The p53 tumor suppressor is activated in the cellular response to genotoxic stress. Transactivation of p53 target genes dictates cell cycle arrest and DNA repair or induction of apoptosis; however, a molecular mechanism responsible for these distinct functions remains unclear. Recent studies revealed that phosphorylation of p53 on Ser46 was associated with induction of p53DINP1 expression, resulting in the commitment of the cell fate to apoptotic cell death. Moreover, upon exposure to genotoxic stress, p33DINP1 was expressed and recruited a kinase(s) to p53 that specifically phosphorylated Ser46. Here, we show that the pro-apoptotic kinase, protein kinase C δ (PKCδ), is involved in phosphorylation of p53 on Ser46. PKCδ-mediated phosphorylation is required for the interaction of PKCδ with p53. The results also demonstrate that p53DINP1 associates with PKCδ upon exposure to genotoxic agents. Consistent with these results, PKCδ potentiates p53-dependent apoptosis by Ser46 phosphorylation in response to genotoxic stress. These findings indicate that PKCδ regulates p53 to induce apoptotic cell death in the cellular response to DNA damage.

The cellular response to genotoxic stress includes cell cycle arrest, activation of DNA repair and, in the event of irreparable damage, induction of apoptosis. The signaling mechanisms responsible for regulation of the DNA damage response are largely unclear. Certain insights have been derived from the finding that the δ isofrom of protein kinase C (PKCδ) is activated in response to DNA damage. Notably, PKCδ is cleaved to a 40-kDa catalytically active fragment by caspase-3 in cells treated with DNA-damaging agents (1, 2). The finding that overexpression of the PKCδ catalytic fragment (PKCδCF) induces chromatid condensation and DNA fragmentation supports a role for PKCδ cleavage in the induction of apoptotic cell death (3). Interaction of PKCδCF with the nuclear DNA-dependent protein kinase catalytic subunit (DNA-PKcs) inhibits the function of DNA-PKcs to form complexes with DNA and to phosphorylate its downstream target, p53 (4). In addition, cells deficient in DNA-PK are resistant to apoptosis induced by PKCδCF overexpression (4). Other studies have shown that PKCδ interacts with the c-Abl tyrosine kinase upon exposure to genotoxic stress (5). c-Abl is a pro-apoptotic tyrosine kinase that targets to the nucleus following genotoxic stress (6–8). Importantly, c-Abl-mediated phosphorylation activates PKCδ and induces translocation of PKCδ to the nucleus (5). In concert with these findings, tyrosine phosphorylation of PKCδ is necessary for its nuclear translocation and subsequent caspase-dependent cleavage in the apoptotic response to DNA damage (9). A recent study demonstrated that nuclear-targeted PKCδ interacts with and phosphorylates a pro-apoptotic molecule, Rad9 (10). PKCδ regulates the interaction of Rad9 with Bcl-2 and the hRad9-mediated apoptotic response to DNA damage (10). Furthermore, previous studies showed that cells derived from PKCδ-null transgenic mice were defective in mitochondria-dependent apoptosis (11). These findings collectively support a pivotal role for PKCδ in the induction of apoptosis in response to DNA damage.

The p53 tumor suppressor functions in the cellular response to stress by inducing cell cycle arrest, DNA repair, senescence, differentiation, or apoptosis (12). Genotoxic stress is associated with stabilization of p53 and induction of p53-mediated transcription. Selective transactivation of p53 target genes dictates cell cycle arrest and DNA repair, or induction of apoptosis (13–15). However, the mechanism by which p53 determines the choice of cell fate is largely unknown. Available evidence suggests that promoter selectivity of p53 is regulated by its phosphorylation. For instance, Ser15 and Ser20 phosphorylation potentiates p53-dependent apoptosis in response to DNA damage (16). In this regard, Ser46 kinase(s) could function in p53-dependent apoptosis.

The present study demonstrates that PKCδ associates with p53. This association is required for PKCδ-mediated phosphorylation of p53 on Ser46 upon exposure to genotoxic stress. Furthermore, PKCδ promotes p53-dependent apoptosis in response to DNA damage.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human MOLT-4 leukemia cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Human U2-OS, MCF-7, 293T, and HCT116 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FBS and antibiotics. Cells were treated with 2 μg/ml adriamycin (ADR, Sigma-Aldrich), 10 μM etoside (Sigma-Aldrich), 500 μM cisplatin (Sigma-Aldrich), and 5 μM rottlerin (Sigma-Aldrich).

