Proteolytic Cleavage and Activation of Protein Kinase C \( \mu \) by Caspase-3 in the Apoptotic Response of Cells to 1-\( \beta \)-D-Arabinofuranosylcytosine and Other Genotoxic Agents

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Protein kinase C (PKC) \( \mu \) is a novel member of the PKC family that differs from the other isozymes in structural and biochemical properties. The precise function of PKC\( \mu \) is not known. The present studies demonstrate that PKC\( \mu \) is cleaved during apoptosis induced by 1-\( \beta \)-D-arabinofuranosylcytosine (ara-C) and other genotoxic agents. PKC\( \mu \) cleavage is blocked in cells that overexpress the anti-apoptotic Bcl-x\(_l\) protein or the baculovirus p35 protein. Our results demonstrate that PKC\( \mu \) is cleaved by caspase-3 at the CQND\( ^{278}\)S site. Cleavage of PKC\( \mu \) is associated with release of the catalytic domain and activation of its kinase function. We also show that, unlike the cleaved fragments of PKC\( \delta \) and \( \theta \), overexpression of the PKC\( \mu \) catalytic domain is not lethal. Cells stably expressing the catalytic fragment of PKC\( \mu \), however, are more sensitive to apoptosis induced by genotoxic stress. In addition, expression of the caspase-resistant PKC\( \mu \) mutant partially inhibits DNA damage-induced apoptosis. These findings demonstrate that PKC\( \mu \) is cleaved by caspase-3 and that expression of the catalytic domain sensitizes cells to the cytotoxic effects of ara-C and other anticancer agents.

Protein kinase C (PKC)\( ^1 \) is a family of phospholipid-dependent serine/threonine kinases that play a major role in regulating a wide variety of physiological processes (1). Based on their structure and cofactor regulation, the PKC isozymes have been divided into the conventional (cPKC; \( \alpha \), \( \beta \), \( \gamma \), novel (nPKC; \( \delta \), \( \epsilon \), \( \eta \), \( \theta \)), and atypical (aPKC; \( \zeta \), \( \delta \alpha \)) subclasses (1). In contrast to other PKC isozymes, PKC\( \mu \) lacks a region homologous to the typical pseudosubstrate domain and contains a pleckstrin homology (PH) domain and thus represents a distinct PKC subclass (2, 3). The recently identified PKC\( \nu \) exhibits a high degree of homology to PKC\( \alpha \) and has been designated to this subclass (4).

Treatment of human tumor cell lines with genotoxic agents is associated with induction of apoptosis (5, 6). Efforts to define the role of PKC in apoptosis have been complicated by the expression of multiple isoforms in different cell types and their involvement in both pro- and antiapoptotic signaling cascades. Studies have demonstrated that PKC\( \delta \) and \( \theta \) selectively interact with Par-4 and abrogate its anti-apoptotic effects (7). PKC\( \alpha \) has been shown to phosphorylate Bcl-2 and suppress apoptosis in Pre-B REH cells (8). Other studies have shown that PKC\( \delta \) and \( \theta \) are proteolytically cleaved and activated by caspase-3 during apoptosis induced by diverse anticancer agents (9–11). Caspase-3-mediated cleavage of PKC\( \delta \) and \( \theta \) occurs in the third variable region, which separates the regulatory and catalytic domains. Cleavage of PKC\( \delta \) and \( \theta \) isoforms by caspase-3 results in release and activation of the catalytic domain (10, 11). The findings that proteolytic cleavage of PKC\( \delta \) and \( \theta \) is inhibited by overexpression of the anti-apoptotic Bcl-x\(_l\) protein or of the baculovirus p35 protein have supported their involvement in apoptosis (9, 11). Other studies have demonstrated that overexpression of the kinase-active PKC\( \delta \) and \( \theta \) catalytic domains, but not full length or kinase-inactive fragments, results in induction of certain features characteristic of apoptosis (10, 11). Conversely, PKC\( \zeta \), which plays a critical role in cell survival, is cleaved and inactivated by caspase-3 during UV-induced apoptosis (12).

