A p53-independent G1 Cell Cycle Checkpoint Induced by the Suppression of Protein Kinase C α and θ Isoforms*

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Linda Deeds, Sanda Teodorescu, Michelle Chu, Qiang Yu, and Chang-Yan Chen‡
From the Cancer Research Center, Boston University School of Medicine, Boston, Massachusetts 02118

The protein kinase C (PKC) family consists of multiple isoforms that are involved in the regulation of diverse cellular responses. Suppression of PKC induces growth arrest in various types of cells. However, the underlying molecular mechanisms have not been thoroughly investigated. In this report, we demonstrated that the concurrent inhibition, rather than separate inhibition, of phorbol ester-dependent PKC α and θ isoforms is crucial for the induction of G1 cell cycle arrest and that this negative cell cycle regulation is via p53-independent mechanisms. PKC suppression-mediated growth arrest is associated with the induction of cell cycle inhibitor p21WAF1/CIP1 and the occurrence of hypophosphorylated Rb. The G1 checkpoint induced by the suppression of PKC occurs not only in murine Swiss3T3 but also in p53-deficient cells and human lung cancer cells containing mutated p53. Luciferase and nuclear run-off assays demonstrated that p21WAF1/CIP1, in part, transcriptionally regulated in response to the suppression of PKC α and θ. However, the stability of p21 mRNA is also augmented after the addition of PKC α and θ antisense oligonucleotides, indicating the involvement of post-transcriptional mechanisms in p21WAF1/CIP1 expression. These data suggest the existence of a cell cycle checkpoint pathway regulated by PKC α and θ isoforms. Furthermore, our findings support the notion that G1 checkpoint control can be restored in tumor cells containing abnormal p53, by targeting the PKC-regulated p21WAF1/CIP1 induction.

PKCas a Ca2+- and phospholipid-dependent serine/threonine kinase that functions as a cellular mediator of signal transduction initiated by various stimuli (1, 2). PKC family consists of at least 11 structurally related serine/threonine protein kinases and can be divided into three subgroups based on differences in their structures and biochemical properties (3, 4). PKC α, βI, βII, and γ are calcium- and phorbol ester/diacylglycerol-dependent. PKC θ, δ, ε, η, and μ only respond to phorbol ester/diacylglycerol. PKC ζ, ι, and λ belong to an atypical PKC subclass that is insensitive to calcium and phorbol ester/diacylglycerol. PKC is also the cellular receptor for phorbol esters and other tumor promoters that can substitute for diacylglycerol and directly activate the enzyme (5, 6). Phorbol esters elicit a variety of physiological responses in different target cells, including modulation of ion fluxes, secretory responses, changes in gene expression, and inhibition of growth (7–12).

Chronic exposure of cells to phorbol esters causes down-regulation of PKC activity, resulting from its prolonging association with the cellular membrane and subsequent rapid degradation (13–15). One of the effects of persistent suppression of PKC activity by either persistent high dose of phorbol esters or PKC inhibitors is to arrest cells in the G1 phase of the cell cycle (15–17). The mechanisms of such growth suppression are not yet clear. The studies of the effect of PKC on cell cycle progression provided very different and, somehow, controversial results. Treatment of phorbol esters stimulated proliferation of mammary epithelial cells and lymphokine production of lymphocytes (16, 18). In tumorigenesis, increase in PKC activity, especially the PKC α isoform, has been observed to be associated with breast cancer development (19, 20). On the contrary, others reported that a low dose of phorbol esters induced transient p21WAF1/CIP1 expression in epithelial cancer or leukaemic cells in a p53-independent fashion (21–23). It is possible that the differences of the phenotypic effects of PKC are dependent upon the cell type and the PKC isoforms being examined.

Cell cycle progression is regulated by cyclin-dependent kinase (CDK) family members and tumor suppressors, such as Rb and p53. Cell growth is blocked when CDK-cyclin complexes cannot form or when the catalytic activity of these complexes is suppressed through binding of a CDK inhibitor (24–28). The p21WAF1/CIP1 gene, which transcribes one of the cell cycle inhibitors, is shown to contain p53-response elements. This cell cycle inhibitor is also a downstream effector of several other tumor suppressors, such as BRCA1 and TGFb (transforming growth factor) (24, 29–35). The effect of p21WAF1/CIP1 is best studied in the p53 pathway, in which it acts as a crucial mediator of p53-dependent G1 cell cycle arrest. In response to DNA damage caused by radiation or chemicals, p21WAF1/CIP1 is rapidly induced in a p53-dependent manner (24, 33, 26, 37). In p21WAF1/CIP1 null cells, the cell cycle arrest mediated by certain DNA damage agents is impaired (38, 39). The study has been performed to examine the expression of p21WAF1/CIP1 in a variety of experimental settings, in vitro or in vivo, in which p53 is absent (40). By comparison of wild-type and p53-deficient mice, it suggests that transcriptional or post-transcriptional regulation of p21WAF1/CIP1 can be accomplished by other factors besides p53 (40). The p53-independent induction of p21WAF1/CIP1 has been shown to inhibit cell division under various circumstances, in which p21WAF1/CIP1 inhibits the activity of CDKs and further affects Rb/E2F activity (22, 40–42).

