe and A549/kdPKC\textsubscript{i} cells were treated with 50 \(\mu\)g/ml TNF\alpha for the indicated times and cell lysates subjected to immunoblot analysis for detection of IkBa, phospho-(Ser32)-IkBa, and actin. D, A549/pBabe and A549/kdPKC\textsubscript{i} cells were treated with 50 \(\mu\)g/ml TNF\alpha for the indicated times and cell lysates subjected to immunoblot analysis for detection of NF\textsubscript{kB} p65, phospho-(Ser536)-NF\textsubscript{kB} p65, and actin. E, transcriptional activity of an NF\textsubscript{kB}-luciferase reporter in A549/pBabe and A549/kdPKC\textsubscript{i} cells in the presence and absence of TNF\alpha. Data represent the mean ± S.E. of three independent determinations.

**FIG. 3.** Rac 1 is a critical downstream target for PKC\textsubscript{i} signaling in human NSCLC cells. A, A549 cell transfectants were assayed for active GTP-bound Rac1 and for total Rac1 expression. B, active and total Rac1 was assessed in A549/LZRS cells, A549 cells treated with the PKC\textsubscript{i}-selective pseudosubstrate peptide inhibitor (PSI), and A549/PKC\textsubscript{i}-(1–113) cells. Expression of PKC\textsubscript{i}-(1–113) was confirmed by immunoblot analysis. C, anchorage-independent growth of A549/LZRS and A549/PKC\textsubscript{i}-(1–113) cell transfectants in soft agar. Data represent the mean ± S.E. of three independent determinations.
Immunoblot analysis confirmed expression of the PKC\textsubscript{\(c\)} (1–113) protein in these cells (Fig. 3B). Furthermore, expression of PKC\textsubscript{\(c\)} (1–113) inhibits anchorage-independent growth in soft agar (Fig. 3C), indicating the importance of the PB1 domain in PKC\textsubscript{\(c\)}-dependent activation of Rac1 and cellular transformation.

The PKC\textsubscript{\(c\)}-Rac1 Signaling Axis Is Required for NSCLC Cell Tumorigenicity in Vivo—We next assessed whether Rac1 is an important downstream effector of PKC\textsubscript{\(c\)}-dependent transformation. Expression of a constitutively active Rac1 allele, RacV12, restores transformed growth of A549/kdPKC\textsubscript{\(c\)} cells in soft agar (Fig. 4A), indicating that Rac1 is critical for PKC\textsubscript{\(c\)}-dependent transformation in vitro. We next assessed the importance of the PKC\textsubscript{\(c\)}-Rac1 signaling axis in A549 cell tumorigenicity in vivo. Athymic nude mice were inoculated subcutaneously with A549/pBabe, A549/kdPKC\textsubscript{\(c\)}, or A549/kdPKC\textsubscript{\(c\)}/RacV12 cells, and tumour growth was assessed over time. A549/kdPKC\textsubscript{\(c\)} cell tumors were significantly growth-inhibited in vivo, whereas A549/kdPKC\textsubscript{\(c\)} cells expressing RacV12 showed tumor growth indistinguishable from A549/pBabe cells (Fig. 4B). Thus, the PKC\textsubscript{\(c\)}-Rac1 signaling axis is required for A549-transformed growth in vitro and tumorigenicity in vivo.

To determine the molecular pathway involved in PKC\textsubscript{\(c\)}-dependent tumorigenesis, we monitored Rac1 and NF\(\kappa\)B activity in tumors derived from A549 cell transfectants. Rac1 activity was assayed by monitoring the activity of the downstream Rac1 effector Mek1,2. We recently demonstrated that Mek1,2 is a critical PKC\textsubscript{\(c\)}- and Rac1-dependent effector of Ras-mediated transformation of rat intestinal epithelial cells (17). A549/pBabe cells exhibit significant levels of Mek1,2 phosphorylated on the Ser-217/221 Raf activation sites (Fig. 4C) and of active, phosphorylated Erk1,2, indicating Mek/Erk activation (Fig. 4C). It was recently shown that Rac1 targets the Mek/Erk pathway by activating the p21-activated kinase, Pak (30). Activated Pak phosphorylates Mek at Ser-298, thereby facilitating Mek/Erk interactions and Erk activation (30). Interestingly, Mek1,2 is phosphorylated on the Pak-specific phosphorylation site Ser-298 in A549/pBabe tumors (Fig. 4C). A549/kdPKC\textsubscript{\(c\)} cell tumors exhibit reduced levels of phospho-Mek at both Raf- and Pak-mediated sites with a concomitant decrease in phospho-Erk levels. Expression of RacV12 in A549/kdPKC\textsubscript{\(c\)} cell tumors restores Pak- and Raf-mediated phosphorylation of Mek and restores Erk phosphorylation to levels indistinguishable from that of A549/pBabe cell tumors. Thus, PKC\textsubscript{\(c\)} regulates the Mek/Erk pathway through activation of Rac1 and Pak in A549 cell tumors in vivo.