**Plasmids**—PKCδ expression plasmids were described previously (17, 18). p53 cDNA was amplified by PCR from human fetal brain cDNA library, then cloned into the pcDNA3-FLAG vector. The N-terminal
region of p53 (amino acids 1–92) was cloned into the pGEX4T-1 vector (Amersham Biosciences). Various mutations were introduced by site-directed mutagenesis and were confirmed by sequencing.

**Cell Transfections**—Cell transfections were performed as described above. The total DNA concentration was kept constant by including an empty vector.

**Immunoprecipitation and Immunoblot Analysis**—Cell lysates were prepared as described above and cleared by centrifugation at 12,000 × g for 15 min. Soluble proteins were incubated with anti-FLAG (Sigma-Aldrich), anti-PKCδ (Santa Cruz Biotechnology (SCBT)), or anti-p53 (SCBT) antibodies for 2 h at 4 °C followed by a 1-h incubation with protein A- (Amersham Biosciences) or G- (Zymed Laboratories) Sepharose beads. The immune complexes were washed three times with lysis buffer. Cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were then incubated with anti-FLAG, anti-Myc (Cell Signaling Technology), anti-GST (Nacalai Tesque), anti-GFP (Nacalai Tesque), or anti-p53DINP1 (Sigma-Aldrich, anti-phospho-p53 (Ser15, Ser20, and Ser46) (Cell Signaling Technology), anti-p53DIINP1 (Sigma-Aldrich), or anti-tubulin (Sigma-Aldrich). The antigen-antibody complexes were visualized by chemiluminescence (PerkinElmer Life Sciences).

**In Vitro Binding Assays**—Cell lysates were incubated with purified GST, GST-PKCδ regulatory domain (RD), or GST-PKCδCF (17) in lysis buffer for 2 h at 4 °C. The adsorbates were resolved by SDS-PAGE and analyzed by immunoblotting with anti-FLAG or anti-GST.

**In Vitro Kinase Assays**—Recombinant PKCδ (Calbiochem) was incubated in kinase buffer (19) with GST, GST-p53-(1–92) wild type, or the GST-p53-(1–92) S46A mutant and ATP for 20 min at 30 °C. Samples were separated by SDS-PAGE followed by immunoblot analysis with anti-phospho-p53 (Ser46), anti-PKCδ, or anti-GST.

Small Interfering RNA (siRNA) Transfections—siRNA duplexes (siRNAs) were synthesized and purified by Invitrogen (Stealth RNAi). Transfection of siRNAs was performed using Lipofectamine 2000 (Invitrogen).

**Assessment of Apoptosis**—Apopotic cells were detected by TUNEL assays using the DeadEnd Colorimetric TUNEL System (Promega).

**RESULTS**

**PKCδ Phosphorylates p53 on Ser46**—To investigate whether PKCδ is involved in phosphorylation of p53, MCF-7 cells were treated with the DNA-damaging agent adriamycin (ADR) in the presence or absence of the PKCδ-specific inhibitor, rottlerin (21). p53 was phosphorylated on Ser15 and Ser20 in response to ADR treatment regardless of PKCδ activity (Fig. 1A). By contrast, phosphorylation on Ser46 was diminished by pretreatment with rottlerin (Fig. 1B). Moreover, consistent with previous results (22), inhibition of PKCδ attenuated the expression level of p53 in relatively later periods following DNA damage (Fig. 1B). Similar results were obtained with U2-OS cells (data not shown). Comparable results were also observed when cells were treated with other DNA-damaging agents, such as etoposide and cisplatin (data not shown). To extend these findings using ectopically expressed p53, 293T cells were transfected with FLAG-tagged p53. Similar to endogenous p53, exogenous p53 was phosphorylated on Ser46 following ADR treatment (Fig. 1C). In contrast, there was little if any phosphorylation of overexpressed p53 on Ser46 in rottlerin-pretreated cells (Fig. 1C). These results indicate that PKCδ is involved in phosphorylation of p53 on Ser46.

