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Protein Kinase C δ Induces Transcription of the TP53 Tumor Suppressor Gene by Controlling Death-Promoting Factor Btf in the Apoptotic Response to DNA Damage[▽]

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Expression of the TP53 tumor suppressor is tightly controlled for its ability to function as a critical regulator of cell growth, proliferation, and death in response to DNA damage. However, little is known about the mechanisms and contributions of the transcriptional regulation of TP53. Here we report that protein kinase C δ (PKCδ), a ubiquitously expressed member of the novel subfamily of PKC isoforms, transactivates TP53 expression at the transcriptional level. Reporter assays demonstrated that PKCδ induces the promoter activity of TP53 through the TP53 core promoter element (CPE-TP53) and that such induction is enhanced in response to DNA damage. The results also demonstrate that, upon exposure to genotoxic stress, PKCδ activates and interacts with the death-promoting transcription factor Btf to co-occupy CPE-TP53. Inhibition of PKCδ activity decreases the affinity of Btf for CPE-TP53, thereby reducing TP53 expression at both the mRNA and the protein levels. In concert with these results, we show that disruption of Btf-mediated TP53 gene transcription by RNA interference leads to suppression of TP53-mediated apoptosis following genotoxic stress. These findings provide evidence that activation of TP53 gene transcription by PKCδ triggers TP53-dependent apoptosis in response to DNA damage.

Apoptosis is a genetically controlled process of cell suicide, essential for the elimination of damaged cells from multicellular organisms. Inappropriate activation or inhibition of apoptosis contributes to the pathogenesis of cancer and autoimmune diseases (7, 16). However, the signaling mechanisms responsible for the regulation of apoptosis remain largely unclear. Protein kinase C δ (PKCδ), a ubiquitously expressed member of the novel subfamily of PKC isoforms, has emerged as a common mediator of apoptosis in response to many stimuli (19, 37). Previous studies have shown that the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate- or oxidative stress-induced mitochondrial translocation of PKCδ is associated with the loss of mitochondrial transmembrane potential and the release of cytochrome *c* (17, 18). These findings suggest that PKCδ is involved in a mitochondrion-dependent apoptotic pathway. Other studies have demonstrated that PKCδ interacts with c-Abl tyrosine kinase upon exposure to genotoxic stress (50). c-Abl is a proapoptotic tyrosine kinase that targets to the nucleus following genotoxic stress and oxidative stress (42, 46, 49). Importantly, activated PKCδ, by c-Abl-mediated phosphorylation, increases c-Abl activity simultaneously through interaction with c-Abl (28). In addition, PKCδ phosphorylates SHP-1 tyrosine phosphatase and decreases its activity (40). Given that c-Abl is negatively regulated by SHP-1 in response to DNA damage (14), PKCδ regulates apoptotic signals of the key apoptotic tyrosine kinase c-Abl not only by its activation but also by inhibiting the activity of SHP-1 tyrosine phosphatase. Furthermore, recent studies have demonstrated that PKCδ interacts with and phosphorylates Rad9, a key factor involved in checkpoint regulation of the DNA damage responses (39, 44). Inhibition of PKCδ attenuates Rad9-mediated apoptosis. These findings collectively support a pivotal role for PKCδ in the induction of apoptosis in response to DNA damage.

The TP53 tumor suppressor gene, frequently mutated in a wide variety of tumors, plays an important role in maintaining genomic integrity (1, 8, 25, 32). Following genotoxic stress, TP53 protein levels are increased, and TP53 functions as a sequence-specific transcription factor that regulates the expression of downstream genes required for cell cycle arrest, DNA repair, or apoptosis (33, 34). Although accumulating studies have revealed the regulation of TP53 expression at the posttranslational level, several reports have raised the possibility that TP53 is also regulated at the transcriptional level in response to genotoxic stress (11, 20, 27). The finding that overexpression of HOXA5 induces wild-type TP53 expression supports the model in which loss of HOXA5-dependent regulation of TP53 gene transcription is an important step in tumorigenesis (22). In addition, overexpression of BCL6 protects cells from DNA damage-induced apoptosis through binding two specific sites within the TP53 promoter region and suppressing TP53 expression (21). Other studies have shown that impaired TP53-mediated autoregulation of TP53 gene transcription results in aberrant cell cycle regulation and suppression of TP53-mediated apoptosis (35). These results collectively support that the transcriptional response to DNA damage is directly related to the functions of TP53; however, mechanisms for the regulation of TP53 gene transcription remain largely unknown.

The present study demonstrates that PKCδ up-regulates

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TP53 gene transcription through the core promoter element (CPE-TP53) in the TP53 promoter. Btf is identified as a candidate transcription factor that transactivates PKCδ-dependent TP53 gene transcription by interacting with CPE-TP53. Furthermore, we provide evidence that disruption of Btf-mediated TP53 gene transcription leads to suppression of TP53-mediated apoptosis in response to DNA damage.

MATERIALS AND METHODS

Cell culture. MOLT-4 (human leukemia) cells and U2OS (human osteosarcoma) cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. MCF-7 (human mammary carcinoma), SaOS-2 (human osteosarcoma), and COS-7 (simian kidney) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Cells were treated with 2 μg/ml adriamycin (ADR; Sigma-Aldrich) or 5 μM rottlerin (Sigma-Aldrich).

Cell transfection. Various deletion mutants of the TP53 promoter were constructed by inserting a PCR-amplified genomic region of the human TP53 promoter (−1053 to +111 [LP1-TP53-Luc], −431 to +111 [SP1-TP53-Luc], −373 to +111 [−373-TP53-Luc], −171 to +111 [−171-TP53-Luc], −157 to +111 [−157-TP53-Luc], −118 to +111 [−118-TP53-Luc], −90 to +111 [−90-TP53-Luc], −60 to +111 [−60-TP53-Luc], or −40 to +111 [−40-TP53-Luc]) into PGL-3 basic vector (Promega), which carries the luciferase gene. Deletion mutants of ΔκB-TP53-Luc, ΔCPE-TP53-Luc, or ΔCPEΔκB-TP53-Luc were constructed by amplifying the SP1-TP53-Luc plasmid that had a deletion of from −45 to −39, from −73 to −54, or from −77 to −39, respectively. Various PKCδ constructs were described elsewhere (43, 44). The green fluorescent protein (GFP)-Btf plasmid has also been described previously (10). Cells were transiently transfected using FuGENE6 (Roche) according to the manufacturer's protocol.

siRNA transfection. Short interfering RNA (siRNA) duplexes targeting for PKCδ or Btf were synthesized and purified by Invitrogen (Stealth RNAi). Transfection of siRNAs was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Immunoblotting and antibodies. Cell lysates were prepared as described elsewhere (38, 45). Briefly, cultured cells were washed twice with chilled phosphate-buffered saline (PBS) and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT], 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1% NP-40). Cell lysates were centrifuged for 10 min at 4°C. The supernatants were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with anti-PKCδ (SC-937; Santa Cruz Biotechnology [SCBT]), anti-TP53 (DO-1; SCBT), anti-Btf (Bethyl), antitubulin (Sigma-Aldrich), anti-PCNA (SCBT), anti-GFP (Nacalai Tesque), or anti-Flag (Sigma-Aldrich) for 1 to 4 h at room temperature. After washing, the membranes were incubated with anti-rabbit or anti-mouse immunoglobulin G (IgG)-peroxidase conjugate (SCBT). The antigen-antibody complexes were visualized by chemiluminescence.

In vitro luciferase assay. Luciferase activities were measured at 48 h post-transfection using the Bright-Glo luciferase assay system (Promega) according to the manufacturer's protocol. The relative increase in activity (*n*-fold) compared with cells transfected with luciferase vector was determined. The data represent means ± standard deviations (SDs) from at least three to four independent transfection experiments, each performed in triplicate.

