Inactivation of DNA-Dependent Protein Kinase by Protein Kinase C δ : Implications for Apoptosis

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Protein kinase C δ (PKC δ) is proteolytically cleaved and activated at the onset of apoptosis induced by DNA-damaging agents, tumor necrosis factor, and anti-Fas antibody. A role for PKC δ in apoptosis is supported by the finding that overexpression of the catalytic fragment of PKC δ (PKC δ CF) in cells is associated with the appearance of certain characteristics of apoptosis. However, the functional relationship between PKC δ cleavage and induction of apoptosis is unknown. The present studies demonstrate that PKC δ associates constitutively with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The results show that PKC δ CF phosphorylates DNA-PKcs in vitro. Interaction of DNA-PKcs with PKC δ CF inhibits the function of DNA-PKcs to form complexes with DNA and to phosphorylate its downstream target, p53. The results also demonstrate that cells deficient in DNA-PK are resistant to apoptosis induced by overexpressing PKC δ CF. These findings support the hypothesis that functional interactions between PKC δ and DNA-PK contribute to DNA damage-induced apoptosis.

The cellular response to ionizing radiation (IR) and other DNA-damaging agents includes cell cycle arrest and activation of DNA repair. In the event of irreparable DNA damage, cells respond with induction of apoptosis. Apoptosis is an ultra-structurally and biochemically distinct form of cell death that occurs in response to a variety of stimuli and is carried out by a genetically determined cell suicide program (21, 23). Cells undergoing apoptosis exhibit morphological and biochemical characteristics that include blebbing of the cell membrane, a decrease in cell volume, nuclear condensation, and internucleosomal cleavage of DNA (26, 57). However, the intracellular signals that control the induction of apoptosis are unclear.

The induction of apoptosis by a variety of stress inducers, including DNA damage, is associated with activation of aspartate-specific cysteine proteases (caspases) (1, 12, 41, 42). Direct involvement of caspases in the induction of apoptosis is supported by studies with the cowpox virus protein CrmA (48), the baculovirus protein p35 (6), and peptide inhibitors (3, 46, 47). CrmA inhibits the induction of apoptosis in cells treated with Fas ligand or tumor necrosis factor (15, 39, 53). By contrast, IR-induced apoptosis involves activation of a CrmA-insensitive pathway (10). These findings have suggested that DNA damage-induced apoptosis is conferred by signals that are distinct from those activated by Fas and tumor necrosis factor (10). The demonstration that IR induces the activation of caspase 3 and that this event, like IR-induced apoptosis, is mediated by a CrmA-insensitive, p35-sensitive pathway (10) has provided support for caspase 3 as a key effector. IR-induced activation of caspase 3 is associated with proteolytic cleavage of poly(ADP-ribose) polymerase (25, 32, 43) and other proteins. Activation of caspase 3 in irradiated cells is

regulated by members of the Bcl-2/Bcl-x_L family (10, 14). Bcl-2 and Bcl-x_L block the release of cytochrome *c* from mitochondria of cells treated with IR and other agents (28, 31, 58). In this context, cytochrome *c* release activates caspase 9, and this event is upstream to activation of caspase 3 (36).

The protein kinase C (PKC) family of serine/threonine kinases consists of multiple isoforms that possess a conserved catalytic domain (29). Studies have demonstrated that the calcium-independent δ isoform is cleaved in cells induced to undergo apoptosis in response to DNA-damaging agents (13, 14). PKC δ is cleaved by caspase 3 at the third variable region (V3) to a 40-kDa catalytically active fragment (13, 14, 17). The finding that overexpression of the PKC δ catalytic fragment (PKC δ CF) is associated with chromatin condensation, nuclear fragmentation, appearance of sub-G₁ DNA, and lethality has supported a role for PKC δ cleavage in induction of apoptosis (17). The ubiquitously expressed PKC δ is unique among the PKC isoforms as a substrate for tyrosine phosphorylation (36). Transformation by Ras (11) or v-Src (60) results in tyrosine phosphorylation of PKC δ . Other studies have demonstrated that PKC δ is phosphorylated and activated by c-Abl during the response to DNA damage (59). PKC δ has been shown to activate the MEK-extracellular signal-regulated kinase (ERK) pathway by a mechanism dependent on Raf and independent of Ras (37). In concert with a potential tumor suppressor function (40), PKC δ has also been linked to induction of growth arrest (16, 54) and apoptosis (17).

The DNA-dependent protein kinase (DNA-PK) is essential in the repair of DNA double-strand breaks that form in irradiated cells and in V(D)J recombination (20, 22, 55). DNA-PKcs is the 470-kDa catalytic subunit of DNA-PK that contains a protein kinase homology domain at the C terminus. DNA-PKcs activity is induced by binding to the 70- and 80-kDa Ku heterodimer (2, 33). Ku binds to DNA in double-strand break repair reactions and thereby recruits DNA-PKcs to sites of DNA damage (5, 34, 50). Recent studies have demonstrated