In contrast to other PKC isoforms, PKC\( \mu \) and its mouse homologue PKD has unique enzymatic features and a distinct substrate specificity (13–15). These findings have suggested that PKC\( \mu \) is involved in novel signaling pathways. PKC\( \mu \) is located in the Golgi bodies and is involved in basal transport processes (16). Recent studies have demonstrated that the 14-3-3\( \gamma \) protein interacts with PKC\( \mu \) and negatively regulates its activity (17). Other studies have shown that overexpression of PKC\( \mu \) reduces sensitivity to tumor necrosis factor (TNF)-induced but not ceramide-induced apoptosis (18). However, the precise role of PKC\( \mu \) in intracellular signaling cascades during apoptosis remains unclear.

The present studies demonstrate that PKC\( \mu \) is cleaved during apoptosis induced by 1-\( \beta \)-D-arabinofuranosylcytosine (ara-C) and other genotoxic agents. The results demonstrate that PKC\( \mu \) is cleaved by caspase-3 at the CQND\( ^{278}\)S site between regulatory and catalytic domains. Cleavage of PKC\( \mu \) results in activation of its kinase function. We also show that overexpression of the cleaved catalytic domain sensitizes cells to the cytotoxic effects of genotoxic agents.

MATERIALS AND METHODS

Cell Culture and Transfection—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 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 2 \( \mu \)M l-glutamine. Human osteosarcoma cell lines SAOS2 and U2OS were

1 The abbreviations used are: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; PH, pleckstrin homology; ara-C, 1-\( \beta \)-D-arabinofuranosylcytosine; PKD, protein kinase D; TNF, tumor necrosis factor; CF, catalytic fragment; PBS, phosphate-buffered saline; IR, ionizing radiation; GFP, green fluorescence protein.
cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. U-937 cells overexpressing Bcl-XL, CrmA, and p35 were prepared as described (19–21). The PKCμ catalytic fragment (CF; amino acids 391–912) generated by polymerase chain reaction from the full length (FL) PKCμ cDNA was subcloned into the pEF-neo vector (21). To generate a PKCμCF-expressing line, U-937 cells were transfected by electroporation (Gene Pulser, Bio-Rad, 0.25 V, 960 μF) with pEF-neo or pEF-PKCμCF. Transfectants were selected in the presence of 400 μg/ml geneticin sulfate. Cells were treated with ara-C (Sigma Chemical Co., St. Louis, MO), etoposide (Bristol-Myers Squibb Co., Princeton, NJ), or cisplatin (Sigma). Irradiation was performed with a γ-ray source (Cesium 137, Gamma Cell 1000, Atomic Energy of Canada, Ltd., Ontario) at a fixed dose rate of 13 Gy/min.

Immunoblot Analysis—Cell lysates were prepared as described (21). 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 PBS (phosphate-buffered saline (PBS)/0.05% Tween 20). The filters were incubated with anti-PKCμ polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-caspase-3 (21). After washing twice with PBS, the blots were incubated with anti-rabbit IgG peroxidase conjugate (Amersham Pharmacia Biotech). The antigen-antibody complexes were visualized using chemiluminescence (ECL detection system; Amersham Pharmacia Biotech).

Apoptosis Assays—Analysis of DNA fragmentation was performed as described (21). Briefly, 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) agarose at low melting point 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. HeLa cells were suspended at a density of 1 × 10^6 cells per ml and transfected by electroporation (0.22 V, 960 μF). Analysis of DNA content was performed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (Becton-Dickinson). The number of cells with sub-G1 DNA content were determined with a MODFIT LT program (Verity Software house, Topsham, ME).

In Vitro Translation and Protease Cleavage Assays—The C-terminal PKCμ fragment (PKCμ (349–912)) generated by polymerase chain reaction from the full length PKCμ cDNA was cloned into the pcDNA3 vector. PKCμ (D348A), PKCμ (D378A), and PKCμ (D391A) were generated in two steps by overlapping primer extension. [35S]Methionine-labeled PKCμ wild type, mutants, and PKCμ (349–912) were synthesized by coupled transcription and translation reactions (Promega, Madison, WI). Labeled proteins were incubated with 5 μg/ml Escherichia coli-derived caspase-3 (in 50 mM HEPEs (pH 7.5), 10% glycerol, 2.5 mM dithiothreitol, and 0.25 mM EDTA at room temperature for 30 min (22)). Cleavage reactions were also performed in the presence of 5 μg of cytoplasmic extract from untreated or ara-C-treated cells. The reaction products were analyzed using electrophoresis in 10 or 12% SDS-polyacrylamide gels and then by autoradiography.