In response to various environmental stimulations, including exposure to intrinsic or extrinsic toxic agents, cells have evolved mechanisms to prevent such damage. One of the pro-
tective mechanisms is cell cycle checkpoints that exert negative controls over cell cycle progression via eliciting growth arrest or apoptosis. These controls allow us to either eliminate tumor-prone cells or repair cellular/genomic damages prior to entering the next phases of the cell cycle. Although the action of PKC activity has been demonstrated to be linked to cell growth and tumor promotion, the mechanism of PKC suppression-induced cell cycle arrest is not clear. We hypothesize that PKC suppression-activated cell cycle arrest may potentially guard normal cells against transformational or tumorigenic effects. Therefore, it is important to understand the underlying mechanisms of the growth arrest mediated by PKC suppression and its impact on cells, especially on tumor cells containing nonfunctional or deleted p53.

In this study, we investigated thoroughly the mechanisms of cell cycle arrest mediated by persistent PKC inhibition. Individual PKC isoform has been shown to possess its own role in the regulation of various cellular responses. PKC α and θ have been shown to regulate cell growth and counteract the apoptotic signaling, such as Fas-mediated apoptotic signals (1–6, 43). Therefore, the phenotypic effects of the suppression of these two PKC isoforms on the cell growth inhibition were primarily examined. We demonstrated that suppression of both PKC α and θ, using the antisense oligonucleotides, arrests cells in the G1 phase of the cell cycle. The cell cycle regulatory signals involving the G1 inhibitor p21WAF1/CIP1 and, perhaps, subsequent retinoblastoma (Rb) pathways are perturbed by the concurrent suppression of these two PKC isoforms. The blockade of cell cycle progression, under such conditions, is largely p53-independent. The induction of p21WAF1/CIP1 involves transcriptional and post-transcriptional mechanisms. We also demonstrated the existence of the restriction cell cycle checkpoint in tumor cells with either mutated or deleted p53, which can be activated by suppression of PKC α and θ expression/activities. Our data suggest a potential G1 checkpoint control regulated by PKC family members, in an Rb-dependent but not p53-dependent fashion.

MATERIALS AND METHODS

Cells and Transfections—Swiss3T3 fibroblasts and human lung cancer cell line 5800 cells were obtained from the American Type Culture Collection. Rb null mouse embryonic fibroblasts (MEF-/-) were obtained from Dr. Jackson (BIB, Cambridge, MA). SE8 cells are Swiss3T3 cells infected with E6 (obtained from Dr. Vaziri, Boston University School of Medicine). All cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum supplemented with glutamine and penicillin/streptomycin.

The concentrations of PKC isoform antisense oligonucleotides were first determined to be 20 μM. The oligonucleotides were mixed with QS2888 cytofertin (2.5 μg/ml, Invitrogen) in serum-free medium and incubated for 10 min at 37 °C. Cells were washed with serum-free medium twice and fed with the medium containing the oligonucleotide mixture and 0.5% serum for 48 h. Cells were refed with growth medium containing a half-dose of the oligonucleotides every other 2 days (44). For transient transfection, cells were transfected with p21WAF1/CIP1 and exposed to various treatments 48 h after the transfection.

DNA Profile Analysis—Cells were harvested, washed twice with cold 1× PBS, and fixed with fixation solution containing 35% ethanol and 65% Dulbecco’s modified Eagle’s medium. Subsequently, the samples were stained with staining solution containing 1× phosphate-buffered saline, 8 mg/ml RNase, and 18 mg/ml propidium iodide and incubated in the dark for over 30 min at room temperature. A Becton Dickinson FACScan machine was used to analyze the samples.

4HThymidine Incorporation Assay—Cells growing in 24-well plates were starved overnight in Dulbecco’s modified Eagle’s medium containing 0.5% serum to synchronize the cells. Two microcuries of 4H-thymidine was added directly to the starvation medium, with or without the treatments. Twenty-four hours later, the medium was aspirated, and cells were fixed. After washing with 1 ml of 5% trichloroacetic acid, cells were solubilized in 1% SDS, 0.3 N NaOH. Incorporated radioactivity was determined by a scintillation counter (45).

Northern Blot Analysis—Total RNA was prepared (17). Fifteen micrograms of total RNA per sample was fractionated in 1% agarose gels containing formaldehyde and ethidium bromide. Equivalent loading of RNA in samples was confirmed by visualization of 18 S rRNA. Gels were transferred to nitrocellulose with 20× SSC. RNA was UV-cross-linked to the filters using a Stratalinker device (Stratagene). Filters were hybridized with random primed 32P-labeled cDNA probes. Autoradiography was performed at −70 °C.

Luciferase Assay—Cells (20 × 10^4) were transiently transfected with 15 μg of the luciferase construct and 2 μg of G-luc for an internal control, using DMRIE-C reagent (Invirott). After the treatment, a luciferase assay was performed using a luciferase assay system kit (Promega, Madison, WI). The luciferase activity was determined using a luminometer.

Immunoprecipitation and Immunoblotting—Following the treatment, cell lysates were prepared, and total protein concentrations from each sample were normalized. Subsequently, lysates were adjusted to 0.4 M NaCl, 0.5% deoxycholate, and 0.05% SDS (46). Each sample was divided into two aliquots for reciprocal immunoblotting. The samples were immunoprecipitated with either anti-p21WAF1/CIP1 Ab or anti-CDK4 Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 4 h at 4 °C. Immunoprecipitates were collected with protein A-Sepharose and separated on a 12% SDS-PAGE gel. CDK4 or p21WAF1/CIP1 was then detected with the corresponding Abs. For cyclin D1 and Rb detection, whole cell lysates were prepared following various treatments. Cyclin D1 and Rb phosphorylation status were determined by immunoblotting using anti-cyclin D1 or Rb Ab (Santa Cruz Biotechnology).