To further define the role for PKCδ in Ser46 phosphorylation, FLAG-p53 was co-transfected into 293T cells together with the Myc vector, Myc-PKCδCF, or the Myc-PKCδCF(K→R) mutant, which is catalytically inactive (18). Expression of catalytically active PKCδ was associated with prominent phosphorylation of p53 on Ser46 (Fig. 2A, upper). Conversely, Ser46 phosphorylation was completely abrogated by expression of the dominant negative PKCδCF(K→R) mutant. Moreover, the level of p53 expression paralleled that of Ser46 phosphorylation (Fig. 2A,
PKCα Regulates p53-dependent Apoptosis

To determine if PKCα functions in DNA damage-induced Ser46 phosphorylation, 293T cells were co-transfected with FLAG-p53 and Myc vector, Myc-PKCαCF, or Myc-PKCαCF(K→R). Immunoprecipitates of lysates with anti-FLAG were analyzed by immunoblotting with anti-phospho-p53 (Ser46) (top panel) or anti-p53 (second panel). Cell lysates were also analyzed by immunoblotting with anti-Myc (third panel) or anti-tubulin (bottom panel). By contrast, co-expression with the GFP-PKCαCF (Fig. 2B). To confirm whether PKCα is responsible for Ser46 phosphorylation in response to DNA damage, we knocked down PKCα by transfection of cells with PKCα siRNAs. Down-regulation of PKCα was associated with attenuation of Ser46 phosphorylation following genotoxic stress (Fig. 2C). To establish a direct role for PKCα in Ser46 phosphorylation, kinase-active recombinant PKCα was incubated with ATP and GST, GST-p53(1–92) wild type or the GST-p53(1–92) mutant in which Ser46 is substituted with Ala. The finding that purified PKCα phosphorylated GST-p53(1–92), and not GST, indicates that PKCα is the Ser46 kinase in vitro (Fig. 2D). Notably, co-incubation of PKCα with the GST-p53(1–92) S46A mutant abrogated reactivity with anti-pSer46 (Fig. 2D). These results collectively support the direct role for PKCα in phosphorylation of p53 on Ser46 in response to DNA damage.

PKCα Interacts with p53—To examine whether PKCα associates with p53, MOLT-4 cells, which highly express p53, were treated with ADR. Lysates were immunoprecipitated with anti-PKCα or, as a control, IgG. Immunoblot analysis of the precipitates with anti-p53 revealed that PKCα associates with p53 (Fig. 3A). Moreover, treatment with ADR was associated with increased formation of PKCα-p53 complexes (Fig. 3A). In the reciprocal experiment, immunoblot analysis of anti-p53 immunoprecipitates with anti-PKCα demonstrated a low, but substantial level of interaction between PKCα and p53 (Fig. 3B). Importantly, the finding that PKCαCF was detectable in anti-p53 immunoprecipitates from ADR-treated MOLT-4 cells indicated that the catalytic fragment of PKCα is responsible for binding to p53 (Fig. 3B, upper). Similar results were obtained with U2-OS cells (data not shown). To identify the region of PKCα that associates with p53, lysates from 293T cells transfected with FLAG-p53 were incubated with purified GST, GST-PKCαCF or GST-PKCα regulatory domain (RD). Analysis of precipitates with anti-FLAG showed the binding of p53 to PKCαCF, but not to PKCαRD (Fig. 3C). These findings indicate that the catalytic domain of PKCα is required for binding to p53.
PKCδ Forms Complexes with p53DINP1 in Response to Genotoxic Stress—A previous study demonstrated that expression of the p53-inducible gene, p53DINP1, by DNA damage enhances Ser46 phosphorylation and leads to apoptotic cell death (23). Notably, p53DINP1 recruits an unknown kinase(s) responsible for Ser46 phosphorylation to p53 (23). These data indicate that p53DINP1 interacts with a Ser46 kinase(s) upon exposure to genotoxic stress. To examine the possibility that PKCδ associates with p53DINP1, 293T cells were co-transfected with GFP-p53DINP1 and FLAG vector, FLAG-PKCδ CF, or FLAG-PKCδ RD. Immunoblot analysis of anti-FLAG immunoprecipitates with anti-GFP demonstrated that FLAG-PKCδ FL, and not CF or RD, was associated with GFP-p53DINP1 (Fig. 4A). To extend these findings to endogenous proteins, MOLT-4 cells were left untreated or treated with ADR. Immunoprecipitates of lysates with anti-p53DINP1 were analyzed by immunoblotting with anti-PKCδ (upper panel) or anti-p53DINP1 (lower panel).
p53DINP1-PKC<sub>δ</sub> complexes (Fig. 4B). Similar results were obtained with U2-OS cells (data not shown). Comparable results were also observed when cells were treated with etoposide (data not shown). These findings demonstrate an inducible binding of PKC<sub>δ</sub> with p53DINP1 following genotoxic stress.