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FIG. 1. Association of DNA-PKcs and PKCδ. (A) Lysates from U-937 cells were subjected to immunoprecipitation with anti-DNA-PK (αDNA-PK), PIRS or anti-PKCδ (αPKCδ). Immunoprecipitates were analyzed by immunoblotting with anti-DNA-PKcs. Whole-cell lysate (Lysate) was used as a positive control for the immunoblot analysis. (B) Soluble proteins from U-937 cells were subjected to immunoprecipitation with anti-PKCδ. Lysates before and after immunoprecipitation were analyzed by immunoblotting with anti-DNA-PKcs (top). The results are expressed as the mean $\pm$ standard deviation (SD) of three independent experiments (bottom). (C) U-937 cell lysates were immunoprecipitated with PIRS, anti-PKCδ, or anti-DNA-PKcs. Immunoprecipitates were analyzed by immunoblotting with anti-PKCδ. Lysate was used as a positive control for the immunoblotting. (D) U-937 cells were treated with 20 Gy of IR and harvested at the indicated times. Lysates were immunoprecipitated with anti-DNA-PKcs, and the precipitates were analyzed by immunoblotting with anti-PKCδ.

that DNA-PKcs is a self-contained kinase that is activated by direct interaction with double-stranded DNA and that the role of Ku is to stabilize the binding of DNA-PKcs to DNA ends (9, 18). DNA-PKcs, but not Ku, is cleaved by caspase-3 during

apoptosis (7, 19, 51). The available evidence indicates that DNA-PKcs is cleaved into 240-, 150-, and 120-kDa fragments and that cleavage is associated with loss of DNA-PK activity (51). The 240-kDa fragment is derived from the N terminus,

FIG. 2. Direct interaction of DNA-PKcs with PKCδ. (A) U-937 cell lysate was incubated with GST, GST-PKCδ FL, or GST-PKCδ CF. The protein adsorbates were analyzed by immunoblotting with anti-DNA-PKcs. (B) Purified DNA-PK (1 μ g) was incubated with GST, GST-PKCδ FL, or GST-PKCδ CF. After extensive washing, the bound proteins were eluted by boiling in SDS sample buffer and analyzed by immunoblotting with anti-DNA-PK. (C) Purified DNA-PK (1 μ g) was resolved by SDS-PAGE and transferred to three nitrocellulose filters. The filters were incubated with GST, GST-PKCδ FL, or GST-PKCδ CF for 1 h at room temperature and then analyzed by immunoblotting with anti-PKCδ antibody.

FIG. 3. PKC δ CF phosphorylates DNA-PKs and releases DNA-PKs from Ku-DNA beads. (A) GST-PKC δ FL or GST-PKC δ CF was incubated with purified DNA-PK-Ku complex (top) or myelin basic protein (MBP) (bottom) in the presence of [γ - 32 P]ATP for 15 min at 30°C. In vitro kinase reactions were analyzed by SDS-PAGE and autoradiography. (B) Purified DNA-PK/Ku was incubated with DNA beads, and the beads were washed and suspended in kinase buffer. Kinase reaction mixtures containing beads, 20 μ M wortmannin, ATP, and GST-PKC δ CF or kinase-inactive GST-PKC δ CF K-R were incubated for 15 min at 30°C. The supernatant fraction was obtained by sedimentation of the beads. The beads and supernatant fractions were boiled in SDS sample buffer. Proteins were separated by SDS-PAGE (5% polyacrylamide) and analyzed by immunoblotting with anti-DNA-PKs.

and the 150-kDa fragment, which contains the kinase homology domain, is from the C terminus (51). The 150-kDa fragment can also undergo further cleavage to a 120-kDa protein that retains the kinase domain (51).

The present studies demonstrate that PKC δ interacts with DNA-PKs. The results show that PKC δ CF phosphorylates the cleaved fragment of DNA-PKs and inactivates it in vitro. Phosphorylation of DNA-PKs by PKC δ also inhibits the binding of DNA-PKs to DNA. We further show that cells deficient in DNA-PK exhibit resistance to apoptosis induced by overexpressing the catalytically active form of PKC δ .

MATERIALS AND METHODS

Cell culture. U-937 monoclastic leukemia cells (American Type Culture Collection, Rockville, Md.) were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 2 mM L-glutamine. The cell lines SCSV3, SCH8-1, CHO, and CHO/V-3 were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated FBS. Irradiation was performed at room temperature with a Gammacell-1000 (Atomic Energy of Canada, Ottawa, Ontario, Canada) under aerobic conditions with a 137 Cs source emitting at a fixed-dose rate of 0.76 Gy/min as determined by dosimetry.

Immunoprecipitation and immunoblot analysis. Cell lysates and immunoprecipitations were prepared as described previously (45). Soluble proteins (150 μ g) were incubated with anti-PKC δ (Santa Cruz Biotechnology, Santa Cruz, Calif.) or anti-DNA-PK (Upstate Biotechnology, Inc., Upstate, N.Y.) for 1 h and precipitated with protein A-Sepharose for an additional 1 h. Preimmune rabbit serum (PIRS) was used as a negative control. The resulting immune complexes were washed three times with lysis buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose filters. Total-cell lysate (30 μ g) was used as a positive control. The residual binding sites were blocked by incubating the nitrocellulose paper in 5% dry milk in phosphate-buffered saline (PBS)-0.05% Tween 20 for 1 h at room temperature and then with anti-PKC δ or anti-DNA-PK antibodies for 1 h. The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL detection system; Amersham). Signal intensities were determined by densitometric analysis (UltroScan; LKB, Bromma, Sweden).

Far-Western analysis. Purified DNA-PK protein (1 μ g; provided by S. P. Lees-Miller) was subjected to SDS-PAGE and transferred to a nitrocellulose filter. Three identical filters were made. The filters were then incubated with purified glutathione S-transferase (GST)-full-length PKC δ (PKC δ FL), GST-PKC δ CF, or GST for 1 h at room temperature. The filters were then analyzed by immunoblotting with anti-PKC δ .