Semiquantitative RT-PCR analysis. Total RNA was isolated from cells with the use of RNeasy spin column kits (Qiagen) according to the manufacturer's protocol. Total RNA (800 ng) was amplified using a Super Script III One Step reverse transcription-PCR (RT-PCR) system with a platinum tag kit (Invitrogen). The reaction for RT-PCR was as follows: cDNA synthesis at 55°C for 30 min and denaturation at 94°C for 2 min, followed by 20 cycles (TP53, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], and β-actin) or 25 cycles (GADD45) at 94°C for 15 s, 55°C for 30 s, and 68°C for 30 s, with a final extension at 68°C for 5 min. The following oligonucleotide primers were used: TP53, 5′-ACCTACCAGGGCAGCTACGGTTTC-3′ and 5′-GCCGCCCATGCAGGAACTGTTACA-3′; GAPDH, 5′-AAGGCTGTGGGCAAGGTCATCCCT-3′ and 5′-TTACTCCTTGGAGGCCATGTGGGC-3′; β-actin, 5′-CAGGGCGTGATGGTGGGCA-3′ and 5′-CAAACATCATCTGGGTCATCTTCTC-3′; GADD45, 5′-ATGACTTTGGAGGAATTCTCGGCT-3′ and 5′-TCACCGTTCAGGGAGATTAATCAC-3′.

DNAP assay. Nuclear extracts were prepared as described elsewhere (4). The biotinylated DNA probes containing the CPE-TP53 element (1 μg) were mixed with nuclear extracts from COS-7 cells, poly(dI · dC) (15 μg), and DNA-affinity precipitation (DNAP) buffer (20 mM HEPES-KOH, pH 7.9, 80 mM KCl, 1 mM MgCl, 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol, 0.1% Triton X-100, and 0.1 mM PMSF), and the mixture was incubated for 30 min on ice. The same mixture without DNA probes was also tested as a negative control. Streptavidin-Dynabeads (Dynal, Great Neck, NY) were then added with mixing by rotation for 30 min. The Dynabeads were collected with a magnet and washed twice with DNAP buffer. The trapped proteins were separated by SDS-polyacrylamide gel electrophoresis, visualized by silver staining, and identified by mass spectrometry.

Chromatin immunoprecipitation (ChIP) assay and re-ChIP assay. MOLT-4 cells (1×10^7 to 5×10^7) were harvested and washed with chilled PBS once followed by incubation in 1% formaldehyde for 15 min at room temperature for chromatin cross-linking. Then the cells were collected and washed with chilled PBS again. After centrifugation, the cell pellets were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A), and the lysates were sonicated to obtain DNA fragments of 200 to 500 bp in length. After centrifugation, 50 μl of the supernatant was used as an input, and the remainder was diluted 2- to 2.5-fold in washing buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% NP-40, and protease inhibitors as described above). This diluted fraction was subjected to immunoprecipitation with 2 μg of indicated antibodies for 2 h to overnight at 4°C with rotation. The immunocomplexes were collected with 30 μl protein A-Sepharose beads (SCBT) for 1 to 2 h at 4°C with rotation. The beads were then pelleted by centrifugation and washed sequentially with 300 μl of the following buffers: wash buffer I (500 mM NaCl, 0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0), wash buffer II (250 mM LiCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1% deoxycholate), and then Tris-EDTA buffer (twice). Precipitated chromatin complexes were removed from the beads by shaking with 150 μl elution buffer (1% SDS and 0.1 M $NaHCO_3$) for 15 min, and this step was repeated. All the eluate was collected, and then the cross-linking was reversed by adding NaCl to a final concentration of 200 mM for overnight at 65°C. The remaining proteins were digested with the extraction buffer (50 mM Tris-HCl, pH 6.8, 10 mM EDTA, 40 μg/ml proteinase K) for 1 h at 45°C. DNA was recovered by phenol-chloroform-isoamyl alcohol (25/24/1) extraction and precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. In re-ChIP experiments, complexes were eluted by incubation for 30 min at 37°C in 25 μl 10 mM DTT. After centrifugation, the supernatant was diluted 20 times with re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0) and subjected to the ChIP procedure again. PCR amplification was performed in chromatin immunoprecipitated-fragments using the following oligonucleotide pairs: CPE (from −157 to +110), 5′-AACTCCATTTCCTTTGCTTCCTCCGGCAGG-3′ and 5′-CAATCCAGGGAAGCGTGTCACCGTCGT-3′; CR (from −998 to −820), 5′-CGAAAGCACTGTGTTCTTAGCACCGCGGGT-3′ and 5′-CCCTAGGGCTTGATGGGAACGGGAAACCTT-3′.

Apoptosis assay. Cells transfected with Btf siRNA were left untreated or treated with 2 μg/ml ADR. Cells were also transfected with the Flag vector or Flag-Btf. The apoptotic effect was measured after 24 h using the DeadEnd Fluorometric TUNEL system (Promega). Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with 4′,6′-diamidino-2-phenylindole (DAPI) as described elsewhere (2).

RESULTS

PKCδ up-regulates TP53 expression at a transcriptional level. Our previous studies have shown that inhibition of PKCδ activity attenuates basal and DNA damage-induced expression of TP53; the underlying mechanism remains unknown (41). To address this issue, MOLT-4 cells were treated with the DNA-damaging agent ADR (doxorubicin) in the presence or absence of the PKCδ-specific inhibitor rottlerin. TP53 protein was significantly induced after DNA damage while inhibition of PKCδ blocked such induction as expected (Fig. 1A, third panel). Notably, the RT-PCR assay revealed that TP53 mRNA was also induced following genotoxic stress and that inhibition of PKCδ also suppressed the expression of TP53 mRNA (Fig. 1A, top panel). Similar results were obtained with MCF-7 cells