Analysis of Kinase Activity—Full length PKCμ proteins prepared by coupled transcription and translation were incubated with caspase-3 alone or in the presence of recombinant p35 (20, 21). Protein kinase assays were performed as described (PKC assay kit; Life Technologies, Inc., Gaithersburg, MD) using glycogen synthase as a substrate. Proteins prepared from U-937 and U-937/ara-C cells were subjected to immunoprecipitation with anti-PKCμ antibody. Immune complex kinase assays were performed by incubating the immunoprecipitates in kinase buffer (50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 4 mM MgCl2, 50 μM ATP, 1 μCi of [γ-32P]ATP, and 5 μg of glycogen synthase substrate) for 10 min at 30 °C. An equal volume of the reaction was spotted on phosphocellulose paper. The filters were then washed twice with 1% phosphoric acid and once with 95% ethanol. The amount of radiolabeled phosphate incorporated into the peptide was quantified by liquid scintillation counting.

RESULTS

Previous studies have demonstrated that ara-C induces apoptosis of human U-937 myeloid leukemia cells (6). To determine whether PKCμ is cleaved during apoptosis, we treated U-937 cells with ara-C and harvested the cells at various times. Immunoblot analysis of the lysates with an anti-PKCμ antibody demonstrated time-dependent decreases in the 110-kDa PKCμ protein and increases in a 60-kDa cleaved fragment (Fig. 1). The kinetics of cleavage of PKCμ coincided with the activation of caspase-3 and the appearance of internucleosomal DNA fragmentation (Fig. 1). Because ara-C incorporates into DNA and induces DNA strand breaks (23, 24), we studied the effects of other classes of DNA-damaging agents on proteolytic cleavage of PKCμ. Ionizing radiation (IR) induces DNA strand breaks either by direct interaction with DNA or through the formation of reactive oxygen intermediates (25). Cisplatin induces DNA intrastrand cross-links (26), whereas etoposide induces DNA strand breaks as a result of forming a complex with topoisomerase II and the DNA 5’-terminus (27). Treatment of cells with IR, cisplatin, or etoposide was associated with the cleavage of PKCμ to a 60-kDa fragment (Fig. 2A). As shown with ara-C, cleavage of PKCμ coincided with the induction of DNA fragmentation by these agents (Fig. 2A). Treatment of osteosarcoma cell lines SAOS2 and U2OS with cisplatin also resulted in cleavage of PKCμ to a 60-kDa fragment (Fig. 2B). These results demonstrate that treatment of different cell types with diverse DNA-damaging agents is associated with the cleavage of PKCμ during apoptosis.

U-937 cells that overexpress Bcl-xL (U-937/Bcl-xL) exhibit resistance to induction of apoptosis by blocking release of mitochondrial cytochrome c and activation of caspase-3 (28). Although exposure of U-937/neo cells to ara-C resulted in cleavage of PKCμ, there was no detectable cleavage in ara-C-treated U-937/Bcl-xL cells (Fig. 3). The cowpox virus protein CrmA and baculovirus protein p35 have been shown to prevent apoptosis by inhibiting caspases. Previous studies have demonstrated that apoptosis induced by genotoxic agents is mediated by a CrmA-insensitive and p35-sensitive mechanism (20, 21). Using the previously characterized U-937 transfectants stably over-expressing CrmA or p35, we found that CrmA expression has
no effect on ara-C-induced proteolysis of PKC\(\mu\) (Fig. 3). By contrast, cleavage of PKC\(\mu\) was inhibited following ara-C treatment of U-937/\(\text{p35}\) cells (Fig. 3). These findings indicated that PKC\(\mu\) is cleaved by a p35-sensitive caspase-like protease.