Generation of expression constructs. FL, CF, and kinase-inactive (CF K-R) PKC were prepared by cloning the appropriate PCR product of human PKC δ into pGEX-2T (Pharmacia Biotech, Uppsala, Sweden). The resultant plasmids,

pGEX-PKC δ FL, pGEX-PKC δ CF, and pGEX-PKC δ CF K-R contain a tac promoter controlling the expression of a fusion protein consisting of GST linked to the N terminus of human PKC δ FL or CF. A similar strategy was used for green fluorescence protein (GFP) fusion constructs by cloning the PCR product of PKC δ into a eukaryotic expression vector, pEGFP-c1 (Clontech, Palo Alto, Calif.). The resultant plasmids, pEGFP-PKC δ CF and pEGFP-PKC δ CF K-R, contain the cytomegalovirus promoter controlling the expression of a fusion protein consisting of GFP linked to the N terminus of PKC δ CF.

Dissociation of DNA-PKs from DNA by PKC δ . DNA-PK/Ku (1 μ g; Promega) was incubated with double-stranded DNA-cellulose (15 μ g; Sigma) in kinase buffer II (KBII) (20 mM Tris-HCl [pH 7.4], 75 mM KCl, 10 mM MgCl $_2$, 1 mM dithiothreitol [DTT], 0.2 mM EGTA, 0.1 mM EDTA) for 30 min at room temperature. The DNA-cellulose beads were then washed and resuspended in kinase buffer. The kinase reaction mixtures containing beads, 100 μ M ATP, and GST-PKC δ CF or GST-PKC δ CF K-R were incubated for 15 min at 30°C. To ensure that phosphorylation was not due to DNA-PKs, wortmannin was added to inhibit DNA-PKs activity (27). The supernatant fraction was obtained by sedimentation of the beads. After the beads were washed with kinase buffer, they and the supernatant fraction were boiled in SDS sample buffer. The proteins were separated by SDS-PAGE (5% polyacrylamide) and analyzed by immunoblotting with anti-DNA-PK.

In vitro phosphorylation of DNA-PKs by PKC δ . The recombinant GST-PKC δ CF or GST-PKC δ CF K-R linked to glutathione beads was resuspended in kinase buffer II (KBII) (20 mM Tris-HCl [pH 7.4], [γ - 32 P]ATP, 20 mM MgCl $_2$, 4 mM DTT). Purified DNA-PK (0.5 μ g) in the absence of sonicated DNA was incubated in kinase buffer containing [γ - 32 P]ATP with GST-PKC δ CF or GST-PKC δ CF K-R at 30°C for 20 min. The reaction was terminated by the addition of SDS-PAGE sample buffer (100 mM Tris-HCl [pH 7.0], 4% SDS, 720 mM 2-mercaptoethanol, 5 mg of bromophenol blue per ml), and the reaction products were analyzed by SDS-PAGE and autoradiography.

In vitro transcription-translation of DNA-PKs fragments. Specific DNA-PKs polypeptides were formed by using a coupled in vitro transcription-translation method (Promega) with templates generated from the cDNA by PCR as described previously (24).

DNA-PKs polypeptide binding assays. PKC δ CF binding to in vitro-translated DNA-PKs polypeptides was tested by incubating GST-PKC δ CF (5 μ g) in 20 μ l of MB (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μ g of leupeptin per ml, 1 μ g of pepstatin per ml, 1 μ g of aprotinin per ml) with equal amounts of each [35 S]DNA-PKs in vitro-translated product for 1 to 2 h at 4°C. A separate incubation of the in vitro-translated [35 S]DNA-PKs product with GST was used as a negative control. The beads were washed four times in 1 ml of MB at 4°C, and the proteins were eluted by boiling in SDS sample buffer. The samples were analyzed by SDS-PAGE and autoradiography. Signal intensities were determined by densitometric analysis.

Proteolysis of DNA-PKs in vitro. Purified DNA-PKs (1 μ g) was incubated with 2.5 μ g of purified recombinant caspase-3 per ml in CB (50 mM HEPES [pH 7.5], 10% glycerol, 2.5 mM DTT, 0.24 mM EDTA) at room temperature. The

FIG. 4. Association of PKC δ CF with specific DNA-PKcs protein fragments. (A) Positions of the DNA-PKcs protein fragments. Protein fragments from various regions of the DNA-PKcs gene were prepared by PCR and in vitro transcription-translation as described in the text. (B) GST-PKC δ CF bound to glutathione-Sepharose was mixed with in vitro-translated products from DNA-PKcs regions to allow binding. After being washed, samples were separated by SDS-PAGE (10% polyacrylamide) and analyzed by autoradiography. (C) GST-PKC δ CF or GST bound to glutathione-Sepharose was mixed with DNA-PKcs fragment 6, 8, or 9. After being washed, the bound proteins were analyzed by SDS-PAGE and autoradiography.

reaction products were analyzed by SDS-PAGE (7.5% polyacrylamide), transferred to nitrocellulose membranes, and immunoblotted with anti-DNA-PKcs.