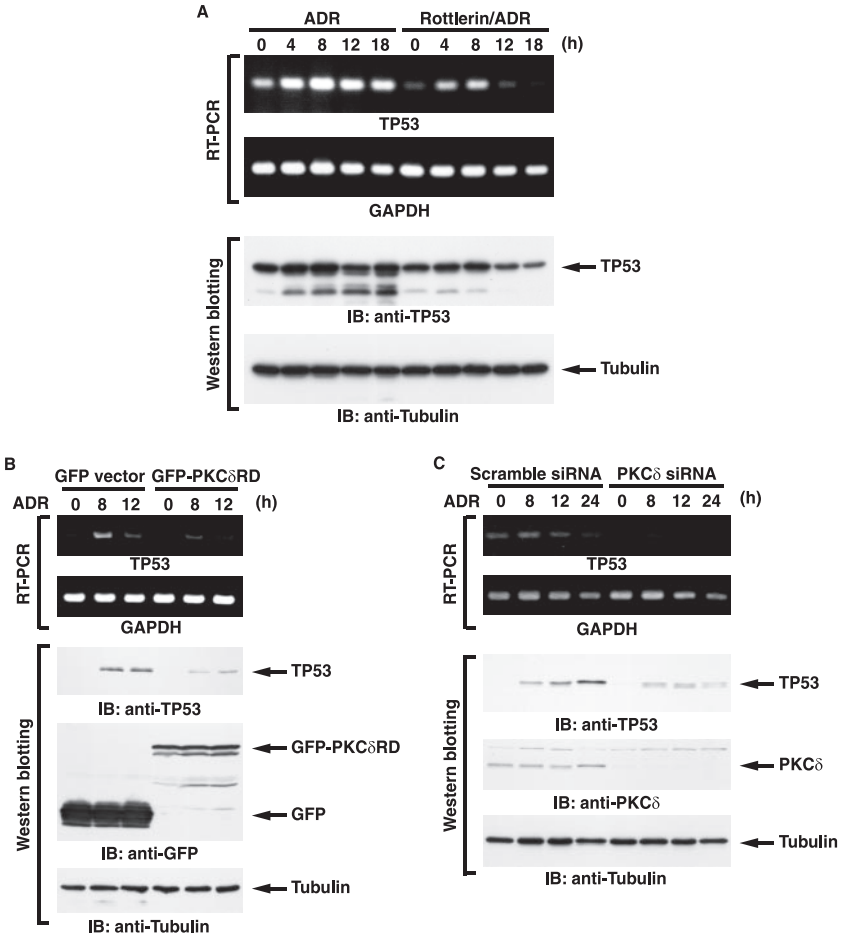

FIG. 1. PKCδ up-regulates TP53 expression at a transcription level. (A) MOLT-4 cells were pretreated with or without rottlerin for 1 h followed by treatment with ADR for the indicated times. TP53 mRNA levels were determined by RT-PCR (top panel), and GAPDH was also amplified as a loading control (second panel). Cell lysates were subjected to immunoblot analysis with anti-TP53 (third panel) or antitubulin (bottom panel). (B) MCF-7 cells transfected with GFP vector or GFP-PKCδRD were treated with ADR for the indicated times and then analyzed as described above. (C) U2OS cells transfected with scramble siRNA or PKCδ siRNA were treated with ADR for the indicated times and then analyzed by RT-PCR and Western blotting.

(data not shown). Comparable results were also obtained in MOLT-4 cells treated with another DNA-damaging agent, etoposide (data not shown). These observations suggest that the reduction of the TP53 protein level might be accompanied by a decline in transcriptional activation resulting from inhibition of PKCδ. Therefore, we hypothesized that PKCδ might regulate TP53 expression at a transcriptional level. To further define the role of PKCδ in the regulation of TP53 gene transcription, MCF-7 cells were transfected with GFP vector or GFP-PKCδ regulatory domain (RD), which functions as a dominant-negative mutant. In contrast to the GFP vector-transfected cells, TP53 mRNA expression was substantially abolished by expression of the dominant-negative PKCδRD (Fig. 1B, first panel). Moreover, the TP53 protein level paralleled the TP53 mRNA level (Fig. 1B, third panel). Comparable results were observed when cells were transfected with the PKCδCF(K-R) mutant, which is catalytically inactive (data not shown). To confirm whether PKCδ is responsible for TP53 mRNA induction in response to DNA damage, we knocked down PKCδ by transfection of U2OS cells with PKCδ siRNAs. Down-regulation of PKCδ was associated with attenuated expression of TP53 mRNA as well as TP53 protein (Fig. 1C). These results collectively indicate that PKCδ up-regulates TP53 expression at a transcriptional level. Activation of TP53 gene transcription by PKCδ is therefore likely to have important functional consequences that result from alteration in protein synthesis.

PKCδ transactivates the human TP53 promoter via CPE-TP53. In order to examine the molecular mechanisms of PKCδ-mediated regulation of TP53 gene transcription, we performed reporter assays to analyze TP53 promoter activity. U2OS cells were transfected with luciferase reporter constructs driven by 1,165-bp (LP1-TP53-Luc) or 543-bp (SP1-TP53-Luc) genomic DNA regions containing the human TP53 promoter (Fig. 2A) Transfected cells were then pretreated with or without rottlerin followed by the treatment with ADR. The luciferase activity of cells transfected with LP1-TP53-Luc or SP1-TP53-Luc increased in response to DNA damage, while inhibition of PKCδ significantly decreased both the basal and DNA damage-induced promoter activity (Fig. 2B). Similar re-

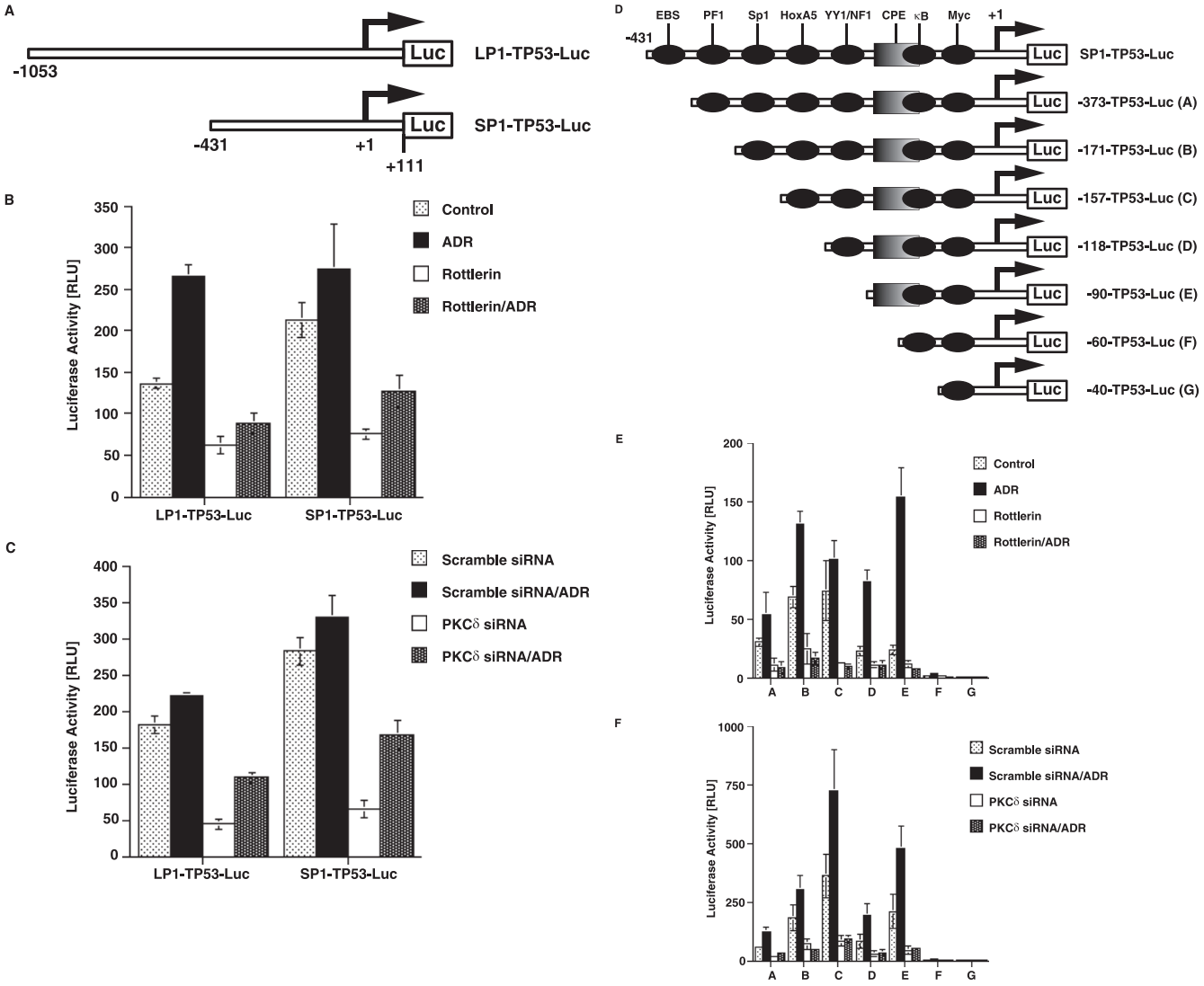

FIG. 2. PKCδ-responsive element is located from −90 to +111 on the TP53 promoter. (A) Schematic representation of two TP53 promoter-driven luciferase reporter constructs. The arrow represents the transcription start site at +1 (30). (B) U2OS cells were transiently transfected with LP1-TP53-Luc or SP1-TP53-Luc and then left untreated or treated with ADR for 24 h in the presence and absence of rottlerin. The luciferase activity was measured 48 h posttransfection. The relative increase in activity (*n*-fold) compared with cells transfected with PGL-3 vector was determined. (C) COS-7 cells transiently cotransfected with LP1-TP53-Luc or SP1-TP53-Luc together with scramble siRNA or PKCδ siRNA were left untreated or treated with ADR for the indicated times. The luciferase activity was measured as described above. (D) Schematic representation of seven 5′-deletion mutants of the TP53 promoter reporter constructs. Various regulatory sites are shown (23). (E) U2OS cells transfected with the deletion mutants were treated with ADR for 24 h in the presence or absence of rottlerin. The luciferase activity was measured as described above. (F) COS-7 cells cotransfected with the deletion mutants together with scramble siRNA or PKCδ siRNA were left untreated or treated with ADR for the indicated times. The luciferase activity was measured as described above.

