Caspase-3-mediated Cleavage of Protein Kinase Cθ in Induction of Apoptosis

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Protein kinase Cθ (PKCθ) is a member of the novel or nPKC family. A functional role for PKCθ is unknown. The present studies demonstrate that PKCθ is cleaved in the third variable region (V3) in apoptosis induced by diverse agents. PKCθ cleavage is blocked in cells that overexpress the anti-apoptotic Bcl-xL, or the baculovirus p35 protein. PKCθ is cleaved by Caspase-3 and by apoptotic cell lysates at a DEVD_{aspartic} site. We also show that overexpression of the cleaved kinase-active PKCθ fragment, but not full-length PKCθ or a kinase-inactive fragment, results in induction of sub-G₁ phase DNA, nuclear fragmentation, and lethality. These findings indicate that proteolytic cleavage of PKCθ by Caspase-3 induces events characteristic of apoptosis.

The 11 known isoforms of the protein kinase C (PKC) family have been divided into the classical (cPKC; α, β, γ), novel (nPKC; δ, ε, η, θ, µ), and atypical (aPKC; ζ, η) groups (1). The Ca^{2+}-dependent cPKCs contain the conserved regulatory regions, C1 and C2, while the Ca^{2+}-independent nPKC and aPKC isoforms lack the C2 domain. Cleavage of cPKCs in the third variable (V3) region by calpains I and II deletes the C1 and C2 regulatory regions and results in catalytically active fragments (2). Other studies have shown that the nPKC isoform is activated by the Caspase-3 cysteine protease in cells induced to undergo apoptosis (3–5). Caspase-3-mediated cleavage of PKCθ at a DMQDV_{aspartic}/Ile site in the V3 region deletes the C1 regulatory domain (3–5). Overexpression of the anti-apoptotic Bcl-2 and Bcl-xL proteins blocks PKCθ cleavage (3, 4). These findings have suggested that PKCθ is functionally involved in the induction of apoptosis.

The PKCθ isoform is structurally related to PKCδ (6–8), although the V3 domain of PKCθ has no significant homology with that in PKCδ or the other PKC isoforms. Few insights are available regarding the functional roles of PKCθ. Whereas PKCθ transcripts are found ubiquitously, PKCθ is predominately expressed in hematopoietic cells and skeletal muscle (6, 8). Studies in T cells have demonstrated that PKCθ is involved in antigen-specific activation (9). PKCθ interacts with 14-3-3 proteins (10) and is involved in AP-1-mediated transcription (11). Other work has shown that the human immunodeficiency virus Nef protein inhibits translocation of PKCθ from the cytosolic to membrane fraction after phorbol ester stimulation (12). Unlike the cPKCs and PKCδ, there are no reports of proteolytic cleavage of the PKCθ isoform.

The present studies demonstrate that PKCθ is cleaved to an activated form in cells induced to undergo apoptosis. The results indicate that PKCθ is cleaved by the Caspase-3 protease. We also show that overexpression of the PKCθ catalytic fragment induces characteristics of apoptosis.

MATERIALS AND METHODS

Cell culture—Human U-937 myeloid leukemia cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/liter L-glutamine. U-937 cells overexpressing bcl-xL, CrmA, and p35 were prepared as described (13–15). Cells were treated with 10 μmol/liter 1,2-dioleoyl-sn-glycero-3-phospho-L-serine and 3 μg/ml etoposide (Bristol-Meyers Squibb Co., Princeton, NJ), and 100 μmol/liter cisplatinum (Sigma). 

Immunoblot Analysis—Cytoplasmic extracts were prepared and fractionated through Q-Sepharose columns as described (3, 4). Proteins were subjected to electrophoresis in 10% SDS-polyacrylamide gels and then transferred to nitrocellulose paper. The residual binding sites were blocked by incubating the filters with 5% dry milk in PBST (phosphate-buffered saline/0.05% Tween 20). The filters were incubated with anti-PKCθ polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After washing twice with PBST, the blots were incubated with anti-rabbit IgG peroxidase conjugate (Amersham Corp.). The antigen-antibody complexes were visualized by chemiluminescence (ECL detection system; Amersham).