Inactivation of the proteolytic fragment of DNA-PKcs by phosphorylation with PKC δ CF. Purified DNA-PKcs was incubated with or without 2.5 μ g of purified recombinant caspase 3 per ml in CB at room temperature for 30 min to 1 h as described above. An aliquot was saved for immunoblotting with anti-DNA-PKcs. The cleaved fragments of DNA-PKcs were mixed with purified GST-PKC δ CF or GST-PKC δ CF K-R linked to GST-beads and further incubated for 15 min at 30°C in KB containing [γ - 32 P]ATP. The GST-PKC δ CF and GST-PKC δ CF K-R were then removed by sedimentation to avoid substrate phosphorylation by PKC δ CF in the next kinase reaction. The supernatants containing the phosphorylated DNA-PKcs fragments were then incubated for an additional 15 min at 30°C with GST-p53 in KBII containing [γ - 32 P]ATP. The kinase reactions were stopped by boiling in 2 $\times$ SDS sample buffer. The eluted proteins were analyzed by SDS-PAGE and autoradiography.

Transient transfections. The cells were transiently transfected with pEGFP, pEGFP-PKC δ CF, or pEGFP-PKC δ CF K-R in the presence of Lipofectamine (Life Technologies, Gaithersburg, Md.). After 12 to 18 h, the cells were harvested and sorted by FACS analysis.

Confocal microscopy. The cells were grown on coverslips and transfected with GFP-PKC δ CF or GFP-PKC δ CF K-R. After 48 h, the cells on the coverslips were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. They were permeabilized with 0.1% Triton X-100 and stained with 0.5 μ g of 4',6-diamidino-2-phenylindole (DAPI) per ml for 10 min at room temperature. The coverslips were mounted on slides with antileach mounting medium (Molecular Probes, Eugene, Ore.) and viewed by confocal microscopy with an LSM410 microscope (Zeiss) equipped with an argon-krypton and a UV laser.

Cell sorting and propidium iodide staining for cells with sub-G $_1$ DNA. At 18 h after transfection, the cells were trypsinized and washed with Dulbecco's modified Eagle's medium. GFP-positive cells were sorted in a Becton-Dickinson

FACS Vantage. The GFP-positive cells were replated in the culture medium–10% heat-inactivated FBS for 40 h and then fixed with 40% ethyl alcohol for 30 min. They were washed with PBS, resuspended in 0.5 μ g of propidium iodide per ml in PBS, and incubated with 50 μ g of RNase per ml at 37°C for 30 min. Numbers of cells with sub-G $_1$ DNA were assessed by FACS analysis.

RESULTS

DNA-PKcs associates with PKC δ in vivo. Whereas IR induces the activation of PKC δ (14), we asked if PKC δ interacts with proteins, such as DNA-PKcs, that are involved in DNA double-strand break repair. Analysis of anti-PKC δ immunoprecipitates with an anti-DNA-PKcs antibody demonstrated reactivity with a protein of >350 kDa (Fig. 1A). PIRS was used as a negative control of immunoprecipitation. As a positive control, analysis of anti-DNA-PKcs immunoprecipitates by immunoblotting with anti-DNA-PKcs demonstrated a similar pattern of reactivity (Fig. 1A). To evaluate the stoichiometry of the interaction between DNA-PKcs and PKC δ , we subjected U-937 cell lysates to immunoprecipitation with anti-PKC δ and analyzed the supernatants and precipitates by immunoblotting with anti-DNA-PKcs. Signal intensities from before and after anti-PKC δ immunoprecipitation were compared by laser densitometric scanning. The results demonstrate that approximately 50% of DNA-PKcs is associated with PKC δ (Fig. 1B). When anti-DNA-PKcs immunoprecipitates were analyzed by immunoblotting with anti-PKC δ in the reciprocal experiment, the results confirmed a constitutive association of DNA-PKcs with PKC δ (Fig. 1C). The interactions between DNA-PKcs and PKC δ are specific, since the anti-DNA-PKcs antibody does not cross-react with PKC δ and the anti-PKC δ antibody does not cross-react with DNA-PKcs (Fig. 1A and C). Activation of caspase 3 in irradiated cells is associated with proteolytic cleavage of PKC δ to an active 40-kDa fragment (hereafter termed PKC δ CF) (14). To assess the interaction of DNA-PKcs with PKC δ CF, U-937 cells were irradiated and harvested at different time intervals. Lysates from control and irradiated cells were subjected to immunoprecipitation with anti-DNA-PKcs. Analysis of the precipitates with anti-PKC δ demonstrated binding between DNA-PKcs and PKC δ CF (Fig. 1D). The finding that DNase has no effect on the coimmunoprecipitation of DNA-PKcs and PKC δ CF indicated that the association between these proteins is not dependent on DNA binding (data not shown).

DNA-PKcs binds directly to PKC δ in vitro. To assess the interaction of DNA-PKcs with PKC δ FL and PKC δ CF in vitro, we incubated GST fusion proteins prepared from PKC δ FL and PKC δ CF with U-937 cell lysates. Analysis of the adsorbates with anti-DNA-PKcs demonstrated that whereas both PKC δ FL and PKC δ CF bind to DNA-PKcs, the apparent affinity of the interaction with PKC δ CF is greater than that with PKC δ FL (Fig. 2A). To determine whether the interaction between DNA-PKcs and PKC δ is direct, purified DNA-PKcs was incubated separately with GST-PKC δ FL, GST-PKC δ CF, or GST. After being washed, the bound proteins were separated by SDS-PAGE (5% polyacrylamide) and analyzed by immunoblotting with anti-DNA-PKcs. Reactivity with anti-DNA-PKcs in the GST-PKC δ CF and GST-PKC δ FL, but not GST, adsorbates supported a direct interaction of DNA-PKcs with PKC δ FL and PKC δ CF (Fig. 2B). To further demonstrate direct interaction between DNA-PKcs and PKC δ , purified DNA-PKcs was resolved by SDS-PAGE, transferred to a nitrocellulose filter, and renatured in aqueous buffer. After incubation with GST-PKC δ FL, GST-PKC δ CF, or GST, the filters were washed and probed with anti-PKC δ . Reactivity with anti-PKC δ at the position corresponding to DNA-PKcs confirmed the direct interaction of DNA-PKcs with PKC δ