sults were obtained in U2OS cells treated with etoposide (data not shown). To extend these findings by knocking down PKCδ, COS-7 cells were transfected with PKCδ siRNA and then treated with ADR. Reduction of PKCδ expression was associated with the attenuation of both LP1-TP53-Luc and SP1-TP53-Luc activities (Fig. 2C). Similar results were obtained with U2OS cells (data not shown). These results imply that PKCδ is involved in DNA damage-mediated TP53 promoter activation and that the −431 to +111 region of the TP53 promoter contains an element(s) required for PKCδ-dependent regulation. In addition, basal promoter as well as DNA damage-mediated luciferase activities were lower with LP1-TP53-Luc than with SP1-TP53-Luc, suggesting that the −1,053-to-−431 region of the TP53 promoter contains a *cis* element(s) that represses the promoter. To map the PKCδ-responsive element, we generated progressive deletion mutants from the 543-bp region in the TP53 promoter region (Fig. 2D). U2OS cells were transfected with these mutants and then treated with ADR in the presence and absence of rottlerin. Deletion of this 543-bp region, leaving only 202 (−90 to +111) bp intact, did not compromise the ability of rottlerin to repress the TP53 promoter (Fig. 2E). These findings indicate that the PKCδ-responsive element is located in the small fragment, spanning from −90 to +111. Similar results were obtained

when COS-7 cells were transfected with PKCδ siRNA to suppress PKCδ expression (Fig. 2F).

Previous studies have demonstrated that the TP53 promoter region extending from −70 to −40 and overlapping the κB motif, which is designated a TP53 core promoter element (CPE-TP53), is required for TP53 basal and DNA damage-induced promoter activity (27). Consequently, abrogation of −431 to −60 resulted in little if any luciferase activity in control as well as complete unresponsiveness to genotoxic stress, thus making it difficult to identify the PKCδ-responsive element (Fig. 2E and F). To resolve this issue, U2OS cells were transfected with three different mutants, which carried deletions of the sequence for κB (ΔκB-TP53-Luc), CPE-p53 (ΔCPE-TP53-Luc), or both (ΔCPEΔκB-TP53-Luc) (Fig. 3A). Transfection with ΔκB-TP53-Luc retained positive responsiveness to the DNA-damaging agent, while inhibition of PKCδ decreased the basal and DNA damage-induced activity (Fig. 3B). By contrast, deletion of CPE-TP53 completely abolished DNA damage-induced activation of the promoter, and inhibition of PKCδ had little if any repressive effect on the promoter activity (Fig. 3B). These results demonstrate that PKCδ requires CPE-TP53 for activation of the TP53 promoter. To confirm these findings, PKCδ was knocked down in COS-7 cells by transfection with PKCδ siRNA. In contrast to ΔκB-TP53-Luc, silencing of PKCδ had no significant effect on the activity with ΔCPE-TP53-Luc or ΔCPEΔκB-TP53-Luc, regardless of ADR treatment (Fig. 3C). Taken together, these results demonstrate that PKCδ up-regulates basal and DNA damage-induced activity of the TP53 promoter through CPE-TP53.

Identification of Btf as a candidate transcription factor that participates in regulation of TP53 gene transcription via CPE-TP53. Given that CPE-TP53 is responsible for PKCδ-mediated induction of TP53 expression, we hypothesized that PKCδ may regulate TP53 gene transcription by activating an unknown transcription factor through this element. However, to our knowledge, the transcription factor(s) responsible for positive regulation via CPE-TP53 remains unclear. To identify the unknown transcription factor(s), we utilized a DNAP assay. Biotinylated DNA probes containing the CPE-TP53 sequence were incubated with nuclear extracts from COS-7 cells in the presence of poly(dI · dC) in the DNAP buffer. The same mixture without DNA probes was used as a negative control. The DNA-protein complexes were isolated with streptavidin-conjugated magnet beads and visualized by silver staining (Fig. 4). Analysis of the complexes by mass spectrometry revealed that Btf (*B*cl-2-associated *t*ranscription *f*actor) was one of these binding proteins (Fig. 4). Previous studies showed that Btf contains putative basic zipper-like (bZIP) and Myb-like DNA-binding domains and thus can bind to DNA in vitro (13, 26). Moreover, overexpression of Btf induces apoptosis, although the mechanisms involved remain unknown.

PKCδ interacts with the TP53 promoter via Btf in vitro and in cells. To determine if PKCδ and Btf can bind to CPE-TP53 in vitro, we performed ChIP assays on the TP53 promoter. Chromatin was isolated from MOLT-4 cells treated with or without ADR and immunoprecipitated with an anti-Btf antibody. Immunoprecipitated DNA was analyzed by PCR with primers amplifying the TP53 promoter region encompassing CPE-TP53 or another control region. DNA fragments containing CPE-TP53, but not the control region, were specifically

