Protein Kinase C-ε Promotes Survival of Lung Cancer Cells by Suppressing Apoptosis through Dysregulation of the Mitochondrial Caspase Pathway*

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The serine/threonine protein kinase C (PKC) has been implicated in the regulation of drug resistance and cell survival in many types of cancer cells. However, the one or more precise mechanisms remain elusive. In this study, we have identified and determined the mechanism by which PKC-ε, a novel PKC isoform, modulates drug resistance in lung cancer cells. Western blot analysis demonstrates that expression of PKC-ε, but not other PKC isoforms, is associated with the chemo-resistant phenotype of non-small cell lung cancer (NSCLC) cell lines. Northern blotting and nuclear run-on transcription analysis further reveals that the failure of expression of PKC-ε in the chemo-sensitive phenotype of small cell lung cancer (SCLC) cells results from transcriptional inactivation of the gene. Importantly, forced expression of PKC-ε in NCI-H82 human SCLC cells confers a significant resistance to the chemotherapeutic drugs, etoposide and doxorubicin. Resistance is characterized by a significant reduction in apoptosis in PKC-ε-expressing cells. Treatment of NCI-H82 cells with etoposide induces a series of time-dependent events, including the release of cytochrome c from the mitochondria to the cytosol, activation of caspase-9 and cleavage of poly(ADP-ribose) polymerase (PARP). All of these events are blocked by PKC-ε expression. Furthermore, caspase-specific inhibitors, z-VAD-fmk and z-DEVD-fmk, significantly attenuate the accumulation of sub-G1 population and block the PARP cleavage in response to etoposide. These results suggest that PKC-ε prevents cells from undergoing apoptosis through inhibition of the mitochondrial-dependent caspase activation, thereby leading to cell survival. Finally, down-regulation of PKC-ε expression by the antisense cDNA in NSCLC cells results in increased sensitivity to etoposide. Taken together, our findings suggest an important role for PKC-ε in regulating survival of lung cancer cells.

Lung cancer is the leading cause of cancer-related deaths in the United States. Human lung cancer is a disease of heterogeneous histology which can be divided into two major categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC represents ~25% of all lung cancer and is characterized by a marked initial response to chemotherapy and radiation. However, nearly all patients with SCLC relapse and develop resistance to cytotoxic therapy and eventually die from the disease. Overall, the 5-year survival rate is only 3–8% (1). The underlying mechanism of developing drug resistance in relapsed SCLC patients is unclear. Studies have suggested that tumor cells that survive chemotherapy display phenotypes distinctly different from those expressed by the primary tumor prior to chemotherapy (2). It is clear that understanding the molecular determinants of chemo-resistance in SCLC upon relapse will lead to more efficient therapy.

Apoptosis, or programmed cell death, is a highly specific and regulated process that plays an important role in the development and homeostasis of multicellular organisms as well as tumorigenesis (3, 4). There is an increasing body of evidence that the control of apoptosis is disrupted in many tumor cells. The dysregulated apoptosis is thought to contribute to enhanced tumor progression and metastasis. Furthermore, it has become evident that resistance to apoptosis is one potential mechanism whereby tumor cells escape from chemotherapy-induced cytotoxicity, leading to cell survival (5). Development of chemo-resistance in tumor cells may, therefore, be due to their selective defects in the intracellular signaling proteins central to apoptotic pathways. These defects may provide a selective advantage for the tumor cells thereby rendering them resistant to chemotherapy.

Recent studies have defined a number of key molecules that are critical in apoptotic process. These include the Bcl-2 family of proteins and a family of aspartate-specific cysteine proteases named caspases. The Bcl-2 family proteins play a critical role in regulation of mitochondria function during apoptosis (6, 7). Members of the Bcl-2 family can have both pro-apoptotic and anti-apoptotic activities. The interactions between the different Bcl-2 family members play an important role in determining cell fate during apoptosis. Caspases are the central engines of the apoptotic process, which are synthesized as latent zymogens and are activated by proteolytic cleavage (8). There are currently two well characterized caspase-activating cascades.

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† The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; PKC, protein kinase C; DAG, diacylglycerol; z-DEVD-fmk, benzoyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone; z-VAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp(Ome) fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase; TPA, 12-O-tetradecanoylphorbol-13-acetate; CMV, cytomegalovirus; COX, cytochrome c oxidase; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; CHAPS, 3-(3-cholamidopropyl)dimethylammonium)-1-propanesulfonic acid; pNA, p-nitroanilide; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; z-IETD-fmk, benzoyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone.

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that regulate apoptosis: one is mediated by cell surface death receptors, and the other is regulated by the mitochondria. In the death receptor pathway, death receptor ligation promotes clustering and activation of caspase-8 and -10, which in turn activate downstream caspases. By contrast, caspase activation via the mitochondrial pathway involves mitochondrial integration of apoptotic signals and subsequent release of cytochrome c into the cytosol. Once released from the mitochondria, cytochrome c initiates formation of a complex (apoptosome) with procaspase-9 and an adaptor molecule, apoptotic protease-activating factor-1 (Apaf-1) thereby activating caspase-9, which in turn activates downstream caspases such as caspase-3, -6, and -7.