FIG. 5. Caspase 3 cleaves DNA-PKs and partially inhibits DNA-PKs activity. (A) Purified DNA-PK was incubated with recombinant caspase 3 (2.5 μ g/ml) (left) or with recombinant ICE (right) at room temperature for 1 h or 30 min, respectively. The reaction products were subjected to SDS-PAGE and analyzed by immunoblotting with anti-DNA-PKs. (B and C) Purified DNA-PK/Ku in the presence of DNA-beads was incubated with recombinant caspase 3 for 1 h at room temperature. In vitro kinase reactions containing [γ - 32 P]ATP were performed in the absence (B) or presence (C) of GST-p53 as the substrate. The reactions were stopped by the addition of SDS sample buffer, and the products were analyzed by SDS-PAGE and autoradiography. DNA-PK CL1 and CL2, DNA-PK-cleaved fragments 1 and 2.

(Fig. 2C). The absence of reactivity when the filters were incubated with GST indicated that binding of DNA-PKs with PKC δ is specific (Fig. 2B).

PKC δ CF-mediated phosphorylation of DNA-PKs inhibits DNA-PK binding to DNA in vitro. To determine whether PKC δ phosphorylates DNA-PK, we incubated GST-PKC δ FL or GST-PKC δ CF with purified DNA-PKs in the presence of [γ - 32 P]ATP. The phosphorylation reactions were carried out in the absence of DNA to inhibit DNA-PKs autophosphorylation. Analysis of the products by autoradiography indicated that DNA-PKs is a substrate for PKC δ (Fig. 3A, top) PKC δ CF is at least 40 times more active than PKC δ FL, as determined by their phosphorylation of myelin basic protein (Fig. 3A, bottom). The present results also demonstrate approximately 10-fold more phosphorylation of DNA-PKs by PKC δ CF (Fig. 3A, top).

Recent studies have demonstrated that c-Abl-mediated phosphorylation of DNA-PK on tyrosine inhibits DNA-PKs activity (27). Other studies have shown that autophosphorylation of DNA-PKs inhibits its activity by inducing the dissociation of DNA-PKs from DNA (8). To further assess the functional significance of the interaction between DNA-PKs and PKC δ , we asked if PKC δ affects the association of DNA-PKs with DNA. To address this issue, DNA-PKs was bound to DNA-beads and incubated in the presence of wortmannin to inhibit DNA-PKs autophosphorylation and hence its autodis-

sociation from DNA. After being washed to remove unbound DNA-PKs, the beads were incubated with GST-PKC δ CF or GST-PKC δ CF K-R in the presence of [32 P]ATP. The bound and supernatant fractions were then analyzed by immunoblotting with anti-DNA-PKs. The results demonstrate that addition of GST-PKC δ CF K-R has no detectable effect on the release of DNA-PKs from DNA (Fig. 3B). By contrast, incubation with GST-PKC δ CF resulted in the release of DNA-PKs from the beads (Fig. 3B). Whereas DNA-PK requires DNA for activity, these results suggest that PKC δ CF inhibits DNA-PK activity by abrogating the ability of DNA-PK to associate with DNA.

PKC δ CF binds to DNA-PKs at its catalytic domain. To determine the regions of DNA-PKs responsible for the association with PKC δ CF, fragments of the DNA-PKs polypeptide were generated from mouse DNA-PKs cDNAs. Fourteen different DNA-PKs fragments, representing the entire open reading frame, were synthesized by in vitro transcription-translation (Fig. 4A) (24). Purified GST-PKC δ CF was incubated separately with the in vitro-translated fragments of DNA-PK, washed, and analyzed by autoradiography. The signal intensities of GST-PKC δ CF-bound DNA-PKs fragments were compared to the total amount of product in the reaction mixture by densitometric scanning. The results demonstrate that DNA-PKs-6 (amino acids 2333 to 2774; 10 to 15% bound to PKC δ CF), DNA-PKs-8 (amino acids 3414 to 3850; 12 to 18%

FIG. 6. Phosphorylation and inactivation of DNA-PKcs by PKC δ CF. (A and B) Purified DNA-PK-Ku in the presence of DNA-beads was incubated with recombinant caspase 3 for 1 h at room temperature. In vitro phosphorylation of the cleaved fragments of DNA-PK was then performed in the presence of [γ - 32 P]ATP and GST-PKC δ CF or GST-PKC δ CF K-R for 15 min at 30°C. After phosphorylation of DNA-PKcs and removal of PKC δ -CF or PKC δ CF K-R by sedimentation, kinase reactions were performed in the absence (A) or presence (B) of GST-p53 for an additional 15 min at 30°C. The reactions were stopped by the addition of SDS sample buffer, and the products were analyzed by SDS-PAGE and autoradiography. Lanes: 1, DNA-PK-Ku with DNA; 2, DNA-PK-Ku without DNA; 3, DNA-PK-Ku with DNA and caspase 3; 4, DNA-PK-Ku with DNA caspase 3, and GST-PKC δ CF; 5, DNA-PK-Ku with DNA, caspase 3, and GST-PKC δ CF K-R; 6, DNA-PK-Ku with DNA and GST-PKC δ CF; 7, GST-p53; 8, buffer with [γ - 32 P]ATP. (C) The percent inhibition of DNA-PKcs-mediated GST-p53 phosphorylation is expressed as the mean $\pm$ SD of four independent experiments.