Nuclear Run-off Analysis—Nuclei were prepared, and in vitro transcription was allowed to proceed in the presence of [32P]UTP (37). Labeled transcripts were hybridized for 3 days with filters containing immobilized DNA samples. Hybridized filters were washed at high stringency and RNase-treated prior to autoradiography.

RESULTS

Suppression of both PKC α and θ Causes Cell Cycle Arrest—It has been reported that suppression of PKC inhibits cell cycle progression (23, 47). However, the underlying molecular mechanisms are not yet clear. Although studies have demonstrated that the PKC family consists of more than 11 members that participate in diverse cellular response, it is not known which isoform(s) of PKC is involved in the regulation of growth arrest. In this study, we intended to address these questions. Previously, we have demonstrated that phorbol ester-dependent PKC α and θ isoforms act as prosurvival factors, counteracting Fas-mediated apoptotic signaling (43). The activity of these two isoforms has also been indicated to be important in the regulation of cell cycle progression in various types of cells (1–6). Furthermore, persistent high dose of phorbol esters preferentially inhibits phorbol ester-sensitive PKC isoforms and subsequently causes cells to arrest in the G1 phase of the cell cycle (13–15). These findings led us to first focus on PKC α and θ isoforms.

We employed antisense oligonucleotides to block expression of various PKC isoforms and thereby determine which PKC isoform(s) is necessary for cell cycle control. The antisense oligonucleotide sequence of each isoform PKC is presented in Table I. Phosphorothioate oligonucleotides were modified by the addition of a propynyl group to the pyrimidine bases, which can augment base stacking and facilitate the sense-antisense interaction (44, 48). The ability of Swiss3T3 cells to proliferate in medium containing PKC α or θ antisense oligonucleotide, alone or in pairwise combinations with various other phorbol ester-dependent PKC isoform antisense oligonucleotides, was tested using incorporation of [3H]thymidine into DNA as an index of cell cycle progression (Fig. 1a). Phorbol 12-myristate 13-acetate (500 nM), 1-0-hexadecyl-2-O-methyl-rac-glycerol (HMG; a PKC inhibitor that preferentially inhibits phorbol ester-sensitive PKC isoforms; Calbiochem), and GO9676 (a specific PKC α, β, and γ inhibitor; Calbiochem) were also employed in the [3H]thymidine incorporation assay. The cells were grown in medium containing 0.5% serum for 48 h to synchronize. Then growth medium containing 10% serum was added to
the cell cultures with or without the treatments. After releasing from serum starvation, control cells incorporated [3H]thymidine into their genomic DNA. However, GO9676, HMG, and G2/M phases. The DNA profile of PKC or α, or β, or ε, or γ, or δ, or η, or μ, or δ, or η, or μ, antisense oligonucleotides were modified by the addition of a propynl group to the pyrimidine bases.

| PKC isoform | Antisense oligonucleotide sequence |
|-------------|----------------------------------|
| PKC α       | 5′-TTGCGCGCTGTAACGTCAAGCCTG-3′   |
| PKC β       | 5′-TTGCAGAAGACCTGACATGTTG-3′     |
| PKC γ       | 5′-GAGAGCCATGCAACACTAGCTTGGTA-3′|
| PKC δ       | 5′-AGATGCGAAGAGGCTACGATGTG-3′   |
| PKC η       | 5′-AATTCTCTCGTCGGCGGACATGGCC-3′ |
| PKC μ       | 5′-AACAGAATCCCTGGCGGACATGGCC-3′ |

The Addition of PKC α and θ Antisense Oligonucleotides Induces p21WAF1/CIP1—Cell cycle transitions are orchestrated by CDKs. p21WAF1/CIP1, a CDK inhibitor, functions as a stoichiometric antagonist of CDKs (29–35). The inhibition of CDK activity by p21WAF1/CIP1 plays an important role in the control of cell cycle progression. Because cell cycle transitions were blocked by the suppression of PKC, it led us to examine the effect of PKC inhibitor HMG or the α and θ antisense oligonucleotides on p21WAF1/CIP1 expression. Cultures of Swiss3T3 cells were treated with 150 nM HMG for 24 h. 20 μM PKC α plus θ antisense oligonucleotides for 48 h, or 0.5 μM doxorubicin for 15 h (as control). After extracting total RNA from the cells, Northern blot was performed (Fig. 2a, upper panel). A basal expression of p21WAF1/CIP1 was detected in control cells. However, p21WAF1/CIP1 transcripts levels were dramatically increased in response to the suppression of PKC treatments (about 10-fold), and a slightly decreased induction of the transcripts was detected after exposure to doxorubicin (about 8.8-fold).