Analysis of DNA Fragmentation—Cells (5 × 10^6) were harvested, washed, and incubated in 50 μl of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K (Sigma) for 6 h at 50 °C. The samples were incubated with 50 μl of 10 mM EDTA (pH 8.0) containing 2% (w/v) low-melting-point agarose and 40% sucrose for 10 min at 70 °C. The DNA was separated in 2% agarose gels. After treatment with RNase, the gels were visualized by UV illumination.

In Vivo Translocation and Protease Cleavage Assay—The full-length (FL) PKCθ cDNA (provided by J. Anthony Ware, Beth Israel Hospital, Boston) was cloned into BamHI sites of a modified pSVβ plasmid (CLONTECH). A PKCθ (D351A and D354A) mutant was generated in two steps: overlapping primer extension, PARP cDNA was generated by polymerase chain reaction cloning (5). [35S]Smethylisobutyl-α-ketoisocaproate-labeled proteins (PKCθFL, PKCθFL(D-A), PARP) were synthesized by coupled transcription and translation reactions (Promega, Madison, WI). Labeled proteins were incubated with 5 μg/ml Escherichia coli-derived Caspase-3, Caspase-1, Caspase-2, Caspase-4, Caspase-6, or Caspase-7 in 50 mM Hepes (pH 7.5), 10% glycerol, 2.5 mM dithiothreitol, and 0.25 mM EDTA at room temperature for 30 min (16). Cleavage reactions were also performed in the presence of 5 μg of cytoplasmic extract from untreated or ara-C-treated cells and in the presence of recombinant CrmA or p35 (14, 15). The reaction products were analyzed by electrophoresis in 10% or 12% SDS-polyacrylamide gels and then autoradiography.

Analysis of Kinase Activity—Recombinant PKCθ proteins were prepared by coupled transcription and translation. A vector expressing a PKCθ fragment (CF; amino acids 355–706) was generated by polymerase chain reaction cloning from the full-length PKCθ cDNA. A mutant PKCθCF with Lys-409 substituted by Arg (K-R) was generated by overlapping primer extension. Protein kinase assays were performed as described (PKC assay kit, Life Technologies, Inc.).

Cell Transfections—PKCθFL, PKCθCF, or PKCθCF(K-R) were cloned into the pEGFP-C1 vector (CLONTECH). HeLa cells were suspended at a density of 1 × 10^6 cells/ml and transfected by electroporation (Gene Pulser, Bio-Rad; 0.22 V, 960 μF). At 40 h post-transfection, cells were sorted by FACScan (Becton Dickinson, Mansfield, MA), and...
viability was checked by trypan blue exclusion. Transfected cells were stained with propidium iodide. FACScan was used to determine sub-G1 content in cells positive for green fluorescence. Chromatin fragmentation was determined by staining methanol-fixed cells with 0.5 μg/ml DAPI (Molecular Probes, Eugene, OR).

**RESULTS AND DISCUSSION**

Treatment of U-937 cells with ara-C and other DNA-damaging agents results in the induction of apoptosis (17, 18). Whereas the V3 region of PKCθ has a DEVD/K site similar to that cleaved in PARP during apoptosis (19, 20), we asked whether PKCθ is also susceptible to cleavage. PKCθ was detectable as a 78-kDa band in control cells (Fig. 1A). By contrast, ara-C-induced apoptosis was associated with cleavage of PKCθ to a 40-kDa fragment (Fig. 1A). Similar results were obtained during apoptosis induced by cisplatinum, etoposide, and 20-gray ionizing radiation (Fig. 1B and data not shown).

To determine whether PKCθ cleavage is associated with induction of apoptosis, we studied cells that overexpress the anti-apoptotic Bcl-xL protein and exhibit resistance to induction of apoptosis (13). Exposure of control U-937/neo cells to ara-C resulted in cleavage of PKCθ, while there was no apparent effect of this agent on PKCθ in the U-937/Bcl-xL transfectant (Fig. 2A). The cowpox protein CrmA (21) and the baculovirus protein p35 (16) block apoptosis in diverse models by associating with the catalytic domain of PKCθ and interfering with its kinase function, we assayed reagents were viable provided further support for the selective effects of PKCθ activation (Fig. 2C).