bound), DNA-PKcs-9 (amino acids 3757 to 4124; 22 to 25% bound), and DNA-PKcs-15 (amino acids 3414 to 4123; 18 to 25% bound) associate with GST-PKC δ CF but not with GST (Figs. 4B and C and data not shown). Thus, PKC δ CF binds to DNA-PKcs through a 1,790-amino-acid region that in part includes the PK homology domain.

Cleavage of DNA-PKcs by caspase 3 and inhibition of DNA-PKcs activity by phosphorylation with PKC δ CF. Recent studies have shown that DNA-PKcs kinase activity is reduced in apoptotic cells and that the inhibition correlates with proteolytic cleavage of DNA-PKcs (51). To investigate which protease is responsible for cleaving DNA-PKcs in vitro, we treated purified DNA-PKcs with caspase 3 or interleukin-converting enzyme (ICE). In contrast to ICE, caspase 3 induced the cleavage of purified DNA-PKcs to 240- and 150-kDa fragments (Fig. 5A). Overexposed gels demonstrate a minor cleaved fragment of 120 kDa (data not shown). To determine whether cleavage of DNA-PKcs by caspase 3 inhibits DNA-PKcs activity in vitro, we incubated purified DNA-PK-Ku-DNA complexes with caspase 3 for 30 min to 1 h and used autoradiography to analyze the products of a kinase reaction performed in the presence of [γ - 32 P]ATP. As assessed by autophosphor-

ylation and/or phosphorylation of the cleaved fragments, the results demonstrate that the 240-kDa cleavage fragment of DNA-PKcs (DNA-PK CL1) is phosphorylated (Fig. 5B). In vitro, DNA-PKcs phosphorylates p53, and this event requires binding of DNA-PKcs to DNA (35). Therefore, to further assess the effect of caspase 3-mediated cleavage on DNA-PK activity, we incubated purified DNA-PK-Ku-DNA complexes with caspase 3 and assessed DNA-PK activity with GST-p53 as a substrate. The results demonstrate that cleavage of DNA-PKcs by caspase 3 inhibits in part the DNA-PKcs activity as assessed by its phosphorylation of p53 (Fig. 5C).

Whereas the present findings demonstrate that cleavage of DNA-PKcs by caspase 3 is associated with partial inhibition of DNA-PKcs activity, we asked whether the cleaved DNA-PKcs fragment is affected by PKC δ CF phosphorylation. To address this issue, we first incubated purified DNA-PK-Ku complexes with in vitro-translated caspase 3 to generate its fragments. The cleaved fragments of DNA-PKcs were then incubated with GST-PKC δ CF, GST-PKC δ CF K-R, or buffer in the presence of [γ - 32 P]ATP. The DNA-PKcs activity in kinase reaction mixtures was assessed by autophosphorylation or phosphorylation of the DNA-PK CL1 or by using GST-p53 as the substrate. The results demonstrate that autophosphorylation of DNA-PKcs, phosphorylation of the DNA-PKcs CL1, or phosphorylation of p53 by PKC δ CF is associated with inhibition of DNA-PKcs activity (Fig. 6). PKC δ CF binds to DNA-PKcs at the catalytic domain (fragments 8, 9, and 15 [Fig. 4]), and this interaction contributes in part to the inhibition of DNA-PKcs (Fig. 6C). Similarly, cleavage of DNA-PKcs by caspase 3 also partially inhibits DNA-PKcs activity (Fig. 5). However, more pronounced inhibition of DNA-PKcs activity was observed upon PKC δ CF-mediated phosphorylation of DNA-PKcs (Fig. 6C). To assess whether cleavage of DNA-PKcs facilitates the inhibition of DNA-PKcs activity by PKC δ CF-mediated phosphorylation, we performed experiments in which uncleaved DNA-PKcs was incubated with PKC δ CF or PKC δ CF K-R and then analyzed for DNA-PKcs-dependent p53 phosphorylation. The results demonstrate that phosphorylation of uncleaved DNA-PKcs by PKC δ CF inhibits DNA-PKcs activity but not to the extent observed when cleaved DNA-PKcs is phosphorylated by PKC δ CF (Fig. 6C and data not shown). Taken together, these findings indicate that the interaction of PKC δ CF with DNA-PKcs and caspase 3-mediated cleavage of DNA-PKcs independently contribute in part to inhibition of DNA-PKcs activity. However, nearly complete inhibition of DNA-PKcs activity was observed when PKC δ CF phosphorylated the cleaved fragment of DNA-PKcs (Fig. 5 and 6).

Functional role of DNA-PK-PKC δ complexes in apoptosis.