To further determine whether p21WAF1/CIP1 expression is regulated at the transcriptional level, we carried out a luciferase assay using a p21WAF1/CIP1 promoter-luciferase reporter gene (p21-Luc) in Swiss3T3 cells. After introducing the p21-Luc construct and treating cells with HMG, the combination of PKC α and θ antisense oligonucleotides, or doxorubicin, luciferase activity was measured (Fig. 2a, lower panel). The enzymatic activity was dramatically elicited by doxorubicin (about 50-fold), whereas the addition of HMG or the PKC isoform antisense oligonucleotides resulted in a smaller increase in luciferase induction (about 30-fold), despite higher levels of the induction of p21WAF1/CIP1 transcripts seen in the upper panel. This suggests the involvement of other regulatory events in p21WAF1/CIP1 expression, such as post-transcriptional mechanisms.

Because the growth arrest and the induction of p21WAF1/CIP1 occurred spontaneously in cells treated with the PKC inhibitor or antisense oligonucleotides, we then tested whether p21WAF1/CIP1 induction is linked to PKC down-regulation-mediated growth arrest. A p21WAF1/CIP1 null, mouse fibroblast cell line (CT2p21+/−) was used. [3H]Thymidine incorporation assay was conducted (Fig. 2b, upper panel). In the absence of p21WAF1/CIP1, the cells continued to uptake [3H]thymidine with or without the addition of HMG or PKC α and θ antisense oligonucleotides. DNA profile analysis was also conducted (Fig. 2b, lower panel). Following the PKC inhibitor or the antisense oligonucleotide treatment, more than 30% of CT2p21+/− cells still presented in S and G2/M phases of the cell cycle, and no G1 arrest occurred. We then introduced p21WAF1/CIP1 into CT2p21+/− cells to test whether forced expression of p21WAF1/CIP1 would regain growth control. Overexpressed p21WAF1/CIP1 was first confirmed by immunoblotting (Fig. 2c, upper panel). The even loading of total lysate protein was verified by reprobing the blot with anti-actin Ab (data not shown). DNA profile analysis was then performed in CT2p21+/− cells overexpressing p21WAF1/CIP1 (Fig. 2c, lower panel). After introducing p21WAF1/CIP1, CT2p21+/− cells became responsive to the treatment of PKC α and θ antisense oligonucleotides and entered a state of quiescence (G0/G1 phases). These results indicate that cell cycle arrest mediated by PKC α and θ antisense oligonucleotides is probably through eliciting p21WAF1/CIP1 expression/function.

Cell Cycle Arrest and p21WAF1/CIP1 Induction Mediated by the Suppression of PKC α and θ in p53 Null Cells—Studies suggest that the p21WAF1/CIP1 gene is a direct transcriptional target for p53 and has an important role in p53-mediated responses to...
Fig. 1. Effect of the suppression of PKC on cell growth. 

a, Swiss3T3 cells were cultured in 0.5% serum for 48 h. The growth medium containing 10% serum and [3H]thymidine (2 μCi/ml) was added to the cultures in the presence or absence of various treatments. Subsequently, trichloroacetic acid-insoluble radioactivity was determined. 

As, antisense oligonucleotide. Serum-starved, cells cultured in medium containing 0.5% serum; Control, cells cultured in the growth medium after serum starvation. The error bars represent the S.E. over five independent experiments.

b, after being released from the starvation medium, the cells were cultured in the growth medium containing HMG or the PKC isoform α and β antisense oligonucleotides, alone or in combination. The cells were then harvested and stained with PI for FACScan analysis. Control, cells cultured in the growth medium after serum starvation. 

c, the cells were cultured in medium containing 20 μM PKC α or β sense and antisense oligonucleotides for 48 h. The whole cell lysate from each treatment was prepared and immunoblotted for the expression of the corresponding PKC isoforms. d, the cells were cultured in medium containing 20 μM PKC α or β sense and antisense oligonucleotides for 48 h. Cell lysate was prepared and immunoblotted for the expression of various other phorbol ester-dependent PKC isoforms.
FIG. 2. Effects of the PKC suppression on \textit{p21}^{\text{WAF1/CIP1}} expression and cell cycle progression. \textit{a}, upper panel, after being released from the starvation medium, the cells were cultured in the growth medium and treated with HMG, PKC α and θ antisense oligonucleotides, or doxorubicin. \textit{p21}^{\text{WAF1/CIP1}} transcripts were detected by Northern blot analysis. \textit{Lower panel}, after transiently co-transfecting with \textit{p21}^{\text{WAF1/CIP1}} promoter-luciferase and β-galactosidase (an internal control) constructs, the cells were stimulated, and the luciferase activity was subsequently...
celular stress (29, 49, 50). The p21<sup>WAF1/CIP1</sup> promoter sequence contains the p53 regulatory motif, and cells lacking p53 express low levels of p21<sup>WAF1/CIP1</sup> (24, 36). Generally, it is believed that p21<sup>WAF1/CIP1</sup> serves as an effector of growth arrest in response to activation of the p53 checkpoint pathways (51). Recently, the p53-independent regulation of p21<sup>WAF1/CIP1</sup> has been reported (40–42). Therefore, it was of interest to us to determine whether p21<sup>WAF1/CIP1</sup> expression occurs via p53-independent mechanisms. The identification of the elements within the p21<sup>WAF1/CIP1</sup> promoter that are responsive to the suppression of PKC α and θ isoforms is under investigation. However, our data do not rule out the possibility of the compensation for p53 deficiency by other p53-related protein (such as p63 or p73).