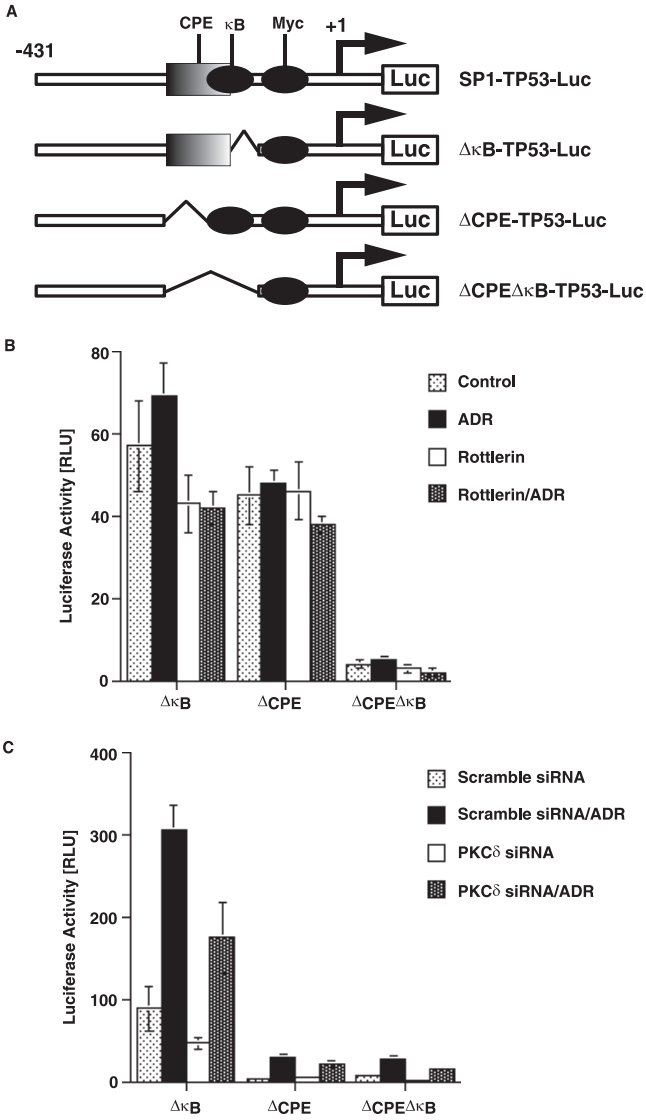

FIG. 3. PKCδ regulates the TP53 promoter activity by means of CPE-TP53. (A) Schematic depiction of the deletion mutants of the TP53 promoter reporter constructs, which had separate deletions of κB (GGGGTTTTCC), CPE (CAGGGGTTGATGGGATTGGGGTTTT), or both. (B) U2OS cells transfected with ΔκB-TP53-Luc, ΔCPE-TP53-Luc, or ΔCPEΔκB-TP53-Luc were left untreated or treated with ADR for 24 h in the presence or absence of rottlerin. The luciferase activity was measured 48 h posttransfection. (C) COS-7 cells were cotransfected with the deletion mutants plus scramble siRNA or PKCδ siRNA and then treated with ADR for 24 h. The luciferase activity was measured after 48 h posttransfection.

immunoprecipitated with anti-Btf, suggesting that Btf actually binds to CPE-TP53 on the TP53 promoter in vivo (Fig. 5A). Isolated chromatin was also immunoprecipitated with an anti-PKCδ antibody, indicating PKCδ occupancy on CPE-TP53 (Fig. 5A). Moreover, occupancy by PKCδ and Btf of CPE-TP53 was substantially increased following DNA damage. These results thus support a model in which PKCδ is associated with Btf to regulate the transcription of the TP53 gene through CPE-TP53. To examine interactions between PKCδ and Btf, we performed re-ChIP assays using untreated or

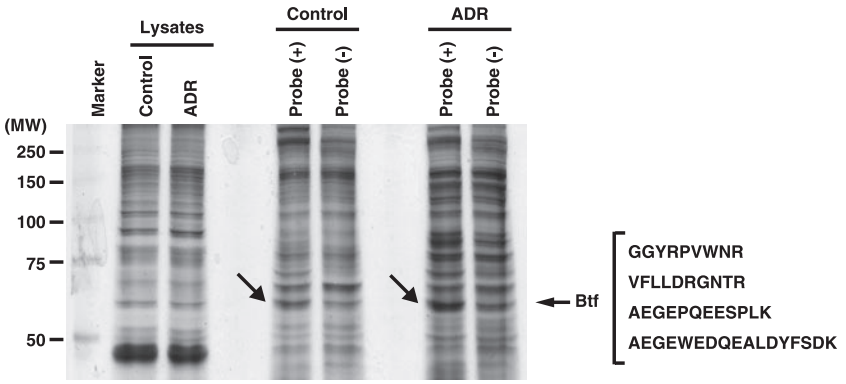

FIG. 4. Identification of CPE-TP53 binding protein by DNAP assay. Nuclear extracts from COS-7 cells treated with or without ADR for 8 h were incubated with biotinylated CPE-TP53 binding sites, DNAP buffer, and poly(dI · dC). DNA-protein complexes were precipitated with streptavidin-Dynabeads. The same mixture without biotinylated DNA probes was used as a negative control. The p70 protein, indicated with the arrow, was identified as one of the CPE-TP53 binding proteins. The peptide sequences identified by mass spectrometry are shown on the right. Numbers at left are molecular weights (MW) in thousands.

ADR-treated MOLT-4 cells. Chromatin was immunoprecipitated with anti-PKCδ as the first antibody, and the eluted samples were then immunoprecipitated with anti-Btf or anti-IgG antibody. Chromatin templates containing CPE-TP53, which were associated with PKCδ, were also immunoprecipitated by anti-Btf, but not by anti-IgG. These findings indicate that PKCδ may form a complex with Btf to occupy CPE-TP53. To confirm these results, we performed reciprocal re-ChIP assays in which anti-Btf was used for the first immunoprecipitation and anti-PKCδ was then used for the second immunoprecipitation. As expected, the results yielded similar conclusions (Fig. 5C). To further determine if occupancy of the TP53 promoter was associated with PKCδ activity, MOLT-4 cells were pretreated with or without rottlerin and then left untreated or treated with ADR. ChIP assays were performed by using anti-PKCδ or anti-Btf. As shown before, isolated chromatin encompassing CPE-TP53 was immunoprecipitated with PKCδ and Btf (Fig. 5D). Notably, inhibition of PKCδ activity by rottlerin decreased the affinity of Btf as well as that of PKCδ for CPE-TP53, indicating that Btf occupancy of CPE-TP53 depends on, at least in part, the kinase activity of PKCδ (Fig. 5D). Taken together, these results demonstrate that PKCδ forms complexes with Btf to occupy CPE-TP53 and that regulation of PKCδ kinase activity is prerequisite to the affinity of these complexes for CPE-TP53.

Btf activates TP53 gene transcription through the CPE-TP53 element in a PKCδ-dependent manner. To establish endogenous Btf occupancy of the TP53 promoter at CPE-TP53, we examined Btf-mediated regulation of TP53 promoter activity. U2OS cells were cotransfected with the deletion mutants of the TP53 promoter reporter constructs plus GFP vector or GFP-Btf, followed by treatment with ADR. Analysis with SP1-TP53-Luc demonstrated that ectopic expression of Btf enhanced TP53 promoter activity under basal conditions, as well as after exposure to genotoxic agents (Fig. 6A). Deletion mutants of −90-TP53-Luc and ΔκB-TP53-Luc retained the ability to enhance TP53 promoter activity in response to Btf. In contrast, deletion mutants of the CPE-TP53 (−60-TP53-Luc, ΔCPE-TP53-Luc, and ΔCPEΔκB-TP53-Luc) completely abolished Btf-mediated promoter activity, indicating that Btf activates the TP53 promoter by occupying the CPE-TP53 element (Fig. 6A). To characterize the role for endogenous Btf in regulating TP53 gene transcription, U2OS cells were cotransfected with the deletion mutants of the TP53 promoter reporter constructs plus scramble siRNA or Btf siRNA. In concert with the ectopic expression of Btf, Btf silencing substantially attenuated the activity of the TP53 promoter containing CPE-TP53 (Fig. 6B). These results demonstrate that Btf positively regulates TP53 expression through CPE-TP53. To further confirm these findings, RT-PCR was performed with MCF-7 cells that were transfected with scramble siRNA or Btf siRNA, followed by treatment with ADR. The results demonstrated that mRNA expression of TP53 was induced after DNA damage, whereas knocking down Btf significantly attenuated the TP53 mRNA level in control and after exposure to cellular stress (Fig. 6C). Notably, silencing of Btf suppressed the mRNA expression of not only TP53 but also the GADD45α gene, a transcriptional target gene of TP53, indicating that the functional consequences of TP53 gene transcriptional regulation result from altering the affinity of TP53 for the promoter of downstream genes. Taken together, these results demonstrate that Btf contributes to increase TP53 expression at a transcriptional level by binding to CPE-TP53. To further define whether the induction of TP53 by Btf requires PKCδ activity, U2OS cells were transfected with GFP vector or GFP-Btf and then treated with ADR in the presence or absence of rottlerin. In contrast to GFP vector-transfected cells, ectopically expressed Btf significantly increased TP53 mRNA levels (Fig. 6D). Notably, rottlerin-induced inhibition of PKCδ profoundly abrogated the enhanced responsiveness to Btf, thus suppressing TP53 expression (Fig. 6D). These findings and the results of ChIP assays indicate that PKCδ kinase activity is required for Btf-mediated TP53 gene transcription.