Protein kinase C (PKC) is a family of at least 11 structurally related serine/threonine protein kinases that play crucial roles in transducing signals that regulate diversified biological functions, including proliferation, differentiation, and apoptosis (9, 10). The PKC family can be divided into three subgroups based on differences in their structures and biochemical properties: the classic isoforms (PKC-α, β, and γ), which are Ca2+- and phorbol ester/diacylglycerol (DAG)-dependent; the novel isoforms (PKC-δ, ε, η, θ, and μ), which are phorbol ester/DAG-dependent but Ca2+-independent; and the atypical isoforms (PKC-ζ and λ), which are Ca2+- and phorbol ester/DAG-independent. The heterogeneous expression profiles of each PKC isoform in tissues strongly suggest that each isoform has a unique individual role. Their different subcellular localization and isoform-specific cofactor and activator requirements indicate each isoform is regulated differently (10, 11). Recent studies using strategies to explore the isoform-specific functions of PKC suggest that specific PKC isoforms may be either anti-apoptotic or pro-apoptotic, which is likely dependent on the nature of the apoptotic stimuli and the specific cell types involved (12). PKC isoforms that appear to be anti-apoptotic include PKC-α, PKC-βII, PKC-ε, and the atypical isoforms PKC-ζ and PKC-λ (13–16). In contrast, PKC-δ has been shown as a common intermediate in the apoptotic pathway (17, 18). However, the precise biochemical mechanisms by which PKC regulates anti-apoptotic effects remain elusive.

Human lung cancer cells express multiple PKC isoforms. It has been reported that the drug-resistant phenotype is associated with expression and/or activity of PKCs in lung cancer cell lines and lung carcinomas (19, 20). However, it is unclear what role(s), if any, a specific PKC isoform may play in this process. In this study, we identify and determine the function of the PKC-ε isoform in lung cancer cells. Using isoform-specific antibodies, we demonstrate that PKC-ε, but not other PKC isoforms, is specifically linked to the chemo-resistant phenotype of the NSCLC cell lines and its induction in SCLC cells confers resistance to chemotherapy. Evidence presented in this study suggests that the chemo-protective effect of PKC-ε is mediated by the inhibition of drug-induced caspase activation through dysregulation of one or more of the mitochondrial apoptotic pathways, thereby leading to cell survival. These observations demonstrate a functional role for PKC-ε in the regulation of survival of lung cancer cells and provide potential novel targets for therapeutic intervention to overcome drug resistance.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human PKC-ε cDNA was obtained from Dr. A. P. Fields (University of Texas Medical Branch, Galveston, TX). The human PKC-ε cDNA was tagged with a FLAG epitope at its N terminus and subcloned into expression vector pCI-neo (Promega, Madison, WI). The plasmid that expresses the antisense PKC-ε cDNA was constructed by subcloning the human full-length PKC-ε cDNA into pcRII-CMV vector (Invitrogen, Carlsbad, CA) in an antisense orientation. Mouse monoclonal antibodies to specific PKC isoforms were purchased from BD Transduction Laboratories (Lexington, KY). Antibodies against cleaved and full-length versions of caspase-3 and caspase-9 were from Cell Signaling (Beverly, MA). Rabbit anti-PARP and goat anti-actin polyclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-cytochrome c (clone 7H8.2C12) and anti-oxidase (COX) subunit IV (clone 20E8) were from BD Pharmingen and Molecular Probes (Eugene, OR), respectively. Caspase inhibitors (z-VAD-fmk and z-DEVD-fmk) were purchased from Enzyme System Products (Livemore, CA). Etoposide and doxorubicin were from Calbiochem (San Diego, CA). G418 sulfate (Geneticin) and LipofectAMINE were from Invitrogen (Rockville, MD). Hoechst 33342 and propidium iodide were obtained from Sigma (St. Louis, MO). [α-32P]CTP and [α-32P]CTP were from Amersham Biosciences (Piscataway, NJ).

**Cell Culture and DNA Transfection**—Human lung cancer cell lines were obtained from ATCC (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 9% (v/v) calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Stable transfection of NCI-H82 cells was carried out using an electroporation method. Transfected cells were selected for G418 resistance. Clonal populations that express the FLAG-tagged PKC-ε were generated and maintained in the culture medium containing 500 μg/ml G418. Stable transfection of NCI-H157 with an antisense PKC-ε cDNA was carried out using LipofectAMINE according to the manufacturer’s recommendation. 48-h post-transfection, cells were selected for G418-resistance. A pool of G418-resistant clones was used for clonal survival assays.

**Northern Blotting and Nuclear Run-on Transcription Analysis**—Total cellular RNA was isolated using the acid phenol-guanidine isothiocyanate method (21). 20 μg of RNA was fractionated on a denaturing 1% agarose gel containing formaldehyde (2.1 M), transferred to a GeneScreen membrane (PerkinElmer Life Sciences, Boston, MA), and hybridized with [32P]-labeled human PKC-ε cDNA probe in ULTRAhyb hybridization buffer (Ambion, Austin, TX). The same membrane was then stripped and re-probed with human β-actin cDNA for loading control.

**Nuclear run-on transcription assays** were carried out as described previously (22). The 32P-labeled nascent RNA transcripts purified from nuclei of representative cell lines were used to hybridize with the immobilized cDNA on Zeta-Probe membrane (Bio-Rad, Hercules, CA) according to the manufacturer’s recommendations. After high stringency washes, membranes were subjected to autoradiography.

**Soft Agar Assay and Growth Studies**—Soft agar assays were carried out in six-well plates as described by Clark et al. (23). Briefly, cells were seeded in 0.3% agarose in growth medium overlaid on a base of 0.6% agarose. Cells were fed weekly. Colonies were scored 21 days later after plating. For growth, 5 × 104 cells per well were plated in six-well plates in complete medium. Media were changed every 2 days. Cell numbers were determined by trypan blue exclusion. Triplicate cultures of each cell clone were prepared and processed in all experiments.