The induction of p21<sup>WAF1/CIP1</sup> and its promoter responsive or induced by the suppression of PKC were further tested in human lung cancer 5800 cells in which endogenous p53 is mutated (Fig. 3b, right panels). A basal line expression of p21<sup>WAF1/CIP1</sup> transcripts was detected in untreated lung cancer cells. After treating the cells with PKC α and θ antisense oligonucleotides, p21<sup>WAF1/CIP1</sup> was significantly induced (8-fold). However, the level of the gene expression was slightly increased by doxorubicin (2.5-fold), indicating incomplete loss of p53 function in the lung cancer cells. Overall, the data suggest the existence of a p53-independent G1 cell cycle checkpoint in lung cancer 5800 cells, which can be elicited by the suppression of PKC activity.

**Interaction of p21<sup>WAF1/CIP1</sup> and CDK4 in Response to PKC Suppression**—The experiments presented above indicate that p21<sup>WAF1/CIP1</sup> is induced in response to the addition of HMG or PKC α and θ antisense oligonucleotides. The results also suggest the connection between p21<sup>WAF1/CIP1</sup> gene induction and growth arrest. Therefore, we further tested the expression/function of p21<sup>WAF1/CIP1</sup> at protein levels. In normal fibroblasts, p21<sup>WAF1/CIP1</sup> exists predominantly in CDks-proliferation complex of nuclear antigen-cyclin-p21<sup>WAF1/CIP1</sup> quaternary complexes, in which p21<sup>WAF1/CIP1</sup> as an universal cell cycle inhibitor, catalytically suppresses cyclin kinase activity and further blocks cell cycle progression signals (36). In addition, another function of p21<sup>WAF1/CIP1</sup> is to promote cyclin-CDK complex assembly. To examine whether p21<sup>WAF1/CIP1</sup> induced by the addition of PKC α and θ antisense oligonucleotides is able to interact with CDK4, the co-immunoprecipitation of p21<sup>WAF1/CIP1</sup> and CDK4 was performed (Fig. 4). There was no co-precipitation of these two molecules in the whole cell lysate from untreated Swiss3T3 cells. An anti-CDK4 immunoblot visualized CDK4 associated with the anti-p21<sup>WAF1/CIP1</sup> co-immunoprecipitate from the cells treated with HMG, the combination of PKC α and θ antisense oligonucleotides, or doxorubicin (as a control) (Fig. 4, first panel). The presence of p21<sup>WAF1/CIP1</sup> in the immunoprecipitates was confirmed by reprobing the blots with anti-p21<sup>WAF1/CIP1</sup> Ab (Fig. 4, second panel). High levels of p21<sup>WAF1/CIP1</sup> protein were recovered by the antibody in the cells treated with HMG or PKC α and θ antisense oligonucleotides, indicating that p21<sup>WAF1/CIP1</sup> is induced not only at the mRNA level but also at the protein level. This similar result was obtained from the reciprocal co-immunoprecipitation and immunoblotting experiments, in which a high level of p21<sup>WAF1/CIP1</sup> was recovered in the anti-CDK4 co-immunoprecipitate under the same experimental conditions (data not shown).

As a control, premunine serum was used for immunoprecipitation, followed by immunoblotting with anti-CDK4 or anti-p21<sup>WAF1/CIP1</sup> Ab to eliminate the possibility of nonspecific binding of these two proteins. There was no co-immunoprecipitation of either CDK4 or p21<sup>WAF1/CIP1</sup> with premunine serum (data not shown). The co-immunoprecipitation and immunoblotting of p21<sup>WAF1/CIP1</sup> and CDK4, in response to the
Fig. 3. p53 dependence of the cell cycle events induced by the suppression of PKC. a, upper panel, after serum starvation, SE6 cells (p53-deficient Swiss3T3) were cultured in the growth medium containing [3H]thymidine (2 μCi/ml) in the presence or absence of various treatments. Subsequently, trichloroacetic acid-insoluble radioactivity was determined. As, antisense oligonucleotide; Serum-starved, cells cultured in medium containing 0.5% serum; Control, cells cultured in the growth medium after serum starvation. Error bars represent the S.E. over five independent experiments. Lower panel, SE6 cells were treated with HMG or the PKC isoform α and β antisense oligonucleotides, alone or in combination, and then stained with PI for FACSscan analysis. b, upper panels, after synchronization, SE6 or human lung cancer 5800 cells were cultured in the growth medium containing HMG, PKC α and β antisense oligonucleotides or doxorubicin. p21WAF1/CIP1 transcripts were detected by Northern blot analysis. Lower panel, SE6 or 5800 cells were transiently co-transfected with p21WAF1/CIP1 promoter-luciferase and β-galactosidase constructs, the cells were stimulated, and the luciferase activity was subsequently assayed. The units on the vertical axis represent the light intensity measured by a luminometer, which is proportional to luciferase concentration. Error bars represent the S.E. over five independent experiments.
Cyclin D-CDK complexes further phosphorylate Rb and promote the entry of S phase. To test whether transcriptional induction of cyclin D1 was affected by the suppression of PKC, a cyclin D-luciferase assay was conducted (Fig. 5a, upper left panel). After transfecting a construct containing the promoter region of the murine cyclin D gene linked to a luciferase gene into the cells, Swiss3T3 cells were exposed to HMG or the PKC isoform antisense oligonucleotides. The responsiveness of luciferase reporter gene was subsequently examined. Cyclin D promoter-driven luciferase activity was moderately high under normal growth conditions (control). The addition of HMG or PKC α and θ antisense oligonucleotides did not affect this cyclin D promoter-driven luciferase activity. The expression level of cyclin D1 protein in response to the suppression of PKC treatments was also examined by immunoblotting (Fig. 5a, upper right panel). After treating Swiss3T3 cells with HMG or PKC α and θ antisense oligonucleotides, similar levels of cyclin D1 protein were revealed by anti-cyclin D1 antibody in untreated or treated cells. The data indicate that cyclin D1 is not a rate-limiting factor for cell cycle progression in cells treated with the PKC inhibitor or the antisense oligonucleotides. It also suggests that the suppression of PKC may interfere with G1 regulatory events that are downstream of cyclin D.