Although PKC\textsubscript{\(c\)} does not regulate NF\(\kappa\)B in A549 cells in vitro, it is possible that it does so in the in vivo setting. However, expression of the NF\(\kappa\)B transcriptional targets cIAP2 and Bcl-X\(_{L}\) were not affected by kdPKC\textsubscript{\(c\)} or RacV12 in A549 cells in vivo (Fig. 4D). We also found no evidence of apoptosis in tumors expressing kdPKC\textsubscript{\(c\)} or RacV12, as measured by caspase-mediated cleavage of PARP (Fig. 4D). Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling analysis revealed very low apoptotic indices (<0.2%) with no significant
A549 cell tumors in the presence of kdPKCmunohistochemical staining for the endothelial cell marker
vascularization due to decreased angiogenesis. However, im-
cell polarity (43) and survival (23, 42, 44, 45).

(33, 38–42). aPKCs have been implicated in the establishment of protein-protein interactions with upstream effectors, including Ras
the phosphoinositide-dependent kinase, PDK1 (35–37), and pro-
can be regulated by 3-phosphoinositides (34), phosphorylation by
pholipid, and diacylglycerol binding motifs (33). aPKC activity
functional divergence is due to the presence of a unique amino-
upon diacylglycerol, calcium, or phosphatidylserine (32, 33). This
from other PKCs in that their catalytic activity is not dependent
and functionally distinct subclass of PKCs. The aPKCs differ
PKC activity is elevated in these cells and can be further activated

PKC activity in NFkB-induced transformation has been unre-
solved. Our data demonstrate that PKCz (but not PKCz) is
involved in NSCLC transformation. Furthermore, our data
provide evidence that the PB1 domain of PKCz is important for its ability to support NSCLC transformation. In addition, our
data demonstrate that Rac1 is a critical downstream target of PKCz-dependent transformation and indicate that the PB1
domain of PKCz mediates Rac1 activation. Interestingly, we
found no evidence for the involvement of PKCz activity in NFkB
activation of A549 NSCLC cells. Indeed, although basal NFkB
activity is elevated in these cells and can be further activated
by TNFz, disruption of PKCz signaling has no effect on either
translational targets cIAP and Bcl-2, both of which have been shown to be important in A549 cell transformation, are not affected by the disruption of PKCz signaling. Our data indicate that, in NSCLC, PKCz is
tightly coupled to Rac1 signaling but not to NFkB activity. Our
data are consistent with recent evidence from mouse embryo
fibroblasts nullizygous for PKCz (the mouse homolog of PKCz)
that showed these cells exhibit an intact TNFz-induced NFkB response (55). In contrast, mouse embryo fibroblasts
nullizygous for PKCz show significant impairment of the NFkB pathway in response to TNFz and chemotherapeutic agents
(56). Taken together with our present data, it appears that, at

**FIG. 5. PKCz is necessary for tumor growth in vivo.** A, immunohistochemical staining of A549/pBabe, A549/kdPKCz, and A549/kdPKCz/ RacV12 cell tumors for BrdUrd. Tumor-bearing animals were injected intraperitoneally with BrdUrd 1 h prior to sacrifice. A labeling index was
determined and analyzed for statistical significance, as described under “Experimental Procedures.” B, immunohistochemical staining of A549 cell
tumors for the endothelial cell marker CD31. C, immunoblot analysis of A549 cell tumors for CD31 and actin. Lanes 1 and 2 indicate extracts from
tworepresentative tumors of each genotype.
PKC in vitro both do not express detectable PKC. It will be of interest to determine whether PKC plays a critical role in the transformed phenotype of other tumor types and, if so, whether Rac1, NFκB or perhaps other as yet unidentified downstream targets of PKC are the critical downstream target(s) in diverse tumor types.

In conclusion, our results provide compelling evidence that PKC is required for the transformed phenotype of NSCLC cells both in vitro and in vivo. Interestingly, our data indicate that PKC is dispensable for adherent cell growth, whereas it is required for anchorage-independent transformed growth of NSCLC cells in vitro and tumorigenesis in vivo. These properties make PKC an attractive candidate for the development of mechanism-based therapy against NSCLC.

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