Whereas PKC<sub>δ</sub> induces p53 and p53DINP1 expression is regulated by p53, it is conceivable that PKC<sub>δ</sub> modulates the expression level of p53DINP1 in response to DNA damage. To examine this hypothesis, MCF-7 cells were treated with ADR in the presence or absence of rottlerin. The expression level of p53DINP1 was enhanced by ADR treatment (Fig. 4C). By contrast, induction of p53DINP1 expression was attenuated by pretreatment of cells with rottlerin (Fig. 4C). Similar results were obtained with U2-OS cells (data not shown). To confirm PKC<sub>δ</sub>-mediated regulation of p53DINP1, 293T cells were co-transfected with FLAG-p53 and GFP vector or GFP-PKC<sub>δ</sub>CF(K<sub>39</sub>R). Immunoblot analysis with anti-p53DINP1 revealed that expression of the dominant negative PKC<sub>δ</sub> mutant inhibited ADR-induced up-regulation of p53DINP1 (Fig. 2B). These findings support the mechanism by which PKC<sub>δ</sub> regulates p53DINP1 expression through p53 activation.

**PKC<sub>δ</sub> Potentiates p53-dependent Apoptosis in Response to DNA Damage**—Previous studies have shown that activation of PKC<sub>δ</sub> following genotoxic stress is associated with the execution of apoptosis (3, 4, 10); however, this mechanism is largely unknown. Importantly, the present study demonstrates that PKC<sub>δ</sub> phosphorylates p53 on Ser<sup>46</sup>, which is responsible for the induction of apoptosis. In this regard, PKC<sub>δ</sub> could be involved in p53-dependent apoptosis following genotoxic stress. To address this issue, U2-OS cells were pretreated with rottlerin followed by the treatment with etoposide for 24 h. Treatment of cells with etoposide increased induction of apoptosis (Fig. 5A). In contrast, pretreatment with rottlerin substantially attenuated etoposide-induced apoptosis (Fig. 5A). Similar findings were obtained with MCF-7 cells (data not shown). Comparable results were also observed when cells were treated with ADR (data not shown). To extend these findings, PKC<sub>δ</sub> was knocked down in U2-OS cells by transfection with PKC<sub>δ</sub> siRNAs. Knocking down PKC<sub>δ</sub> attenuated the induction of apoptosis elicited by etoposide treatment (Fig. 5B). These results suggest that etoposide-induced apoptosis is, at least in part, a PKC<sub>δ</sub>-dependent mechanism. To further define the role for PKC<sub>δ</sub> in p53-dependent apo-
ptosis, p53-deficient HCT116 cells (HCT116/p53−/−) (24) were transfected with FLAG vector, FLAG-p53 wild type (wt), or the FLAG-p53 S46A mutant, in which Ser46 was replaced with Ala. Cells were then treated with etoposide for 24 h in the presence or absence of rottlerin. TUNEL assays demonstrated that treatment of vector-expressing cells treated with etoposide for 24 h in the presence or absence of rottlerin. Furthermore, etoposide-induced apoptosis was enhanced by ectopic expression of p53 wt, and not the p53 S46A mutant (Fig. 5, C and D). Importantly, pretreatment with rottlerin was substantially attenuated etoposide-induced apoptosis regardless of p53 expression (Fig. 5, C and D). These findings provide support for the involvement of PKCδ phosphorylation of p53 on Ser46 in the apoptotic response to DNA damage.