Btf silencing diminishes TP53-dependent apoptosis. The impact of Btf levels on the transcriptional induction of TP53 prompted us to investigate the cellular function that promotes TP53-mediated apoptosis in response to DNA damage. In this context, previous studies have shown that overexpression of Btf in HeLa cells induces apoptosis; the responsible mechanism is largely unknown (13). To determine if Btf is involved in TP53-

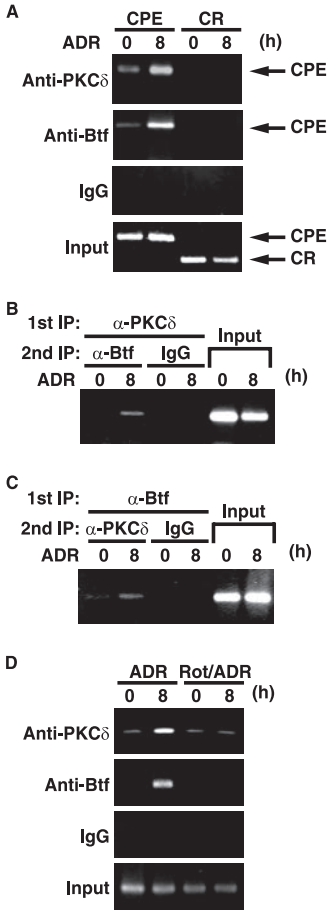

FIG. 5. PKCδ interacts with the TP53 promoter via Btf in cells. (A) MOLT-4 cells were left untreated or treated with ADR for 8 h. ChIP assays were performed using primer sequences containing CPE-TP53 (CPE) or other control regions of the TP53 promoter (CR) as a negative control. PCR was performed with immunoprecipitated chromatin fragments using anti-PKCδ, anti-Btf, or anti-IgG. The inputs represent PCR amplification of total chromatin before immunoprecipitation. (B) MOLT-4 cells were treated as described above. Re-ChIP assays were performed with the use of anti-PKCδ, and the eluted samples were then immunoprecipitated with anti-Btf or anti-IgG. Precipitated chromatin was analyzed by PCR using primer sequences containing the CPE-TP53 region. (C) MOLT-4 cells were treated as described above. Re-ChIP assays were performed by using anti-Btf for the first immunoprecipitation and anti-PKCδ for the second immunoprecipitation. (D) MOLT-4 cells were pretreated with or without rottlerin followed by treatment with ADR for 8 h. ChIP assays were performed by using anti-PKCδ or anti-Btf. Immunoprecipitated chromatin was analyzed by PCR using primer sequences containing CPE-TP53.

dependent apoptosis, U2OS cells and SaOS-2 cells were transfected with scramble siRNA and Btf siRNA, followed by treatment with ADR. Previous studies have shown that ADR-induced apoptosis in U2OS cells was largely dependent on TP53 function since knocking down TP53 was associated with substantial attenuation of apoptosis induction (29). In concert with these findings, treatment of U2OS cells with ADR resulted in the induction of TP53 and apoptosis (Fig. 1C and 7A). By contrast, induction of apoptosis was significantly reduced in SaOS-2 cells (Fig. 7A), mainly owing to the absence of TP53 (29). Importantly, the finding that knocking down Btf prominently attenuated DNA damage-induced apoptosis in U2OS cells but not in SaOS-2 cells indicates that the contribution of endogenous TP53 to apoptosis is triggered by Btf expression. Similar results were obtained with another apoptotic assay by using DAPI staining (Fig. 7B). To further determine if PKCδ is required for Btf-mediated apoptosis, U2OS cells were transfected with the Flag vector or Flag-Btf. Cells were then left untreated or pretreated with rottlerin followed by treatment with ADR. Ectopic expression of Btf enhanced the induction of apoptosis following ADR treatment (Fig. 7C). In contrast, inhibition of PKCδ activity by rottlerin attenuated Btf-mediated apoptosis (Fig. 7C). These findings provide evidence that activation of TP53 gene transcription by PKCδ-Btf signaling has important functional consequences with respect to DNA damage-induced apoptosis.

DISCUSSION

Since TP53 protein is a critical regulator of cell growth, its expression must be tightly regulated to ensure normal cell division as well as its ability to function as a tumor suppressor. However, little is known about the mechanisms and consequences of the transcriptional regulation of TP53. In the present study, we showed that expression of TP53 at both mRNA and protein levels increased after treatment of cells with the genotoxic agent ADR. Down-regulation of TP53 at mRNA levels was associated with reduction of TP53 expression at protein levels (Fig. 1). In this context, transcriptional control mechanisms are critically important in modulating cellular levels of the TP53 tumor suppressor protein after DNA damage. In addition, knock-down of Btf, a transcriptional regulator of TP53, substantially abolished TP53-dependent apoptosis, indicating that the function of TP53 as a tumor suppressor is regulated, at least in part, at a transcriptional level (Fig. 7A). Previous studies have shown that CPE-TP53, covering the region from −70 to −46 in the TP53 promoter, is essential for TP53 promoter activity induced by genotoxic stress as well as maintained at steady-state level (27). In this regard, a novel 21-bp motif named PE21, which overlaps with the CPE-TP53 region, was identified as the sequence responsible for UV-induced transcription and oncostatin M-mediated transcriptional repression (15, 20). These findings collectively suggest a critical role for CPE-TP53 in the regulation of TP53 gene expression and stress response. In concert with these results, our experiments with mutational analysis also showed that the small fragment containing CPE-TP53 (−90-TP53-Luc) revealed dramatically increased responsiveness to ADR, whereas deletion of 10 bp from CPE-TP53 (ΔCPE-TP53-Luc) was sufficient to completely abrogate the TP53 promoter responsiveness to DNA damage as well as the basal promoter activity (Fig. 2E and F and 3B and C). Thus, consistent with the results of previous studies, our findings suggest that a positive regulator participates in the transcriptional regulation of TP53 via CPE-TP53. Whereas recent studies have shown that transcription factor KLF4 acts directly on PE21 and causes resistance to DNA damage-induced apoptosis and that MUC1 associates with KLF4 to down-regulate TP53 expression (24, 36), positive regulation via CPE-TP53 has not been demonstrated previously. In this context, the present study demonstrates that PKCδ binds to and activates a transcription factor, Btf. PKCδ