**Cell Viability and Clonogenic Survival Assays**—Cells (1 × 105 cells) were cultured in six-well plates in the presence of either etoposide or doxorubicin at indicated concentrations for 96 h. Control cells were treated with the vehicle, dimethyl sulfoxide (MeSO). Cell viability was determined by trypan blue dye exclusion and presented as the ratio of viable cell numbers in drug-treated cultures relative to that in MeSO-treated controls times 100. Clonogenic assays were performed by plating cells (5000 cells/well) in six-well plates, treating with etoposide or vehicle (0.1% MeSO) for 48 h. Cells were then cultured in drug-free media for 12 days and stained with 0.5 mg/ml crystal violet in 20% methanol. Drug-resistant colonies were scored, and cloning efficiency was expressed as a ratio of the number of colonies formed in the anti-sense-transfected cells to that in the vector-transfected cells times 100. All treatments were performed in triplicate.

**Measurement of Apoptosis**—Control or treated cells were collected and stained by incubating in a staining solution of 100 μg/ml Hoechst 33342 and 20 μg/ml propidium iodide in phosphate-buffered saline (PBS) at 37 °C for 15 min in dark. Stained cells were viewed under the Zeiss Axioplan-2 fluorescence microscope with the appropriate filters to simultaneously visualize the blue fluorescence from Hoechst 33342 and the red fluorescence from propidium iodide. Apoptotic cells were defined by condensed and/or fragmented chromatin of blue fluorescence-emitting nuclei. To quantify apoptotic cells, a least 500 cells per preparation were scored for the incidence of apoptotic chromatin changes. Results are expressed as a ratio of apoptotic cells to the total number of blue nuclei examined times 100.

Flow cytometry analysis was used to determine the sub-G0 populations of etoposide-treated cells. Cells were fixed in 90% methanol,
treated with RNase A (100 μg/ml) at 4 °C for at least 4 h, and stained with 50 μg/ml propidium iodide in PBS on ice for 15 min. DNA contents were assessed by fluorescence-activated cell sorting (FACS) using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). A total of 50,000 cells was analyzed in every event. Data were evaluated using CellQuest software (Becton Dickinson) and represent the average of three measurements.

Detection of Internucleosomal DNA Fragmentation—Cells (1 × 10⁶) were harvested and lysed in a buffer of 50 mM TrisCl (pH 9.0), 20 mM EDTA, 10 mM NaCl supplemented with protease K (1 mg/ml) and 1% SDS at 37 °C overnight. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), followed by ethanol precipitation. DNA was recovered by centrifugation at 13,000 × g for 30 min and dissolved in LTE buffer (3 mM TrisCl (pH 8.0) and 0.3 mM EDTA) containing 500 μg/ml RNase A. 20 μg of DNA per sample was digested with RNase A at 37 °C for 1 h and separated on a 1.8% agarose gel. DNA fragmentation was examined under UV lights after ethidium bromide staining and photographed.

Colorimetric Caspase-3 Activity Assay—Caspase-3 activity was assessed using a Caspase-3 Cell Death Detection Kit (Calbiochem) according to the protocol suggested by the manufacturer with minor modifications. Briefly, cells were resuspended and incubated at 5 × 10⁶ cells/ml in a lysis buffer of 50 mM HEPES (pH 7.4), 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% CHAPS, and 0.1% Nonidet P-40 for 30 min at 4 °C. Cell lysates were cleared at 10,000 × g for 10 min at 4 °C. Caspase-3 activity was measured in the supernatant using colorimetric Ac-DEVD-pNA as a substrate. The cleavage of the substrate was determined using a kinetic microplate reader ( Molecular Devices Corp., Sunnyvale, CA) at a wavelength of 405 nm. Caspase-3 activity was calculated according to the slope of the A₄₀₅ versus time plot from linear regression analysis. Data were expressed as picomoles of the colorimetric substrate pNA liberated per minute and microgram of protein.

Subcellular Fractionation—Cells were treated with etoposide or Me₂SO for 96 h and harvested by centrifugation at 900 × g for 5 min at 4 °C. Cell pellets were washed twice with ice-cold PBS and resuspended in buffer A (20 mM HEPES (pH 7.5), 250 mM sucrose, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After 10-min incubation on ice, cells were homogenized using a Dounce tissuemizer grinder. Cell homogenates were centrifuged at 1,000 × g for 10 min at 4 °C to discard unbroken cells and nuclei. Supernatants were subjected to additional centrifugation at 10,000 × g for 25 min at 4 °C. The resulting pellets (rich in mitochondria) were resuspended in buffer A and designated as the heavy-membrane fraction. The supernatants were further centrifuged at 150,000 × g for 90 min at 4 °C and collected as the cytosolic fraction.

Western Blot Analysis—Whole cell lysates were prepared in a modified radioimmune precipitation assay buffer as described previously (24). Equal amounts of lysates were resolved on a SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. Protein expression was determined by immunoblotting with appropriate antibodies as described previously (24). The protein was visualized by using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

Statistics—All data are presented as means ± S.D. Differences in anchorage-independent colony formation and effects of caspase inhibitors on the sub-G₀ population were determined by Student’s t test. SigmaPlot (SPSS Inc., Chicago, IL) was used to perform statistical analyses. The difference was considered to be statistically significant if the p value was <0.05.