DISCUSSION

The mechanisms by which genotoxic stress is converted into intracellular signals that control cellular fate are largely unknown. Certain insights have been derived from the findings that PKCδ is activated in the response of cells to agents that arrest DNA replication or induce DNA lesions (1, 2). The available evidence indicates that full-length PKCδ is activated as an early event within 1 h of exposure to genotoxic agents (5, 17). Phosphorylation of PKCδ on tyrosine is a mechanism for PKCδ activation by DNA-damaging agents (5, 9). In this context, activation of PKCδ is induced, at least in part, by c-Abl-dependent phosphorylation (5). Previous studies have also shown that treatment of cells with DNA-damaging agents is associated with translocation of PKCδ to the nucleus (10, 25). Inhibition of PKCδ kinase activity attenuates nuclear targeting of PKCδ. Whereas nuclear PKCδ associates with DNA-PKcs and hRad9 (4, 10), the nuclear targets of PKCδ are otherwise largely unknown. The present findings demonstrate that nuclear PKCδ also interacts with p53. Binding of PKCδ to p53 was detectable constitutively and increased in response to DNA damage. In this context, nuclear targeting of PKCδ and induction of p53 expression following genotoxic stress were both associated with increases in the formation of PKCδ-p53 complexes. Significantly, the present studies demonstrate that the catalytic fragment of PKCδ is responsible for binding to p53. Previous studies have shown that PKCδ is activated as a later event in the genotoxic stress by caspase-3-mediated cleavage (1, 2, 26). The cleaved C-terminal 40-kDa fragment contains the ATP binding and kinase domains, which is constitutively active. Interestingly, a recent study demonstrated that PKCδ activates caspase-3 by its phosphorylation (27). Thus, caspase-3 could be activated by PKCδ-dependent phosphorylation, then cleaved PKCδ-CF fragment by activated caspase-3 might potentiate interaction of PKCδ with p53. In addition to the interaction, we found that PKCδ was associated with induction of p53 expression in later periods following DNA damage. In concert with these results, a previous study showed that inhibition of PKCδ activity attenuated basal level of p53 expression (22). By contrast, another study demonstrated that treatment of HeLa cells with rottlerin increased cisplatin-mediated p53 level (28). Obviously, further studies will be needed to clarify a mechanism by which PKCδ regulates p53 expression.

In contrast to many p53 phosphorylation sites, Ser46 is phosphorylated in a later period following genotoxic stress (16). Moreover, this phosphorylation is required for expression of p53AIP1 that functions in induction of apoptosis (16). These findings thus suggest that Ser46 phosphorylation is essential for p53-dependent apoptosis in response to DNA damage. However, little is known about Ser46 kinase(s). Available lines of evidence revealed that HIPK2 phosphorylated Ser46 upon exposure to UV, but not ionizing radiation (29, 30). p38 MAPK was also reported as a Ser46 kinase; however, this phenomenon is controversial (16, 31–33). In this context, the present studies demonstrate that PKCδ is a novel candidate for Ser46 kinase upon exposure to genotoxic stress. The evidence that PKCδ is fully activated after cleavage by caspase-3 might be associated with a delayed phosphorylation of p53 on Ser46. Moreover, the present findings that the catalytic fragment of PKCδ phosphorylation is essential for p53 further support a role for PKCδ in Ser46 phosphorylation. We also show that PKCδ associates with p53DINP1 following DNA damage. A previous study demonstrated that p53DINP1 recruits kinase(s) responsible for Ser46 phosphorylation to p53. In this regard, inducible association of PKCδ with p53DINP1 provided a further support for the involvement of PKCδ in Ser46 phosphorylation. Importantly, coimmunoprecipitation studies indicate that fragments of PKCδ such as PKCδCF or RD was insufficient for binding to p53DINP1. A potential explanation is that the binding domain extends to both regions of PKCδ. Another possibility is that specific tertiary structure is required for the binding. Whereas PKCδCF can interact with p53, this interaction might be independent of the recruitment by p53DINP1. However, given the recent finding that active caspase-3 translocates to the nucleus after induction of apoptosis (34), it is conceivable that PKCδ is cleaved in the nucleus after recruitment of PKCδ by p53DINP1 to p53.

Upon exposure to genotoxic stress, p53 functions in both cell cycle arrest and induction of apoptosis. However, the mechanism by which p53 regulates these distinct outcomes is largely unknown. A previous study suggested that, for repairable DNA damage, p53 is phosphorylated on Ser15 and Ser20 and induces G1 arrest genes, such as p21. If DNA damage is severe and irreversible, Ser46 is phosphorylated, which then triggers induction of pro-apoptotic genes, such as p53AIP1 (16). Thus, Ser46 kinase(s) are activated in response to DNA damage and induce apoptosis, at least in a p53-dependent mechanism. In this context, the present results demonstrate that PKCδ phosphorylates p53 on Ser46 and induces apoptosis, at least in part, in a Ser46 phosphorylation-dependent manner. Moreover, PKCδ induces p53 expression in later periods following DNA damage. These findings thus support a model in which activation of PKCδ by genotoxic stress induces phosphorylation of p53 on Ser46, resulting in the commitment of cell fate to apoptosis.

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