DNA damage-induced apoptosis is associated with the activation of caspase-3 (20, 21). Other studies have shown that caspase-3 is insensitive to CrmA but is inhibited by p35 (21, 22, 29). To determine whether PKC\(\mu\) is cleaved by caspase-3, PKC\(\mu\)FL labeled with \(\text{S}^{35}\text{S}\)methionine was incubated with purified recombinant caspase-3. PKC\(\mu\) was cleaved to 60- and 50-kDa fragments by caspase-3 (Fig. 4A). In addition, incubation of PKC\(\mu\) with cytosol from ara-C-treated apoptotic U-937 cells resulted in the appearance of similarly cleaved PKC\(\mu\) fragments, whereas lysates from untreated cells had little, if any, effect (Fig. 4A). The finding that the apparent molecular masses of the two cleaved fragments (60 and 50 kDa) were together approximately equal to the full length PKC\(\mu\) suggested that one site in PKC\(\mu\) is sensitive to protease cleavage. Caspase-3 prefers a DXXD-like substrate with an Asp residue at both the P1 and P4 positions (30). PKC\(\mu\) has two DXXD sites between the cysteine-rich and PH domains, either of which can yield cleaved fragments of approximately the same size as those observed on immunoblots (Fig. 4B; schematic of PKC\(\mu\) structure). To define the caspase-3-mediated cleavage site in PKC\(\mu\), we constructed a C-terminal (\(\mu\)349–912) PKC\(\mu\) fragment (Fig. 4B). Incubation of \textit{in vitro} translated C-terminal PKC\(\mu\) fragment (m349–912) with purified recombinant caspase-3 resulted in cleavage to a 60-kDa fragment (Fig. 4C). Similar findings were obtained with lysates from ara-C-treated cells but not after incubation of the C-terminal PKC\(\mu\) fragment with control lysates (Fig. 4C). Immunoblot analysis of caspase-3-cleaved \(\mu\)349–912 with an antibody reactive at the C terminus (amino acids 893–912) demonstrated detection of the 60-kDa cleaved fragment (data not shown). These findings indicated that the PKC\(\mu\) cleavage site is located at the N terminus of \(\mu\)349–912. Consequently, to identify the cleavage site in PKC\(\mu\), we generated three PKC\(\mu\) mutants with substitution of Asp residues in DDND 348S, CQND 378S, and DHED 391S by Ala (D348A, D378A, and D391A). Incubation with caspase-3 resulted in cleavage of wild type, D348A and D391A mutants to the predicted size fragment (Fig. 4D). By contrast, there was no detectable caspase-3-mediated cleavage of the D378A mutant. Taken together, these findings demonstrate that PKC\(\mu\) is cleaved by caspase-3 at the CQND378S site (Fig. 4D).

Cleavage of PKC\(\mu\) at the CQND378S Site Is Associated with Separation of the Regulatory and Kinase Domains—To determine whether cleavage of PKC\(\mu\) is associated with activation of the kinase function, we incubated \textit{in vitro} translated PKC\(\mu\)FL with recombinant caspase-3 and assessed activity by phosphorylation of glycogen synthase kinase peptide. The results demonstrate that cleavage of PKC\(\mu\)FL with caspase-3 is associated with increases in the PKC\(\mu\) kinase function (Fig. 5A). By contrast, preincubation of recombinant caspase-3 with p35, which inhibits PKC\(\mu\) cleavage, blocked the increase in kinase activity (Fig. 5A). To define the functional significance of PKC\(\mu\) cleavage, we transfected HeLa cells with PKC\(\mu\)CF cloned into a vector expressing the green fluorescence protein (GFP). GFP-positive transfectants were selected by flow cytometry and assayed for sub-G1 DNA content. There were no apparent effects of PKC\(\mu\)CF expression on growth or apoptosis (data not shown). U-937 cells were also transfected with the PKC\(\mu\)CF cDNA inserted in the pEF-Neo expression plasmid. Cells transfected with pEF-PKC\(\mu\)CF overexpressed PKC\(\mu\)CF protein as compared with cells transfected with vector alone (Fig. 5B). Anti-PKC\(\mu\) immunoprecipitates from U-937/\(\text{neo}\) and U-937/\(\mu\)CF cells were assayed for phosphorylation of the glycogen synthase kinase peptide. The results demonstrate over a 2-fold increase in PKC\(\mu\) activity in U-937/\(\mu\)CF as compared with U-937/\(\text{neo}\) cells (Fig. 5C). Taken together, these findings support activation of PKC\(\mu\) activity by caspase-3-mediated cleavage of PKC\(\mu\)FL to PKC\(\mu\)CF.