RESULTS

Expression of PKC-ε Isoform Is Phenotype-specific and Regulated Primarily at the Level of Gene Transcription—The underlying mechanism of developing drug resistance in relapsed SCLC patients is unclear. This resistance may be due, in part, to a transition of the SCLC toward the NSCLC histology (2, 25), which shows poor response rates to chemotherapy. Furthermore, it has been reported that the drug-resistant phenotype is associated with expression and/or activity of PKCs in lung cancer cell lines and lung carcinomas (19, 20). Therefore, expression profiling of PKC isoforms was determined by Western blot analysis using isoform-specific antibodies in a panel of human lung cancer cell lines, which includes eight NSCLC lines and four SCLC lines. As shown in Fig. 1A, a striking differential expression pattern for the PKC-ε isoform was observed between the NSCLC and SCLC phenotypes: its expression was detected in all of the NSCLC lines but none of the SCLC lines. Expression of other PKC isoforms (α, β, βII, γ, δ, γ, and η) shows no specific association to either phenotype, and expression of PKC-ζ and ε isoforms was undetectable (Fig. 1A and data not shown). Furthermore, NCI-H22 SCLC cells, when phenotypically transformed to the NSCLC-like phenotype by oncogenic Ras, express PKC-ε (Fig. 1A, H82/RasV12 lanes) (24), suggesting that PKC-ε expression is associated with the NSCLC-phenotype in lung cancer cells. Expression of PKC-ε was further examined at levels of steady-state mRNA and gene transcription to determine the molecular basis of the phenotype-specific expression. Northern Blot analysis (Fig. 1B or data not shown) demonstrates that the lack of PKC-ε protein

FIG. 1. Phenotype-specific expression of the PKC-ε isoform in human lung cancer cell lines. A, differential expression of PKC isoforms. Equal amounts of whole-cell lysates prepared from log-phase growing cells were subjected to Western blot analysis with antibodies against specific isoforms, including α, ε, β, βII, and δ. B, Northern blot analysis. Total cellular RNAs (20 μg) were separated on a denaturing agarose gel, transferred to nylon membrane, and hybridized to 32P-labeled PKC-ε cDNA probe. The membrane was stripped and re-probed with the β-actin cDNA (middle panel). Lower panel, photograph of 18 S RNA on 1% formaldehyde-agarose gel in the presence of ethidium bromide. C, nuclear run-on transcription assay. The 32P-labeled RNA transcripts isolated from nuclei of representative cell lines were hybridized to the immobilized cDNAs as indicated. β-Actin and 18 S ribosomal cDNA were used as positive controls. H82/RasV12, two clonal lines of c-Ha-Ras(V12)-transfectants of NCI-H82 (H82) cells (24).
expression in SCLC cell lines is associated with a deficiency in the expression of steady-state PKC-ε mRNA in these cells. Undetectable expression of PKC-ε mRNA in SCLC cells may be due to changes in the message half-life or transcription of the gene. To distinguish between these two possibilities, nuclear run-on transcription experiments were performed to determine the transcription rate for the PKC-ε gene. As shown in Fig. 1C, PKC-ε gene transcription was undetectable in SCLC lines (H82 and H69), whereas NSCLC lines (H23 and H157) were capable of transcribing the gene. It appears that in SCLC cells the PKC-ε gene is transcriptionally inactive. Together, these data suggest that the phenotype-specific expression of PKC-ε is controlled primarily at the level of gene transcription.

Expression of PKC-ε Enhances Cell Survival to Chemotherapeutic Drugs—It has been reported that the Myc/Ras-mediated phenotypic transition of SCLC toward NSCLC-like phenotype is associated with an acquired resistance to 2-difluoromethoxybenzamide (25). Additionally, we have found that induction of PKC-ε expression in Ras-transformed NCI-H82 SCLC cells is correlated with altered drug sensitivity to N\textsubscript{2},N\textsubscript{12}-bis(ethyl)-spermine, an anti-cancer polyamine analogue.\textsuperscript{2} These data suggest that phenotype-specific expression of PKC-ε may be important for cellular response to drug treatment. To explore the functional significance of PKC-ε expression on chemosensitivity, NCI-H82 (H82) human SCLC cells that do not express endogenous PKC-ε (Fig. 1) were stably transfected with an expression plasmid encoding the FLAG epitope-tagged full-length human PKC-ε. Several G418-resistant clones were isolated and expanded, in which PKC-ε expression was confirmed by Western blot analysis using anti-PKC-ε antibody (Fig. 2A).

FIG. 2. PKC-ε expression enhances cell survival to chemotherapeutic drugs. A, Western blot analysis of PKC isoform expression. Equal amounts of whole-cell lysates were analyzed for the expression of PKC-ε, -δIII, and -δ isoforms using isoform-specific antibodies. B, growth curves in complete media. Cells were plated at 5 × 10\textsuperscript{4} cells/well in six-well plates. Cell numbers were determined every 2 days for 8 days. Data shown are the means of triplicate culture from one representative experiment; bars, S.D. At least three independent experiments were performed with similar results. C and D, dose-dependent effects of etoposide and doxorubicin on cell survival. Cells were treated with etoposide (C) or doxorubicin (D) at indicated concentrations for 96 h. Control cells were treated with vehicle, Me\textsubscript{2}SO (final concentration: <0.1%). Cell viability was determined by trypan blue exclusion. Results are expressed as a percentage of viable cell numbers in treated cells compared with that of untreated control cells. Data are means ± S.D. from three independent experiments performed in triplicate. H82-PKC-ε-1, -7, and -12 are clonal populations of PKC-ε-transfected NCI-H82 cells. H82-Vector is a pool of vector-transfected NCI-H82 cells.

Three PKC-ε-expressing clonal cell lines (1, 7, and 12) were used in this study. As a control, a pool of G418-resistant clones from the empty vector-transfected NCI-H82 cells was generated and designated as H82-Vector. Compared with the control (H82-Vector) cells, PKC-ε-expressing clonal cell lines displayed very similar growth kinetics in complete medium (Fig. 2B) but showed an increased ability to grow on soft agar (Table I). Differences in the anchorage-independent colony formation between PKC-ε-expressing cells and H82-Vector cells were statistically significant (p values were 0.0034, 0.0004, and 0.014 for PKC-ε clones #1, #7, and #12, respectively, compared with H82-Vector). It should be noted that forced expression of PKC-ε did not result in an alteration of expression levels of other PKC isoforms and had no significant effect on cell cycle progression (Fig. 2A and data not shown).