To determine if PKCθ contributes to apoptosis, we transfected HeLa cells with PKCθFL, PKCθCF, or PKCθCF(K-R) cloned into vectors expressing the green fluorescence gene. Positive transfecants were selected by flow cytometry, reseeded in medium, and assayed for viability by trypan blue exclusion. Over 90% of the PKCθFL transfecants were viable, while only 10–15% of the PKCθCF(K-R) transfecants were viable provided further support for the selective effects of PKCθCF expression (Fig. 4A). To assess whether transfection of PKCθCF induces apoptosis, we monitored the appearance of green fluorescence-positive cells with sub-G1 DNA content in cells positive for green fluorescence. Chromatin fragmentation was determined by staining methanol-fixed cells with 0.5 μg/ml DAPI (Molecular Probes, Eugene, OR).

**FIG. 1.** Proteolytic cleavage of PKCθ by DNA-damaging agents. U-937 cells were treated with 10 μM ara-C (A) or 3 μg/ml etoposide (ETOP) or 10 μM cisplatinum (CDDP) (B) for 6 h. Lysates were subjected to immunoblot analysis using anti-PKCθ antibody (upper panel). DNA fragmentation was assessed by electrophoresis in 2% agarose gels (lower panel). FL, full-length; CF, cleaved fragment.

**FIG. 2.** Effects of overexpression of bcl-xL, CrmA, or p35 on ara-C-induced cleavage of PKCθ. U-937 and U-937/bcl-xL (A) or U-937, U-937/CrmA, and U-937/p35 (B) cells were treated with ara-C for 6 h. Immunoblot analysis of the lysates was performed with anti-PKCθ antibody (upper panel). DNA was analyzed for fragmentation in agarose gels (lower panel). FL, full-length; CF, cleaved fragment.
DNA content. Transfection of PKC\textsubscript{\textalpha} FL or PKC\textsubscript{\textalpha} CF(K-R) was associated with 7–10% of cells with sub-G\textsubscript{1} DNA (Fig. 4\textit{B}). Significantly, transfection of kinase-active PKC\textsubscript{\textalpha} CF resulted in 48% of cells with sub-G\textsubscript{1} DNA (Fig. 4\textit{B}). Cells were also stained with DAPI to assess nuclear morphology (25). Transfection of PKC\textsubscript{\textalpha} CF, but not PKC\textsubscript{\textalpha} FL or PKC\textsubscript{\textalpha} CF(K-R), was associated with nuclear fragmentation (Fig. 4\textit{C}).

Recent studies have demonstrated that the \(\alpha\)PKCs (PKC\textsubscript{z} and \(\xi\)) interact with Par-4 and abrogate the ability of Par-4 to induce apoptosis (26). These findings have suggested that the \(\alpha\)PKCs exhibit an anti-apoptotic function. By contrast, the present results and previous work on PKC\textsubscript{\textdelta} (3, 4) support a potential role for at least certain nPKCs in promoting apoptosis. The absence of detectable cleavage of PKC\textsubscript{\textalpha}, \(\beta\), \(\epsilon\), and \(\zeta\) further supports the selective involvement of PKC\textsubscript{\textalpha} and \(\delta\) in apoptosis (3, 4). Previous studies have demonstrated that Caspase-3 cleaves PARP (22, 23), DNA-PK (27, 28), D4-GDI (29), U1 small nuclear riboprotein (27), and PKC\textsubscript{d} (5). We show that PKC\textsubscript{\textalpha} is also cleaved by Caspase-3 and that Bcl-x\textsubscript{L} functions upstream to this event. The finding that the cleaved fragment of PKC\textsubscript{\textalpha} induces characteristics typical of apoptosis further supports a role for PKC\textsubscript{\textalpha} in mediating apoptotic events and not simply a bystander effect of Caspase-3 activation.

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