Because exposure of cells to genotoxic agents induces cleavage of PKC\(\mu\), we asked if PKC\(\mu\)CF affects the sensitivity of cells to DNA damage-induced apoptosis. The U-937/\(\text{neo}\) and U-937/\(\mu\)CF cells were treated with 100 nm ara-C and assayed for DNA fragmentation. In U-937/\(\text{neo}\) cells, internucleosomal DNA damage-induced apoptosis is associated with the activation of caspase-3 (20, 21). The results indicate that one site in PKC\(\mu\) is sensitive to protease cleavage. Caspase-3 prefers a DXXD-like substrate with an Asp residue at both the P1 and P4 positions (30). PKC\(\mu\) has two DXXD sites between the cysteine-rich and PH domains, either of which can yield cleaved fragments of approximately the same size as those observed on immunoblots (Fig. 4B; schematic of PKC\(\mu\) structure). To define the caspase-3-mediated cleavage site in PKC\(\mu\), we constructed a C-terminal (\(\mu\)349–912) PKC\(\mu\) fragment (Fig. 4B). Incubation of \textit{in vitro} translated C-terminal PKC\(\mu\) fragment (m349–912) with purified recombinant caspase-3 resulted in cleavage to a 60-kDa fragment (Fig. 4C). Similar findings were obtained with lysates from ara-C-treated cells but not after incubation of the C-terminal PKC\(\mu\) fragment with control lysates (Fig. 4C). Immunoblot analysis of caspase-3-cleaved \(\mu\)349–912 with an antibody reactive at the C terminus (amino acids 893–912) demonstrated detection of the 60-kDa cleaved fragment (data not shown). These findings indicated that the PKC\(\mu\) cleavage site is located at the N terminus of \(\mu\)349–912. Consequently, to identify the cleavage site in PKC\(\mu\), we generated three PKC\(\mu\) mutants with substitution of Asp residues in DDND 348S, CQND 378S, and DHED 391S by Ala (D348A, D378A, and D391A). Incubation with caspase-3 resulted in cleavage of wild type, D348A and D391A mutants to the predicted size fragment (Fig. 4D). By contrast, there was no detectable caspase-3-mediated cleavage of the D378A mutant. Taken together, these findings demonstrate that PKC\(\mu\) is cleaved by caspase-3 at the CQND378S site (Fig. 4D).
DNA cleavage was observed at 24 h after ara-C treatment (Fig. 6A). However, exposure of U-937/mCF cells to ara-C resulted in induction of DNA cleavage, which was detectable as early as 6 h (Fig. 6A). To assess whether the expression of PKC\textsubscript{m} sensitizes cells to other genotoxic agents, we treated the transfecants with 20 ng/ml etoposide or 10 \textmu M cisplatin and assayed for DNA fragmentation. Although there was no apparent effect of etoposide or cisplatin on U-937/neo cells at 14 h, these agents induced characteristic DNA ladders in U-937/mCF cells (Fig. 6B). Apoptosis was also monitored by analyzing cells for sub-G\textsubscript{1} DNA content. Treatment of U-937/mCF cells with ara-C, cisplatin, or etoposide was associated with increases in the percentage of cells with sub-G\textsubscript{1} DNA as compared with that found following transfection of empty vector (Table I). Similar results were obtained in another cell population, designated U-937/mCF\textsuperscript{-1}, which expresses a lower level of PKC\textsubscript{m} and kinase activity (Table I and data not shown). To define further the role of PKC\textsubscript{m} in apoptosis, we transfected HeLa cells with wild type PKC\textsubscript{m} or the caspase-3-resistant PKC\textsubscript{m}D378A mutant and vector expressing GFP. After 24 h, the transfected cells were exposed to cisplatin and incubated for additional 14 h. GFP-positive cells were then analyzed for sub-G\textsubscript{1} DNA content. Compared with cells transfected with wild type PKC\textsubscript{m}, overexpression of PKC\textsubscript{m}D378A partially inhibited cisplatin-induced apoptosis (Fig. 6C). Taken together, these results indicate that the cleavage of PKC\textsubscript{m} contributes to DNA damage-induced apoptosis and that PKC\textsubscript{m}CF expression sensitizes cells to the apoptotic effects of diverse genotoxic drugs.