Strikingly, significantly increased cell survival was observed in PKC-ε-expressing cells relative to control cells (H82-Vector) in response to the chemotherapeutic drugs, etoposide and doxorubicin, at all concentrations tested (Fig. 2, C and D). Cell viability studies indicate that induction of PKC-ε expression enhanced cell survival in a dose- and time-dependent manner (data not shown). The IC\textsubscript{50} values were increased by 4- to 5-fold for etoposide and 2-fold for doxorubicin in PKC-ε-expressing cells, compared with H82-Vector cells (Table I). At the concentration of 200 nM, etoposide exposure led to a significant cell death (30% viability) in control cells (H82-Vector), whereas 80%
of PKC-ε-expressing cells remained viable. At higher concentrations of the drug, i.e., at 1 μM etoposide, although the viability of PKC-ε-expressing cells decreased to 28–35%, they maintained the survival advantage over H82-Vector cells, compared with 2% viability (Fig. 2C). Although the PKC-ε expression level varied in three clonal cell lines (Fig. 2A), no significant differences were observed with respect to chemo-resistance. These data demonstrate that PKC-ε has protective effects on drug-induced cytotoxicity and that its induction contributes to the development of drug resistance in NCI-H82 SCLC cells.

Expression of PKC-ε Suppresses Etoposide-induced Apoptosis—Etoposide has been shown to induce both necrosis and apoptosis in cancer cells. To determine the nature of etoposide-induced cell death in H82 cells, we assessed morphological changes in the nuclear/chromatin structure of cells in response to etoposide by double-staining cells with Hoechst 33342 and propidium iodide, followed by fluorescence microscopy. It was found that, in H82-Vector cells, etoposide induced morphological alterations typical for apoptosis (26), including chromatin condensation and fragmentation, nuclear swelling, and the appearance of apoptotic bodies (Fig. 3A, upper panel). However, etoposide-treated PKC-ε-expressing cells retained much of their original morphological features with few condensed nuclei (Fig. 3A, lower panel). Furthermore, time course studies indicate that expression of PKC-ε profoundly delayed the onset of apoptosis induced by etoposide (Fig. 3B). Consistent with this result, internucleosomal DNA fragmentation was detected at early time points between 48 and 72 h in H82-Vector cells treated with etoposide but was not detected until later points (>96 h) in PKC-ε-expressing cells (Fig. 3C). Importantly, the integrity of the genomic DNA from PKC-ε-expressing cells was largely retained, whereas that of H82-Vector cells was significantly affected in response to etoposide, showing a clear sign of degradation in the large-size genomic DNA in addition to the internucleosomal DNA fragmentation. These data indicate that PKC-ε-mediated cell survival is associated with the inhibition of apoptosis.

It was reported that exposure to topoisomerase II inhibitors, such as etoposide, induce S phase delay in SCLC cells that may contribute to differential sensitivity to the drug (27). The protective effect of PKC-ε against etoposide-induced apoptosis may reflect the ability of PKC-ε to disrupt cell cycle control mechanisms. Effects of PKC-ε expression on cell cycle progression were determined by flow cytometry analysis to examine DNA contents of etoposide-treated and untreated cells. As shown in Fig. 3D, untreated H82-Vector and H82-PKC-ε-expressing cells were distributed very similar among different phases of cell cycle. However, marked differences were observed in etoposide-treated cells. The sub-G1 population (DNA content <2N) was increased in H82-Vector cells from 6.7% to 20.4%, reflecting increases in the proportion of apoptotic cells, whereas that of PKC-ε-expressing cells remained basically unchanged (4.6% versus 7.1%). Time course studies indicate that increases in sub-G1 population was time-dependent, which occurred at 24 h of post-treatment and progressively increased over the 96-h treatment period (data not shown). It appears that in response to etoposide, the increased sub-G1 population was accompanied by a decrease in the G1 population in H82-Vector cells, whereas populations in the S and G2/M phases were very similar among PKC-ε-expressing cells and H82-Vector cells. These results further confirm a role of PKC-ε on the inhibition of the etoposide-induced apoptosis, suggesting that the influence of PKC-ε expression on cell survival may affect events that control the G1/S phase checkpoint function in response to DNA damage.

Expression of PKC-ε Results in Inhibition of Etoposide-induced Caspase Activation—Caspase activation plays a central role in the execution of apoptosis. Studies have suggested that genotoxic agents such as etoposide induce apoptosis via the mitochondrial/caspase-9-mediated pathway, which involves sequential activation of caspase-9 and caspase-3 (8, 28). It is possible that the protective effect of PKC-ε on etoposide-induced apoptosis may result from blocking mitochondria-dependent caspase activation. Therefore, activation of procaspase-9 was evaluated in response to etoposide treatment by Western blot analysis using antibodies that specifically detect the 37-kDa cleaved caspase-9 (the active form). As shown in Fig. 4A, treatment of H82-Vector cells with etoposide induced the proteolytic cleavage of procaspase-9 into a 37-kDa fragment, whereas the etoposide-induced procaspase-9 cleavage was blocked in PKC-ε-expressing cells. Consistent with caspase-9 activation, etoposide treatment in H82-Vector cells led to caspase-3 activation, evident by the appearance of the cleaved caspase-3 product (19-kDa). By contrast, the proteolytic cleavage of caspase-3 was abrogated in PKC-ε-expressing cells. Consistently, we found that the cellular activity of caspase-3 was attenuated in PKC-ε-expressing cells in response to etoposide treatment with etoposide. Together, these findings suggest that PKC-ε is involved in the regulation of etoposide-induced activation of caspase-9 and caspase-3. It should be noted that a faint band corresponding to the 37-kDa cleaved caspase-9 product was detected in both H82-Vector and PKC-ε-expressing cells in the absence of etoposide, suggesting that there is a basal level of caspase-9 activation. This may be due, in part, to the basal level of apoptosis seen in these cells (Fig. 3D, sub-G1 populations in untreated cells).

Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as poly(ADP-ribose) polymerase (PARP). Intact PARP (116 kDa) is cleaved into 24- and 89-kDa fragments representing the N-terminal DNA binding domain and the C-terminal catalytic domain of the enzyme. To further validate the induction of apoptosis by etoposide, Western blot analysis was performed to examine the cleavage of PARP in response to drug treatment. Consistent with caspase-3 activa-

### Table I

**Effects of PKC-ε expression on cell growth and drug sensitivity**

| Cell line | Growth on soft agar[^a] | Etoposide | Doxorubicin |
|-----------|------------------------|-----------|-------------|
| H82-Vector | 46 ± 24                | 100       | 50          |
| H82-PKCε-1 | 106 ± 47               | 640       | 22          |
| H82-PKCε-7 | 120 ± 44               | 490       | 26          |
| H82-PKCε-12 | 76 ± 23               | 450       | 22          |

[^a]: Numbers of colonies formed on 0.3% agar per 5000 cells plated. Colonies with ≥0.2 mm in diameter were scored. Data are the mean ± S.D. of three independent experiments performed in triplicate.

[^b]: p values were determined by Student’s t test in comparison to H82-Vector cells.

[^c]: Concentrations (nM) that result in 50% of cell death at 96 h. The values are the averages of results from at least three independent experiments.

[^d]: Resistance was calculated as the IC50 value of PKC-ε-expressing cells divided by that of H82-Vector cells treated with the indicated drugs.
tion, etoposide treatment resulted in the cleavage of PARP only in H82-Vector cells, as shown by the appearance of an 89-kDa fragment (Fig. 4B). To determine if the cleavage of PARP is caspase-dependent, the cell-permeable, irreversible, caspase inhibitors z-VAD-fmk and z-DEVD-fmk were used. H82-Vector cells were pretreated with the inhibitors for 1 h prior to the addition of etoposide. As seen in Fig. 5A, inclusion of either caspase inhibitor significantly reduced the PARP cleavage in response to etoposide, although z-VAD-fmk was more potent. z-VAD-fmk shows a broad range of specificity against multiple caspases and is thought to inhibit apoptosis at an early stage, prior to activation of caspase-2, -3, -6, or -7, which may explain its higher potency (29). Consistently, we found that z-VAD-fmk was more efficient in decreasing the accumulation of the sub-G₁ population in response to etoposide in H82-Vector cells (Fig. 5B). At the concentration of 20 μM, treatment with z-VAD-fmk resulted in a significant decrease (50% of reductions, p < 0.02) in the sub-G₁ population as compared with that of the etoposide treatment alone, whereas z-DEVD-fmk had less effect (35% of reduction, p < 0.05). Together, these observations suggest that PKC-ε-mediated cell survival to etoposide is due, at least in part, to the inhibition of drug-induced activation of caspase-dependent apoptotic signaling pathway(s).

PKC-ε Blocks Etoposide-induced Release of Cytochrome c—Apoptosis induced by chemotherapeutic agents or γ-irradiation is likely mediated by the mitochondria pathway (28). Recent studies have demonstrated that the primary regulatory step for mitochondria-mediated caspase activation might be at the level of cytochrome c release from the mitochondrial intermembrane space to the cytosol (8). Therefore, levels of cytochrome c in the cytosol were determined by subcellular fractionation followed by Western blot analysis. As shown in Fig. 6A, cytochrome c release in H82-Vector cells resulted in an increased level of cytochrome c in the cytosolic fraction, whereas levels of cytochrome c in PKC-ε-expressing cells were not affected by etoposide. Interestingly, low levels of cytochrome c were readily detected in the cytosolic fraction of PKC-ε-expressing cells in the absence of etoposide. It is unlikely that this was due to mitochondrial contaminations, because the expression of cytochrome c oxidase subunit IV (COX IV), an integral mitochondrial membrane protein, can not be detected in the cytosolic fraction. It may, therefore, reflect a basal level of cytochrome c release in cells that is independent of etoposide treatment. These data suggest that one possible mechanism underlying PKC-ε-mediated cell survival is to prevent cytochrome c translocation to the cytosol, thereby inhibiting etoposide-induced caspase activation.

Down-regulation of PKC-ε Expression with Antisense cDNA Sensitizes NSCLC Cells to Etoposide—The effect of PKC-ε induction on survival of SCLC cells suggests that the status of PKC-ε expression may correlate to how well the cells respond to chemotherapeutic drugs. This hypothesis prompted us to investigate the effect of specific down-regulation of PKC-ε expression on drug sensitivity in NSCLC cells. NCI-H157 human NSCLC cells, expressing a high level of PKC-ε (Fig. 1A, H157 lane), were transfected with an empty vector or a plasmid
RESULTS (Fig. 7) demonstrate that PKC-ε survival assays were performed to determine the etoposide sensitivity. Results (Fig. 7) demonstrate that PKC-ε survival assays were performed to determine the etoposide resistance assays. Whole-cell lysates were prepared and subjected to immunoblotting analysis with antibodies against PARP. The cleavage of PARP is quantified by densitometry and expressed as the fold induction relative to that in untreated controls designated as 1. B, caspase inhibitors significantly reduce the accumulation of sub-G1 populations in response to etoposide. H82-Vector cells were pretreated with indicated caspase inhibitors (20 μM) for 1 h prior to etoposide treatment (200 nM, 96 h). After 48-h incubation, another aliquot of inhibitors was added to cells. Cells were collected, fixed, and stained with propidium iodide, followed by FACS. Sub-G1 populations are defined as the portion with DNA content less than 2N. Data are means ± S.D. from four independent experiments, *p < 0.05 in comparison to the etoposide-treated cells, as assessed by Student’s t test.

that the expression level of PKC-ε is a key determinant of cellular susceptibility to etoposide in lung cancer cells.