Through physical interaction, cyclin D activates CDKs and subsequently phosphorylates Rb, leading to G1 progression (52). To test whether the suppression of PKC affects Rb phosphorylation status, we conducted immunoblot analysis of Rb in whole cell lysates from the cells without or with HMG or PKC α and θ antisense oligonucleotide treatment (Fig. 5b, upper panel). In control cells, the majority of Rb was phosphorylated and presented as a slowly migrating band. In contrast, the suppression of PKC treatments resulted in increase amounts of fast migrating, hypophosphorylated Rb proteins. The amount of total proteins loaded in each lane was normalized by reprobing the blot with anti-actin antibody (Fig. 5c, lower panel). Together with the data presented above, this suggests that PKC α and θ, once their expressions/activities are suppressed, may activate a negative cell cycle control machinery through p21WAF1/CIP1 induction, which inhibits CDKs and further represses Rb phosphorylation.

To further define the role of Rb in growth arrest induced by the suppression of PKC, we tested the induction of p21WAF1/CIP1 in Rb-deficient, mouse embryo fibroblasts (MEF Rb−/−), using Northern blot analysis (Fig. 5c). Low levels of p21WAF1/CIP1 transcripts were detected in control cells. Upon HMG or PKC α plus θ antisense oligonucleotide treatment, p21WAF1/CIP1 was induced in the cells with Rb null background. The interaction of p21WAF1/CIP1 and CDK4 was also examined in MEF Rb−/− cells, by co-immunoprecipitation and immunoblotting. CDK4 was recovered from anti-p21WAF1/CIP1 immunoprecipitated complexes in response to HMG or PKC α plus θ antisense oligonucleotide treatment in Rb-deficient MEF Rb−/− cells (Fig. 5c, upper panel). There was no such association between these two molecules in control cells. The presence of p21WAF1/CIP1 protein in the complex was confirmed by reprobing the blot with anti-p21WAF1/CIP1 Ab. Consistently, high levels of p21WAF1/CIP1 protein were present in the complexes from the treated cells. (Fig. 5d, lower panel).

DNA profile analysis was also conducted under the same conditions. Less than 20% of untreated cells progressed in the cell cycle phases beyond G1 phase (Fig. 5c). In contrast, after the addition of HMG or PKC α plus θ antisense oligonucleotides, a higher percentage of MEF Rb−/− cells were distributed in S and G2/M phases (>30%), which may be due to relative higher E2F activity in an Rb null background. MEF Rb−/− cells did not arrest in the G1 phase of the cell cycle in response to the
suppression of PKC. Overall, the data suggest that, in the PKC suppression-induced cell cycle arrest, Rb may act as a downstream effector of p21WAF1/CIP1.

**Induction of p21WAF1/CIP1 by the Suppression of PKC Isoforms Results from Increases in Transcriptional Activity**—The increased p21WAF1/CIP1-regulated luciferase activity in the cells treated with PKC α and β antisense oligonucleotides or HMG, as presented in Fig. 2a, suggests that this cell cycle inhibitor is regulated through transcriptional mechanisms. To further confirm this regulation, nuclear run-off assays were carried out with the mouse p21WAF1/CIP1 cDNA immobilized on filters to detect newly transcribed 32P-labeled RNA from nuclei isolated from the cells treated with either HMG or the combination of PKC α and β antisense oligonucleotides. 32P-labeled p21WAF1/CIP1 transcripts derived from treated Swiss3T3 nuclei were hybridized strongly (Fig. 6, left panel). In contrast, there was almost no hybridization in control cells. Nuclear run-off assay was also performed in p53-deficient SE6 cells under the same experimental conditions. Similar results were obtained, in which 32P-labeled p21WAF1/CIP1 transcripts derived from treated SE6 cells significantly hybridized on the filters, but those from untreated, control cells did not (Fig. 6, right panel). Together with the luciferase data presented in Fig. 2a, we conclude that the expression of p21WAF1/CIP1 induced by the suppression of PKC α and β isoforms results, at least partially, from transcriptional mechanisms. This transcriptional regulation is via p53-independent mechanisms.