**DISCUSSION**

PKC isoforms function in signal transduction pathways that regulate cell growth, differentiation, and apoptosis (1, 31). The classic, novel, and atypical PKCs all possess a highly conserved catalytic domain (1). The catalytic domains of PKC\textsubscript{m} and its mouse homologue PKD, however, exhibit little similarity to the other PKC family members (2, 3). PKC\textsubscript{m} and PKD also exhibit a distinct substrate specificity (13–15). Nonetheless, PKC\textsubscript{m} contains a tandem-repeat of cysteine-rich, zinc-finger-like motifs that bind phorbol esters (2). In addition, PKC\textsubscript{m}, like members of the PKC family, is activated by phorbol esters and phospholipids (14, 32). The activity of most PKC family members is controlled by a pseudosubstrate region in the regulatory...
domain that functions as an inhibitor of the active site in the catalytic domain (1). PKC_\mu/PKD, however, lacks the typical pseudosubstrate region, and in contrast to the other PKC isoforms, contains a PH domain (2, 3). Diverse signaling and cytoskeletal proteins contain PH domains that regulate their subcellular localization and activation (33). Studies of PKC_\mu have shown that mutants with deletions or amino acid substitutions in the PH domain exhibit increased basal kinase activity (34). These findings have indicated that the PH domain functions as a negative regulator of the catalytic domain. The present studies demonstrate that PKC_\mu is cleaved between the cysteine-rich and PH domains during induction of apoptosis. The results also demonstrate that the C-terminal cleavage product, which contains the catalytic domain, exhibits an increased basal kinase activity. Together, these results suggest that cleavage in this region relieves the inhibitory effects of PH domain on the catalytic function.

Previous work has demonstrated that the PKC_\delta and PKC_\theta, but not the classic or atypical, isoforms are cleaved in apoptotic cells (9, 11). The Ca^{2+}-dependent classic PKCs contain the conserved regulatory regions C1 and C2, whereas the Ca^{2+}-independent novel PKCs, including PKC_\delta and PKC_\theta, lack the C2 domain. Cleavage of the classic PKCs in the third variable (V3) region by calpains I and II deletes the C1 and C2 regulatory regions and results in catalytically active fragments (35). By analogy, cleavage of PKC_\delta in the V3 region by the caspase-3 cysteine protease deletes the C1 regulatory region and releases an active catalytic fragment (10). Similar findings have been reported for caspase-3-mediated cleavage of the PKC_\theta V3 region (11). The present results demonstrate that PKC_\alpha is cleaved in cells induced to undergo apoptosis by ara-C and other genotoxic agents. The finding that expression of the baculovirus p35 protein blocks cleavage of PKC_\mu supported involvement of a cysteine protease. In addition, the demonstration that CrmA expression had no effect on PKC_\mu cleavage indicated lack of involvement of a caspase-1-like protease. In this context, previous work has demonstrated that DNA damage-induced apoptosis is mediated by the CrmA-insensitive, p35-sensitive pathway (20, 21). We also found that overexpression of the anti-apoptotic Bcl-x\_\text{L} protein blocks PKC_\mu cleavage. Bcl-x\_\text{L} inhibits cytochrome c release from mitochondria in response to genotoxic stress (28). Cytochrome c activates caspase-9 by an Apaf-1-dependent mechanism and thereby activation of caspase-3 (36). Taken together, our findings in cells treated with genotoxic agents suggested that PKC_\alpha, like PKC_\delta and PKC_\theta, is cleaved by a caspase-3-dependent mechanism.

Caspases have an absolute requirement for an Asp residue at the P1 position in their substrates. Moreover, caspase-3 prefers an Asp residue at both the P1 and P4 positions and cleaves most known substrates at DXXD motifs (30). Previous studies have shown that PKC_\delta and PKC_\theta are cleaved by caspase-3 at DMQD^{350/351}N and DEVD^{355/356}K, respectively (10, 11). Based on these findings, we predicted that PKC_\mu would be cleaved by caspase-3 at one or both of the two consensus DXXD sites (DDND^{344/345}S and DHED^{393/394}S). However, mutation of Asp residues at the P1 positions and incubation of mutant proteins with caspase-3 revealed that PKC_\mu is not cleaved at these two consensus DXXD motifs. Subsequent site-directed mutagenesis studies showed that caspase-3 cleaves PKC_\mu at an unconventional CQND^{377/378}S site. In concert with these results, recent studies have demonstrated caspase-3-mediated cleavage of other proteins, such as DNA topoisomerase I, amyloid-\beta-precursor protein, and p21-activated protein kinase, also occurs at unconventional sites (37–39). The present findings thus demonstrate that, despite the presence of two DXXD motifs, PKC_\mu is cleaved by caspase-3 at the CQND^{377/378}S site.