DISCUSSION

In this study, we have revealed a phenotype-specific expression pattern for the PKC-ε isoform in human lung cancer cells. Our studies indicate that the transcriptional control of the PKC-ε gene is a critical step for its phenotype-specific expression. Furthermore, induction of PKC-ε expression renders cells resistant to chemotherapy, whereas its down-regulation (depletion) by antisense cDNA results in an enhanced chemo-sensitivity. These results suggest that regulation of the PKC-ε gene expression is a key event involved in cellular response to chemotherapeutic drugs. It is predictable that changes in PKC-ε gene expression would lead to alternations of the expression of many cellular genes, which may be the genetic determinants of chemo-sensitivity and chemo-resistance. Therefore, transcriptional activation of PKC-ε gene provides an intriguing mechanism by which drug resistance might be regulated.

PKC-ε is a member of the novel PKC subgroup, which appears to play an important role in growth regulation. Among PKC isoforms, PKC-ε is the only isoform that has been demonstrated to promote proliferation and transformation in both fibroblast and epithelial cells upon overexpression (30, 31). However, we find that expression of PKC-ε in NCI-H82 SCLC cells does not lead to profound changes in cell proliferation and serum dependence. Consistently, PKC-ε-expressing cells display very similar patterns of cell cycle progression as that of H82-Vector cells (Fig. 3D and data not shown). One explana-
PKC-ε on Cell Survival

FIG. 6. Expression of PKC-ε blocks etoposide-induced cytochrome c release. A, cells were treated with either vehicle or 200 nM etoposide for 96 h, followed by subcellular fractionation. Equal amounts of cytosolic and heavy membrane (mitochondria) proteins were separated by SDS-PAGE, transferred to membrane, and subjected to immunoblot analysis with an antibody against cytochrome c (Cyt c, top). The cytosolic fraction was confirmed to be free of mitochondria by blotting with an antibody to cytochrome c oxidase (COX IV, an integral mitochondrial membrane protein). PKC-ε expression was detected in both cytosolic and heavy membrane fractions. Actin expression was detected for loading controls. B, quantification of cytochrome c release. Levels of cytochrome c in cytosolic fractions were determined by densitometric analysis and normalized to the actin levels. Results are expressed as a ratio of the level of cytochrome c in treated cells to that of the respective untreated control. Data are the average of two independent experiments.

FIG. 7. Down-regulation of PKC-ε expression with antisense cDNA sensitizes NSCLC cells to etoposide. Clonogenic survival assays were performed to determine etoposide sensitivity. NCI-H157 cells transfected with empty vector (black bar) or antisense PKC-ε cDNA (open bar) were treated with either vehicle or etoposide at indicated concentrations for 48 h, followed by the incubation in the drug-free medium for 12 days. Colonies were then scored. Results are expressed as the ratio of numbers of colonies formed in the antisense-transfected cells relative to that in the vector-transfected cells × 100. Error bars: S.D. The inset shows the representative Western blotting of PKC-ε levels in transfected cells. Actin expression was detected for loading controls.

For PKC-ε on cell proliferation in our system might be due to the difference in the genetic background of the cells studied. Furthermore, the activation of the mitogen-activated protein kinase (MAPK) pathway, the Raf-ERK pathway, is critical for PKC-ε-mediated cell proliferation and transformation in fibroblasts and epithelial cells (30, 32). However, we do not observe the activation of the ERK pathway in response to PKC-ε expression (data not shown), suggesting that the inability of PKC-ε to activate the ERK pathway might account for its little effect on proliferation. However, PKC-ε expression results in increased anchorage-independent growth (Table I), suggesting that PKC-ε may promote cell survival.

Resistance to apoptosis has been implicated as one potential mechanism whereby tumor cells escape death induced by chemotherapy. In this study, we show that induction of PKC-ε expression renders cells resistant to the chemotherapeutic drug etoposide, which is accompanied with a significantly reduction in apoptosis. This suggests that the pro-apoptotic effect of PKC-ε is mediated, at least in part, by suppression of drug-induced apoptosis. Etoposide induces the activation of caspase-9 and caspase-3, along with evidence of PARP cleavage. These events are correlated with the release of cytochrome c from the mitochondria to the cytosol, suggesting that mitochondrial pathways are involved in apoptosis induced by etoposide. In contrast, the death receptor-mediated pathway is unlikely to play a role in this process, because etoposide does not induce caspase-8 activation. Moreover, the caspase-8-specific inhibitor, z-IETD-fmk, has no effect on PARP cleavage and the accumulation of the sub-G1 population in response to etoposide (data not shown). That the pan-caspase inhibitor, z-VAD-fmk, blocks the cleavage of PARP and attenuates the accumulation of the sub-G1 population suggests that etoposide-induced apoptosis is caspase-dependent. In any detectable etoposide-induced events, including changes in nuclear/chromatin morphology, accumulation of the sub-G1 population, activation of caspase-9 and -3, and the mitochondrial release of cytochrome c, were blocked or significantly reduced by the expression of PKC-ε. These findings suggest that PKC-ε inhibits etoposide-induced apoptosis at the level of, or upstream from, the mitochondria by preventing the release of apoptosis-promoting factors, such as cytochrome c.