**Induction of p21WAF1/CIP1 by the Suppression of PKC Isoforms Results from Increases in RNA Stability**—That the PKC suppression-induced luciferase activity, as an indicator of p21WAF1/CIP1 transcriptional activity, was relatively lower compared with the extent of p21WAF1/CIP1 transcript induction (see Fig. 2a), which suggests the involvement of other mechanisms in the regulation of p21WAF1/CIP1 expression. This led us to examine whether the expression of this cell cycle inhibitor is regulated at the post-transcriptional level. To monitor the half-life of p21WAF1/CIP1, Swiss3T3 cells were treated with 150 nM MEF8−/− cells, with or without the PKC α and β antisense oligonucleotides or HMG treatment, were prepared and immunoprecipitated with anti-p21WAF1/CIP1 Ab. Subsequently, the immunoprecipitates were examined for the presence of CDK4 by immunoblotting with anti-CDK4 Abs. The same blots were also reprobed with the same Ab as that used in the immunoprecipitation. e, after serum starvation, the cells were cultured in the growth medium and treated with HMG or the PKC isoform α and β antisense oligonucleotides. The cells were then harvested and stained with PI for FACScan analysis. Control, cells cultured in the growth medium after serum starvation.
HMG for 24 h or the combination of PKCα and θ antisense oligonucleotides for 48 h, or they were left untreated. Subsequently, the cells were cultured with actinomycin D (a RNA synthesis inhibitor) for various times. Total RNAs extracted from each sample with or without actinomycin D treatment were tested for p21WAF1/CIP1 expression by Northern blot (Fig. 7, upper panels). In control cells, p21WAF1/CIP1 transcripts were dramatically reduced at 2 h. In contrast, the messages of the inhibitor in the treated cells started to significantly reduce 4 h later after the termination of RNA synthesis. These data suggest that the induction of p21WAF1/CIP1 in response to the suppression of PKCα/H9251 and H9258 antisense oligonucleotides stabilized the p21WAF1/CIP1 transcripts in the p53-deficient cells (Fig. 7, lower panels). The data indicate that a post-transcriptional, regulatory machinery appears to be activated in response to the suppression of PKC and operates in a p53-independent manner.

DISCUSSION

In this study, we have investigated the mechanisms of growth arrest induced by PKC down-regulation. Using antisense oligonucleotide technique, we have demonstrated that the suppression of phorbol ester-dependent PKC isoforms α and θ, not other isoforms, is crucial for the induction of cell growth arrest in mouse Swiss3T3 fibroblasts. This negative cell cycle control, induced by the suppression of PKC activity, is regulated possibly through the expression of the cell cycle inhibitor p21WAF1/CIP1. It is known that phorbol ester-sensitive PKC isoforms, as the cellular receptor for phorbol esters and other tumor promoters, play a significant role in transmitting mitogenic signaling and promoting cell proliferation (5, 6). Thus, the PKC suppression-induced p21WAF1/CIP1 expression further emphasizes the connection between cell cycle regulation and tumorigenesis.

p21WAF1/CIP1 was initially identified as a p53-inducible protein. In response to DNA damage, it has been demonstrated that p21WAF1/CIP1 binds to various cyclin-CDK complexes and inhibits the activity of these complexes, resulting in suppression of cell cycle progression, in a p53-dependent manner. Recently, it has also been shown that up-regulation of p21WAF1/CIP1 can be accomplished by other cis or trans elements besides p53 (40). This p53-independent regulation of p21WAF1/CIP1 can be via either transcriptional or post-transcriptional mechanisms, depending upon the specific signaling pathways being activated or different cell types being examined (40). TGFβ or MyoD transcriptionally regulates p21WAF1/CIP1 expression, whereas in certain leukemia cell lines, the induction of p21WAF1/CIP1 by phorbol ester is via post-transcriptional mechanisms, in a p53-independent fashion (29–35, 53, 54). Using nuclei run-off assays, we found that the suppression of both PKC α and θ expression/function...
signals new $p21^{WAF1/CIP1}$ transcript synthesis, which is consistent with the data from the $p21^{WAF1/CIP1}$ promoter-luciferase reporter activity assay, and p53-independent mechanisms account for such induction. However, less efficient activation of $p21^{WAF1/CIP1}$ reporter gene and higher expression of the mRNA transcripts led us to further examine the post-transcriptional mechanisms. Measuring $p21^{WAF1/CIP1}$ transcript half-life demonstrated that $p21^{WAF1/CIP1}$ is in part post-transcriptionally regulated, as presented by an increase in half-life from 1 h in the control to more than 4 h in the treated cells. The PKC suppression-induced accumulation of the gene is not affected in cells that lack functional p53. Our study indicates that, in the regulation of the expression of $p21^{WAF1/CIP1}$ by the suppression of PKC $\alpha$ and $\theta$, different transcriptional and post-transcriptional factors other than p53 are involved. Given the fact that $p21^{WAF1/CIP1}$ is a cyclin-CDK assembly factor and cell cycle inhibitor, it is not surprising that this cell cycle inhibitor is subjected to many levels of regulation.