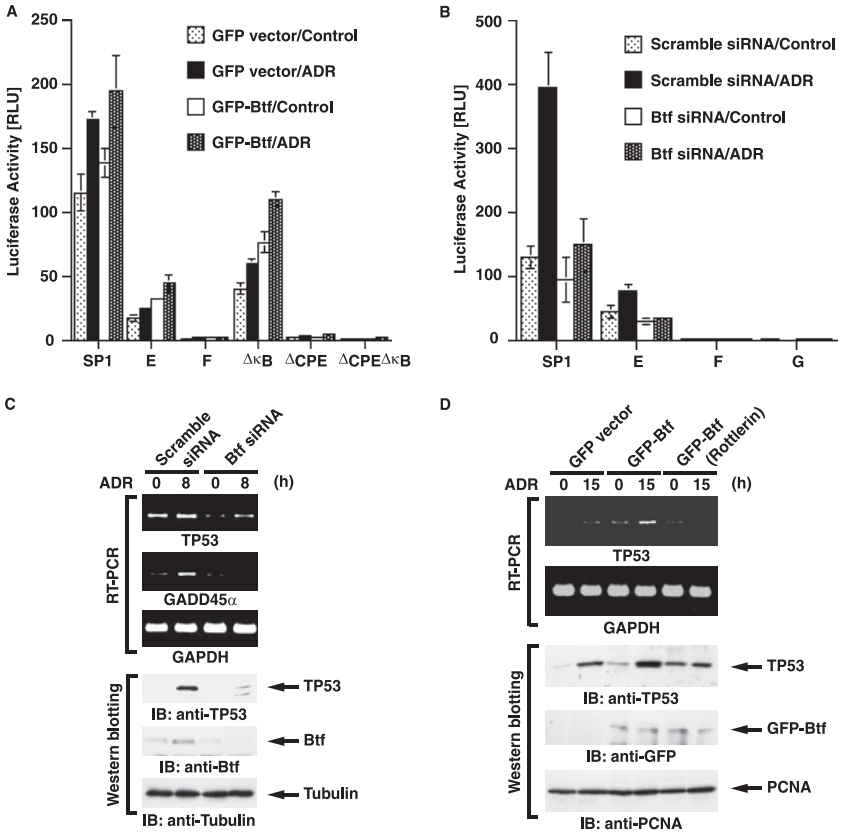

FIG. 6. Btf activates the TP53 promoter activity and induce TP53 expression in a PKCδ-dependent manner. (A) U2OS cells cotransfected with the deletion mutants of the TP53 promoter reporter constructs together with GFP vector or GFP-Btf were left untreated or treated with ADR for 24 h. The luciferase activity was measured at 48 h posttransfection. (B) U2OS cells cotransfected with the deletion mutants of the TP53 promoter reporter constructs plus scramble siRNA or Btf siRNA were left untreated or treated with ADR for the indicated times. The luciferase activity was measured at 48 h posttransfection. (C) MCF-7 cells transfected with scramble siRNA or Btf siRNA were left untreated or treated with ADR for the indicated times. Total RNA was analyzed by RT-PCR using specific primers for TP53 (top panel), GADD45α (second panel), or GAPDH (third panel). Cell lysates were analyzed by immunoblotting with anti-TP53 (fourth panel), anti-Btf (fifth panel), or antitubulin (bottom panel). (D) U2OS cells transfected with GFP vector or GFP-Btf were treated with ADR for the indicated times in the presence or absence of rottlerin. Total RNA was analyzed by RT-PCR using specific primers for TP53 (top panel) or GAPDH (second panel). Cell lysates were subjected to immunoblot analysis with anti-TP53 (third panel), anti-GFP (fourth panel), or anti-PCNA (bottom panel).

occupies CPE-TP53 with Btf and increases Btf occupancy of CPE-TP53, thereby activating TP53 gene transcription in response to DNA damage. To our knowledge, this is the first study to identify Btf as a positive regulator of TP53 gene transcription and to show that Btf transactivates PKCδ-dependent TP53 gene transcription.

PKCδ seems to be involved in the basal level of TP53 and not DNA damage-induced TP53 expression in MOLT-4 and COS-7 cells (Fig. 1A, 2C, and 3C). On the other hand, down-regulation of PKCδ significantly attenuated TP53 expression and TP53 promoter activity following DNA damage in U2OS and MCF-7 cells (Fig. 1B and C, 2B, and 3B). In this context, the promoter analysis revealed that there is HOXA5-dependent regulation of TP53 gene transcription after DNA damage in COS-7 cells (Fig. 2F, compared to −157-TP53-Luc [Fig. 2C] and −118-TP53-Luc [Fig. 2D]; also see Fig. 2D) but not U2OS cells (Fig. 2E compared to 2C and 2D). Interestingly, previous studies have demonstrated that HOXA5 is detectable in only one-third of the primary tumors (22). This suggests the possibility that loss of HOXA5 is associated with substantial PKCδ-dependent regulation of TP53 expression at a steady-state level and in response to DNA damage, in cancer cells (for example, U2OS cells) but not normal cells (for example, COS-7 cells). Furthermore, other studies have demonstrated that Homeobox genes are a family of regulatory genes that act as transcription factors and that the aberrant expression of HOX genes is associated with the leukemic phenotype (3). In this regard, it is plausible that aberrant expression of HOX proteins in MOLT-4 cells is, at least in part, required for DNA damage-induced expression of TP53 (Fig. 1A). Obviously, given that a lot of transcription factors participate in regulation of TP53 gene transcription, further analyses are needed to clarify these mechanisms.

Previous studies have established that PKCδ is involved in transcriptional regulation. For instance, PKCδ-dependent phosphorylation of STAT1 at Ser^{727} is required for the transcriptional regulation of interferon-sensitive genes, whereas PKCδ-dependent phosphorylation of STAT3 at Ser^{727} reduces its DNA-binding and transcriptional activity. p300 is a transcriptional coactivator/histone acetyltransferase that is also phosphorylated at Ser^{89} by PKCδ (12, 31, 48). These findings demonstrate that PKCδ regulates transcription factors in a