In H82 cells, PKC-ε effectively blocks the etoposide-induced release of cytochrome c, thereby resulting in the inhibition of caspase-9 activation. However, PKC-ε seems to have little effect on the basal level of release of cytochrome c, which was detected in PKC-ε-expressing cells as well as H82-Vector cells in the absence of etoposide (Fig. 6A and data not shown). This is likely due to basal levels of apoptosis occurring independently of PKC-ε expression. It was observed that log phase growing cells display a basal level of caspase-9 activation, and a small portion of cells (5–7%) were accumulated in the sub-G1 fraction (Fig. 3D). This suggests that the anti-apoptotic function of PKC-ε with etoposide may require additional steps such as activation of PKC-ε and/or other signaling molecules. In support of this notion, a recent study (33) reported that the V1–2 peptide derived from the V1 region of PKC-ε blocked the protective effect of TPA in tumor necrosis factor α and calphostin C-induced apoptosis in U937 cells. The V1–2 peptide has been shown to specifically inhibit PKC-ε translocation by interfering with its anchoring (34). A plasma membrane translocation mechanism for PKC activation is well described (10), suggesting TPA-induced activation of PKC-ε is required for the suppression of apoptosis. It should be noted that, in the absence of etoposide, there is a higher level of cytosolic cytochrome c in...
PKC-ε-expressing cells than in H82-Vector cells. However, PKC-ε-expressing cells do not show an increased level of apoptosis (Fig. 3, A and D), suggesting expression of PKC-ε may raise the apoptotic threshold of a cell. It is possible that PKC-ε may employ one or more different mechanisms to promote the basal level of survival from that in response to etoposide.

Interestingly, a significant fraction of exogenously expressed PKC-ε is localized to the mitochondria-rich heavy membrane (Fig. 6A). It is unclear how PKC-ε is localized to the mitochondria. This may result from cytosolic to mitochondrial translocation due to the mitochondrial generation of diacylglycerol as reported previously (35). Several recent studies have suggested that the mitochondrial recruitment of PKC-ε may be associated with its activation. In PKC-ε transgenic mice, it was found that PKC-ε was colocalized with the mitogen-activated protein kinase (MAPK) in the murine heart mitochondria, which appears to be responsible for the increased PKC-ε-MAPK signaling (36). Ohnuma et al. (37) reported that PKC-ε activation by ischemic preconditioning in rabbit hearts is involved in the direct translocation of PKC-ε to the mitochondria. Additionally, we have observed that the PKC activator TPA can induce PKC-ε translocation from the cytosol to the heavy membrane in lung cancer cells expressing endogenous PKC-ε. Furthermore, Ruvolo et al. (38) reported that either the treatment of human pre-B REI cells with the PKC agonist Bryostatin-1 or forced expression of exogenous PKC-ε induces mitochondrial localization of PKC-ε, which is tightly associated with increased Bcl-2 phosphorylation, and enhanced resistance to chemotherapy. This study has defined a functional role for mitochondrial associated PKC-ε in Bcl-2 phosphorylation and in suppressing drug-induced apoptosis (38). Therefore, the mitochondrial associated PKC-ε may have a functional significance in mediating its anti-apoptotic function.

A growing body of evidence has suggested a general anti-apoptotic role for PKC-ε in different cell types in response to a variety of apoptotic stimuli. For examples, PKC-ε has been implicated in the protection of cardiac myocytes from hypoxia-induced cell death (34). Overexpression of PKC-ε in human TF-1 cells suppressed apoptosis upon IL-3 withdrawal and increased Bcl-2 expression (40). In particular, enhanced PKC-ε expression has been linked to a decreased cellular sensitivity to chemotherapeutic drugs, possibly through inhibition of drug-induced apoptosis. It has been reported that PKC-ε overexpression in R6 rat embryo fibroblasts prevented cisplatin-induced apoptosis and protected cells against cisplatin cytotoxicity (41). Additionally, compared with the cisplatin-sensitive ovarian carcinoma 2008 cells, the resistant-variant 2008/C13*25 cells displayed increased PKC-ε expression and a significant reduction in PKC-ε expression (42). Consistent with our findings, Wu et al. (43) recently demonstrated that overexpression of PKC-ε rendered LNCaP human prostate cancer cells resistant to apoptosis, which was accompanied with a lack of stimulation of caspase-3 proteolytic activity in response to apoptotic stimuli. In addition, we find that expression of a dominant negative mutant of PKC-ε in NSCLC cells results in apoptosis instead of a G2/M cell cycle arrest in response to paclitaxel. Apoptosis is accompanied with the activation of caspase-9 and the cleavage of PARP. These data suggest that inhibition of caspase activation may be a common mechanism by which PKC-ε protects cells from death. Interestingly, proteolytic activation of PKC-ε during chemotherapeutic agent-induced apoptosis by caspase-3 has been reported in U937 cells (44). However, the functional significance of this proteolytic activation of PKC-ε is unclear.

In summary, we have defined an anti-apoptotic mechanism by which PKC-ε promotes survival of human lung cancer cells to chemotherapeutic drug etoposide. PKC-ε induction in H82 SCLC cells confers drug resistance without affecting cell proliferation. The influence of PKC-ε on the sensitivity to etoposide is mediated by one of the inhibition of caspase activation through one or more of the mitochondrial pathways. Although other mechanisms potentially involved in drug resistance (e.g. modulation of P-glycoprotein expression) might also be affected by PKC-ε expression (39), our findings suggest that the abnormal expression of PKC-ε could raise the apoptotic threshold of cells, thereby leading to resistance to chemotherapy. Elucidating the precise mechanism(s) of drug-induced apoptosis and how PKC-ε may affect this process in mediating lung cancer cell survival will likely lead to better therapeutic regimens that target the apoptosis machinery to overcome drug resistance.

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