The role of PKC in cell cycle regulation has been reported with different conclusions, which may be due to different experimental settings or different cell types being examined. $p21^{WAF1/CIP1}$ is transiently induced in hematopoietic or leukemic cell lines after a low dose and short pulse of phorbol esters. This specific phorbol ester treatment in these cells activates PKC, and the cells progress through S-phase with the disappearance of $p21^{WAF1/CIP1}$ (22). Other studies also demonstrated that vascular endothelial or hematopoietic cells exhibited altered levels of cell cycle regulators in response to activation of PKC (55, 56). In HC11 mammary epithelial cells, studies have shown that the PKC $\alpha$ isoform translocates from the cytosol to the nucleus, following low dose phorbol ester treatment (23). Subsequently, the activated PKC $\alpha$ mediates transient growth inhibition and $p21^{WAF1/CIP1}$ induction. The implication of such growth arrest in connection to the nature of phorbol esters as tumor promoter is not clear. However, our present report demonstrated a very different pattern of cell cycle regulation mediated by suppression of both PKC $\alpha$ and $\theta$ isoforms or by exposure to PKC inhibitor, in which the cell cycle arrest and the induction of $p21^{WAF1/CIP1}$ are persistent, with no occurrence of apoptosis (data not shown). It is reasonable to believe that an acute, intensive increase of PKC activity may transiently switch on intracellular phosphorylation events. Among many phorbol ester-activated events, $p21^{WAF1/CIP1}$ may thus be temporarily induced. Because PKC is an important mitogenic signal transducer during cell proliferation, it is possible that persistent suppression of PKC is seen as an abnormal and dangerous signal by the cellular surveillance system. Subsequently, checkpoint mechanisms are mobilized, further halting cell cycle progression.

In order to block cell cycle progression, $p21^{WAF1/CIP1}$ interacts with cyclin-CDK complexes via its amino-terminal binding sites (57). It has also been shown that the protein binds to proliferation complex of nuclear antigen, a co-activator of DNA polymerase $\delta$ and $\epsilon$ activities through the carboxyl terminus, which suppresses the DNA synthesis (58). Furthermore, it has been suggested that $p21^{WAF1/CIP1}$ is phosphorylated at two sites (Thr$^{145}$ and Ser$^{146}$) of its C terminus by protein kinase B (59). The phosphorylation of Ser$^{146}$ regulates $p21^{WAF1/CIP1}$ stability. It is possible that, in PKC activation-mediated $p21^{WAF1/CIP1}$ expression, phorbol esters elicit protein kinase B activity and subsequently cause a transient increase in $p21^{WAF1/CIP1}$ protein expression by affecting its C-terminal phosphorylation status. In the case of persistent suppression of PKC $\alpha$ and $\theta$ isoforms, cellular protective mechanisms may be activated, resulting in $p21^{WAF1/CIP1}$ induction and subsequently causing prolonged growth arrest, perhaps by blocking the CDK/Rb/E2F axis of cell cycle progression machinery. It has been observed that the inhibition of PKC activity increases p53 stability, whereas the activation of PKC by phorbol esters blocks the DNA damage-induced accumulation of p53 (60). It is conceivable that PKC inhibition activates the counteracting phosphatase activity, which affects protein stability, through an unknown mechanism.

Tumors use a diversity of genetic mechanisms to escape immune surveillance for survival advantage. The most effective modifications are found in dismantling the expression/function of various tumor suppressors that are involved in the regulation of cell cycle progression or apoptotic process. Loss of p53 is a common phenomenon in tumor cells, resulting in changes in the cellular response to radiation or chemotherapy. Cells that lack p53-mediated cell cycle checkpoints become increasingly unstable in their genome and less prone to apoptosis (61). In this study, we demonstrated that the suppression of PKC $\alpha$ and $\theta$ expression elicits a G1 cell cycle checkpoint response in p53-deficient cells and that $p21^{WAF1/CIP1}$, as a key player, is involved in this negative cell cycle regulation. In PKC suppression-mediated cell cycle control, cyclin D, an early G1 regulator, is not a rate-limiting factor for cell cycle progression. Rb and cyclin A have been found to be perturbed in this cell cycle regulatory process. Previously, we reported that cells expressing oncogenic ras and mutated p53 are susceptible to Ras-mediated apoptosis, once endogenous PKC activity is suppressed (17). Therefore, it is tempting to suggest that cell cycle checkpoints are able to be restored under various circumstances and that the suppression of PKC elicits the G1 restriction point.

PKC has been shown to be involved in the regulation of various apoptotic processes, such as Fas- or ceramide-mediated apoptosis (43, 62–64). Fas, as an important apoptotic regulator, operates the apoptotic process through multiple pathways, mainly by eliciting caspase cascade. We previously have demonstrated that, to ensure transmission of the apoptotic signaling, Fas-mediated apoptotic signaling suppresses protective mechanisms, such as PKC activation (43). In contrast, PKC activation partially blocks Fas-induced apoptosis. In this protective action, PKC $\alpha$ and $\theta$ have been proven to be involved. Interestingly, in the present study, we demonstrated that the same isoforms, once their activities are suppressed, elicit a G1 cell cycle checkpoint. These findings indicate that the activity of PKC, especially that of the $\alpha$ and $\theta$ isoforms, is essential for cell proliferation as well as maintaining homeostasis.

In summary, the regulation of $p21^{WAF1/CIP1}$ in response to genotoxic damage, such as ionizing radiation, is important to our understanding of p53-induced cell cycle arrest. However, the regulation of $p21^{WAF1/CIP1}$ by factors independent of p53 indicates that this cell cycle inhibitor is a more universal regulator. Under the conditions in which PKC $\alpha$ and $\theta$ are suppressed, $p21^{WAF1/CIP1}$ is induced and activated in the absence of p53. This regulation operates by both transcriptional and post-transcriptional mechanisms. The identification of the factors that elicit the restriction checkpoint, as a result of becoming activated by PKC suppression, will improve the understanding of how different signals affect growth arrest. Moreover, it will provide knowledge about restoring the G1 checkpoint controls under the conditions in which p53 is deficient, especially in certain p53-mutated/deleted human tumors.

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