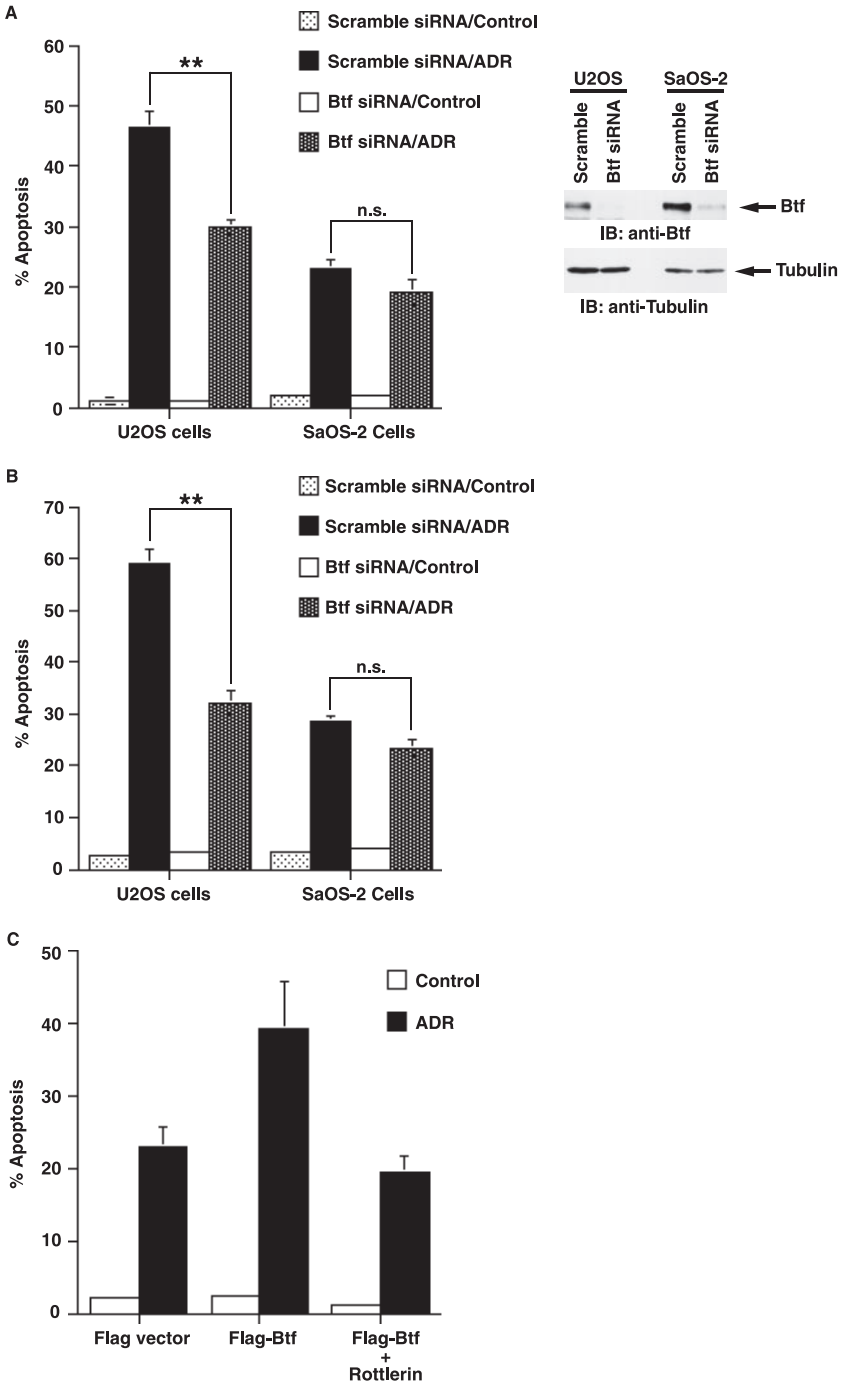

FIG. 7. Down-regulation of endogenous Btf diminishes TP53-dependent apoptosis. (A and B) U2OS cells and SaOS-2 cells were transfected with scramble siRNA or Btf siRNA and then left untreated or treated with ADR for 24 h. The percentage of apoptotic cells was determined by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling assays (A) or DAPI staining (B). The results are represented as means ± SDs obtained from five fields of 100 to 300 cells each used in at least three independent experiments. Asterisks and n.s. indicate $P < 0.01$ and "not significant," respectively. Cell lysates were subjected to immunoblot analysis with anti-Btf (upper panel) or antitubulin (lower panel). (C) U2OS cells were transfected with the Flag vector or Flag-Btf and then left untreated or treated with ADR for 15 h. Rottlerin was pretreated in specifically indicated cells. The results are represented as means ± SDs obtained from five fields of 100 to 300 cells each used in at least three independent experiments.

phosphorylation-dependent manner. The present study revealed that Btf binds directly to the TP53 promoter at the CPE-TP53 region and activates TP53 gene transcription. Notably, Btf interacts with PKCδ, and inhibition of PKCδ kinase activity attenuates the affinity of Btf for CPE-TP53, regardless of Btf expression (Fig. 5 and 6). These findings indicate that PKCδ-dependent activation of Btf is required for transcriptional regulation of the TP53 gene and thus suggest the pos-

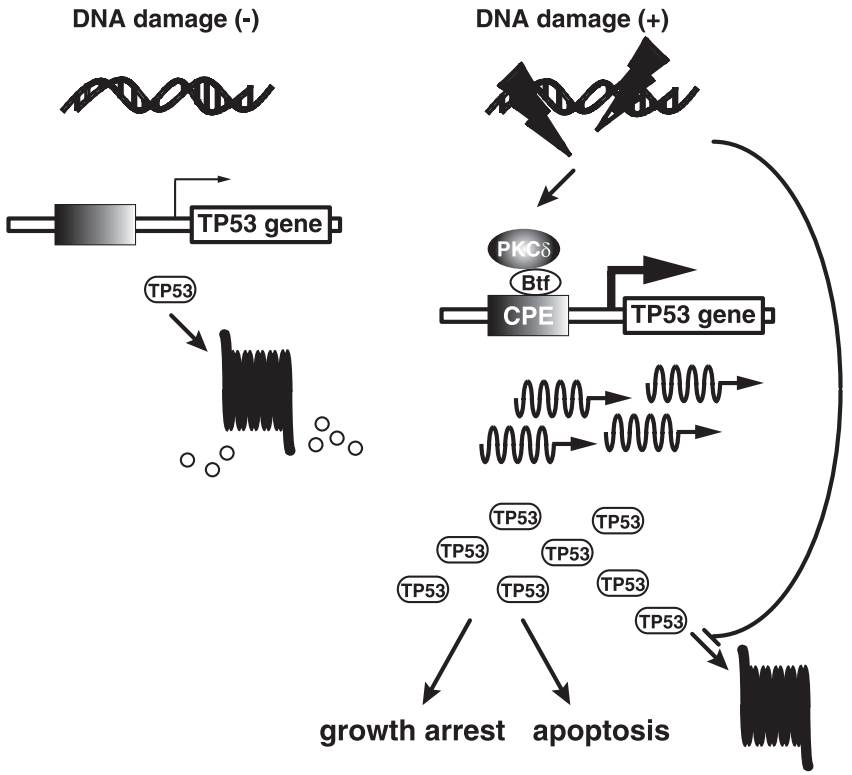

FIG. 8. Schematic depiction of the regulation of TP53 gene transcription by PKCδ and Btf. In control cells, expressed TP53 is immediately degraded by the MDM2-mediated ubiquitination and proteasome system (left). Upon exposure to genotoxic stress, activated PKCδ induces Btf for co-occupancy with CPE-TP53, thereby up-regulating the expression of TP53 at mRNA levels (right). TP53 is also stabilized at protein levels by abrogating ubiquitin-dependent degradation.

sibility that Btf might be a novel phosphorylation substrate for PKCδ. More importantly, these results provide another potential model for transcriptional regulation pathways mediated by PKCδ.

Btf is a newly identified transcription factor with respect to its role as a CPE-TP53 binding protein. Previous studies showed that Btf is a death-promoting transcription factor that contains putative basic zipper-like and Myb-like DNA-binding domains, allowing Btf to bind to DNA in vitro (13). However, the downstream targets and mechanisms of Btf-induced apoptosis remain poorly understood. Our results show that Btf directly interacted with the TP53 promoter in vivo (Fig. 5A) and that this interaction was associated with the activation of TP53 gene transcription (Fig. 6). Moreover, silencing of Btf expression reduced TP53-dependent apoptosis (Fig. 7A). These findings provide evidence that TP53 is a downstream target of Btf and suggest that Btf induces apoptosis in a TP53-dependent manner. Interestingly, in contrast to the results of previous studies, our findings demonstrate that Btf is involved in apoptosis not only by repressing the transcription of survival genes but also by directly activating the expression of genes promoting apoptosis. We also found that activation of Btf by PKCδ is critical for TP53 gene transcription; however, the precise mechanisms involved remain to be elucidated. Since PKCδ is a serine/threonine kinase, it is conceivable that PKCδ activates Btf by phosphorylation. In this regard, Btf contains plenty of serine/threonine residues that can be modified by phosphorylation; however, whether PKCδ activates Btf by phosphorylation is beyond the scope of the present study. Further analyses are needed to more clearly define regulatory mechanisms involving Btf and PKCδ.

PKCδ is required for apoptosis induced by a wide variety of stimuli in many cell types, but many mechanistic details remain to be elucidated. Recent studies have shown that PKCδ is proteolytically cleaved to a 40-kDa catalytically active fragment by caspase-3 in response to apoptotic stimuli (5, 6). The importance of the PKCδ catalytic fragment in apoptotic activity was demonstrated by the finding that overexpression of the PKCδ catalytic domain is targeted into mitochondria and nuclei to induce nuclear fragmentation and cell apoptosis (9, 44). Other studies have indicated that the aberrant activation of topoisomerase IIα by PKCδ is associated with the induction of apoptosis upon exposure to genotoxic agents (47). Thus, DNA damage-induced nuclear targeting of PKCδ contributes to the induction of an intrinsic apoptotic pathway. In the present study, we further demonstrated that TP53 functions as a novel nuclear effector of PKCδ-mediated apoptosis. We also showed that PKCδ activates transcription factor Btf to bind to the TP53 promoter (Fig. 5). Inhibition of PKCδ activity decreased the affinity of Btf for the TP53 promoter, resulting in reduction of TP53 protein expression (Fig. 6). Moreover, suppression of transcriptional activation by Btf silencing diminished TP53-dependent apoptosis (Fig. 7A and B), implying that regulation of Btf by PKCδ also has a critical role in determining the fate of cells with DNA damage. Taken together, these findings support a model in which the activation of TP53 gene tran-

scription by PKCδ results in TP53-dependent apoptosis in response to DNA damage (Fig. 8).

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