Both PKC δ and DNA-PKcs are cleaved after apoptotic stimuli of cells (14, 51). If the cleavage and regulation of these proteins is associated with functions in apoptosis, mutant cells of DNA-PKcs may show alterations in apoptotic pathways that are dependent on PKC δ . DNA-PK-deficient (ScSV3, scid) and DNA-PK $^{+}$ (SCH8-1, scid + human DNA-PKcs) cells (5) were transiently transfected with GFP vectors expressing PKC δ CF. After 48 h, the morphology of GFP-positive cells was analyzed. PKC δ was found to stimulate the disintegration or shrinkage of nuclei of DNA-PK $^{+}$ cells, indicative of apoptosis (Fig. 7). In striking contrast, DNA-PK-deficient cells failed to demonstrate nuclear shrinkage in response to PKC δ CF. To confirm whether the differential response was due to DNA-PK mutations, we examined a second set of DNA-PK mutant and control Chinese hamster ovary cell lines in the same way. DNA-PK-deficient CHO V-3 cells (52) failed to form PKC δ CF-dependent apoptosis, unlike the DNA-PK $^{+}$ parental CHO

FIG. 7. Transient overexpression of PKC δ CF in SCH8-1 (DNA-PK $^{+/+}$) and ScSV3 (DNA-PK $^{-/-}$) cells. GFP-tagged PKC δ CF was transiently transfected in SCH8-1 (DNA-PK $^{+/+}$) and ScSV3 (DNA-PK $^{-/-}$) cells. The cells were stained with DAPI, and the GFP-positive cells were analyzed by confocal microscopy. The results are shown as overlay photographs of DAPI and GFP staining. Arrows indicate apoptotic cells. Bar, 10 μ m.

cells (Fig. 8). Thus, DNA-PK is linked to apoptotic mechanisms via PKC δ .

If phosphorylation by PKC δ is required in apoptosis, a PKC δ mutant protein inactivated in kinase activity would be expected to differ in its properties in the above assay. To test this, SCH8-1 and ScSV3 cells were transiently transfected with GFP vectors expressing PKC δ CF or the kinase-inactive mutant PKC δ CF K-R. After 48 h, the cells were sorted for GFP positivity and analyzed for sub-G $_1$ DNA content. Approximately 60% of the cells were apoptotic when PKC δ CF was overexpressed in DNA-PK $^{+/+}$ cells, in contrast to 20% in DNA-PK $^{-/-}$ cells (Fig. 9). By contrast, only 10% cells were apoptotic when PKC δ CF K-R was transfected into DNA-PK $^{+/+}$ cells (Fig. 9). Taken together, these findings demonstrate that interaction of DNA-PKcs with PKC δ CF contributes to apoptosis.

DISCUSSION

Caspase-mediated proteolysis in apoptosis. In addition to PKC δ , substrates that are activated by caspase-mediated cleavage in apoptosis include the p21-activated kinase 2 (PAK2) (49), cytosolic phospholipase A2 (56), sterol regulatory binding proteins (44), the 45-kDa subunit of DNA fragmentation factor (38), and PITSLRE kinase α 2-1 (4). Expression of the cleaved fragments of PKC δ or PAK2 in cells induces certain characteristics of apoptosis (17, 49). However, the precise role of these cleaved proteins in apoptosis is unclear. By contrast, other proteins are inactivated by caspases. Previous studies have demonstrated that DNA-PKcs is a substrate of caspase 3 and that cleavage is accompanied by loss of DNA-PKcs activity (7, 19, 51). The functional role of DNA-PK cleavage in the

induction of apoptosis, like that for many of the other substrates of caspases, is unknown.

DNA-PK $_{cs}$ is cleaved by caspase 3 at a DEVD/N $_{2713}$ site into N-terminal 240-kDa and C-terminal 150-kDa fragments. The 150-kDa fragment contains another DWVD/G site that predicts the generation of an additional fragment of 120 kDa (51). In the present studies, cleavage of DNA-PKcs to the 240- and 150-kDa fragments by caspase 3 resulted in partial loss of catalytic activity in the presence of Ku and DNA. Moreover, cleavage of DNA-PKcs also partially inhibited the DNA-PKcs-mediated phosphorylation of p53. These findings suggest that mechanisms other than cleavage of DNA-PKcs by caspase 3 are responsible for the loss of DNA-PKcs activity that has been observed in cells induced to undergo apoptosis (51).

Regulation of DNA-PKcs activity. DNA-PKcs autophosphorylation inactivates DNA-PKcs by a mechanism in which DNA-PKcs disassociates from Ku (8). Other studies have demonstrated that the c-Abl tyrosine kinase negatively regulates DNA-PKcs activity in the response to DNA damage (27). c-Abl phosphorylates the C-terminal region of DNA-PKcs and induces the disassociation of DNA-PKcs from the DNA-PKcs-Ku complex (24, 27). c-Abl and Ku both bind to the C terminus of DNA-PKcs near the kinase domain, and c-Abl phosphorylates the region of DNA-PKcs to which Ku binds (24). Thus, autophosphorylation and c-Abl-mediated phosphorylation regulate DNA-PKcs activity by mechanisms that oppose the activation of DNA-PKcs through binding to the Ku-DNA complex.

The present studies demonstrate that DNA-PKcs is also regulated by PKC δ . DNA-PKcs constitutively associates with the full-length form of PKC δ in nonapoptotic cells and with

FIG. 8. Transient overexpression of PKC δ CF in DNA-PK $^{-/-}$ and DNA-PK $^{+/+}$ CHO cells. GFP-tagged PKC δ CF was transiently transfected in CHO (DNA-PK $^{+/+}$) and CHO V-3 (DNA-PK $^{-/-}$) cells. The cells were stained with DAPI, and the GFP-positive cells were analyzed by confocal microscopy. The results are shown as overlay photographs of DAPI and GFP staining. Arrows indicate apoptotic cells. Bar, 10 μ m.