The available evidence indicates that PKC_\mu is involved in diverse cellular events. In B cells, PKC_\mu activity is up-regulated after cross-linking of CD19 with the B cell receptor complex (40). PKC_\mu associates with the Syk tyrosine kinase, phospholipase C\_\gamma (40), type II phosphatidylinositol 4-kinase and type I phosphatidylinositol-4-phosphate 5-kinase (15). Other studies have demonstrated that PKC_\mu is negatively regulated by the 14-3-3 signaling protein (17). The findings that mouse PKC_\mu localizes to Golgi\_\text{L} and functions downstream of the \(\beta\_\gamma\) subunits of heterotrimeric G proteins have also suggested that PKC_\mu is involved in protein secretion (16, 41). The present results demonstrate that PKC_\mu is cleaved in the apoptotic response to genotoxic stress. The functional significance of PKC_\mu cleavage is supported by the demonstration that cells expressing the PKC_\mu catalytic fragment are more sensitive to the apoptotic effects of genotoxic agents. Studies in cells ex-

**TABLE I**

Effects of PKC_\mu CF overexpression on DNA damage-induced apoptosis

Cells were treated with 100 nm ara-C, 10 \(\mu\)M cisplatin, or 20 ng/ml etoposide (ETOPO) for 14 h. DNA was isolated and analyzed for fragmentation in agarose gels. C, HeLa cells were transiently transfected with wild type (WT) or the caspase-3-resistant PKC_\mu(D377A) mutant and vector expressing GFP. At 24 h post-transfection, the cells were treated with 10 \(\mu\)M cisplatin and incubated for 14 h. The GFP-positive cells were analyzed for DNA content by flow cytometry. The results (mean \pm S.D. of two independent experiments, each performed in triplicate) are presented as percentage of apoptotic cells with sub-G1 DNA content. Values obtained were significant at \(p < 0.01\) in a Student’s \(t\) test.

| Cell type          | Untreated | ara-C | Cisplatin | Etoposide |
|--------------------|-----------|-------|-----------|-----------|
| U-937              | 4.3 \(\pm\) 0.4 | 8.1 \(\pm\) 0.2 | 12.8 \(\pm\) 1.4 | 8.5 \(\pm\) 1.2 |
| U-937/\mu CF       | 5.4 \(\pm\) 0.8 | 15.8 \(\pm\) 1.2 | 52.9 \(\pm\) 5.6 | 29.8 \(\pm\) 2.1 |
| U-937/\mu CF-1     | 4.9 \(\pm\) 0.3 | 12.5 \(\pm\) 0.7 | 27.2 \(\pm\) 1.7 | 23.5 \(\pm\) 3.6 |

**FIG. 6. Role of PKC_\mu cleavage in the apoptotic response to DNA damage.** A, U-937 and U-937/\mu CF cells were exposed to 100 nm ara-C for indicated times or B, 10 \(\mu\)M cisplatin (CDDP) or 20 ng/ml etoposide (ETOPO) for 14 h. DNA was isolated and analyzed for fragmentation by agarose gels. C, HeLa cells were transiently transfected with wild type (WT) or the caspase-3-resistant PKC_\mu(D377A) mutant and vector expressing GFP. At 24 h post-transfection, the cells were treated with 10 \(\mu\)M cisplatin and incubated for 14 h. The GFP-positive cells were analyzed for DNA content by flow cytometry. The results (mean \pm S.D. of two independent experiments, each performed in triplicate) are presented as percentage of apoptotic cells with sub-G1 DNA content. Values obtained were significant at \(p < 0.01\) in a Student’s \(t\) test.
pressing full-length PKC\(\mu\) have demonstrated that TNF-induced apoptosis is inhibited by enhanced expression of NF-\(\kappa\)B-dependent protective genes, including the inhibitor of apoptosis protein 2 (18). These findings in cells expressing full length PKC\(\mu\) or the catalytic fragment suggest that caspase-3-mediated cleavage of PKC\(\mu\) reverses a protective function and confers sensitivity to an apoptotic response. In studies of PKC\(\delta\) and PKC\(\theta\), cleavage by caspase-3 results in the release of catalytic fragments that contribute to induction of apoptosis (10, 11). By contrast, although cleavage of PKC\(\mu\) is not sufficient to induce apoptosis, our findings indicate that expression of PKC\(\mu\)-C-terminal domains that are active in the absence of lipid second messengers.

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Proteolytic Activation of PKC\(\mu\) in Apoptosis

18481
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J. Biol. Chem. 2000, 275:18476-18481.
doi: 10.1074/jbc.M002266200 originally published online April 6, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002266200

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