PKC δ CF in cells induced to undergo apoptosis. The results indicate that PKC δ , like c-Abl, binds directly to the C terminus of DNA-PKcs at the catalytic domain. PKC δ CF phosphorylates DNA-PKcs and results in the disassociation of DNA-PKcs from DNA. These findings are in concert with the demonstration that interaction of DNA-PKcs with PKC δ inhibits

DNA-PKcs activity. Taken together with the finding that cleavage of DNA-PKcs by caspase 3 is partially sufficient to inhibit DNA-PKcs activity, the results support a model in which cleavage of PKC δ to the catalytically active fragment results in association and phosphorylation of DNA-PKcs and thereby complete inhibition of DNA-PKcs activity. Thus, on the basis of these findings, we would propose that in addition to auto-phosphorylation (8) and c-Abl-mediated phosphorylation (24, 27), DNA-PKcs is downregulated by PKC δ . To our knowledge, DNA-PKcs may be the first substrate found to be regulated by PKC δ CF-mediated phosphorylation.

Recent studies have shown that PKC δ associates constitutively with c-Abl (59). Activation of c-Abl by DNA damage results in c-Abl-dependent phosphorylation of PKC δ . Also, c-Abl-mediated phosphorylation of PKC δ results in activation of PKC δ in vitro and in irradiated cells (59). Whereas c-Abl functions in the downregulation of DNA-PKcs by mediating direct phosphorylation of DNA-PKcs (27), it may also contribute to the interaction of PKC δ and DNA-PKcs by activating PKC δ . In this context, c-Abl associates with PKC δ in a complex that includes DNA-PKcs (27, 59). The activation of PKC δ by c-Abl in the response to DNA damage (59) thus provides a mechanism by which both c-Abl and PKC δ can downregulate DNA-PKcs activity. Subsequent cleavage of PKC δ to the catalytic fragment by caspase 3 would preclude further activation by a c-Abl-dependent mechanism. The constitutive activation of PKC δ CF and thereby the phosphorylation of DNA-PKcs and/or the cleaved CL1 fragment is sufficient to inhibit disassociation of DNA-PKcs from the Ku-DNA complex.

Functional interaction of DNA-PKcs and PKC δ in apoptosis. Overexpression of PKC δ CF in cells is associated with

FIG. 9. Transient overexpression of PKC δ CF and not PKC δ CF K-R in DNA-PK $^{+/+}$ cells is associated with induction of apoptosis. GFP-tagged PKC δ CF or PKC δ CF K-R mutant was transiently transfected in SCH8-1 (DNA-PK $^{+/+}$) (lane 1, GFP; lane 2, GFP-PKC δ CF; lane 3, GFP-PKC δ CF K-R) or ScSV3 (DNA-PK $^{-/-}$) (lane 4, GFP; lane 5, GFP-PKC δ CF; lane 6, GFP-PKC δ CF K-R) cells. As controls, cells were transfected with GFP-expressing empty vector. The GFP-positive cells were sorted by FACS analysis and analyzed for DNA content by flow cytometry. The results are expressed as the percentage (mean $\pm$ SD of three independent experiments, each performed in duplicate) of cells with sub-G $_1$ DNA content.

chromatin condensation, nuclear fragmentation, induction of sub-G₁ DNA, and lethality (17). These findings have indicated that cleavage of PKC δ to a kinase-active fragment by caspase 3 contributes to multiple changes that are characteristic of apoptosis. Whereas the mechanistic basis for the proapoptotic effects of PKC δ CF are unclear, the demonstration that PKC δ CF interacts with DNA-PKcs prompted studies on the functional significance of this interaction. Cells deficient in DNA-PKcs were transfected to express PKC δ CF, and the findings were compared to those obtained with cells that express DNA-PK. The results obtained with ScSv3 (DNA-PK $^{-/-}$) and SCH8-1 (DNA-PK $^{+/+}$) cells demonstrate that PKC δ CF-induced apoptosis is abrogated in part in DNA-PK-deficient cells. Similar results were obtained with CHO V-3 (DNA-PK $^{-/-}$) and CHO (DNA-PK $^{+/+}$) cells transfected to express PKC δ CF. These findings suggest that PKC δ CF induces apoptosis, at least in part by a DNA-PK-dependent mechanism.

The demonstration that cells deficient in DNA-PK are hypersensitive to DNA-damaging agents has supported a direct role for DNA-PKcs in DNA repair (5, 30, 34). The inhibition of DNA-PKcs by PKC δ CF would be expected to inhibit DNA repair and thereby facilitate the DNA fragmentation that is induced in apoptosis. Thus, given that PKC δ inhibits DNA-PKcs activity, it is not evident why DNA-PK $^{-/-}$ cells would be less sensitive to PKC δ CF-induced apoptosis. One potential explanation is that the direct binding of PKC δ CF to DNA-PKcs provides access for DNA-PKcs substrates to phosphorylation by PKC δ CF. In this context, DNA-PKcs associates with and phosphorylates the p53 tumor suppressor and other proteins. The present experiments have not addressed whether the pools of DNA-PKcs that associate with PKC δ are the same as those that form complexes with other proteins. However, it is conceivable that in the absence of DNA-PKcs, PKC δ would not be positioned to interact with a protein that is central to the apoptotic response. Insights into this potential mechanism could be obtained from an analysis of other proteins associated with the PKC δ -DNA-PKcs complex.

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