

Proteolytic Activation of Protein Kinase C δ by an ICE/CED 3-like Protease Induces Characteristics of Apoptosis

By Tariq Ghayur,* Margaret Hugunin,* Robert V. Talanian,* Sheldon Ratnofsky,* Christopher Quinlan,* Yutaka Emoto,† Pramod Pandey,‡ Rakesh Datta,‡ Yinyin Huang,‡ Surender Kharbanda,‡ Hamish Allen,* Robert Kamen,* Winnie Wong,* and Donald Kufe‡

From *BASF Bioresarch Corporation, Worcester, Massachusetts 01605; and †Division of Cancer Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Summary

Recent studies have shown that protein kinase C (PKC) δ is proteolytically activated at the onset of apoptosis induced by DNA-damaging agents, tumor necrosis factor, and anti-Fas antibody. However, the relationship of PKC δ cleavage to induction of apoptosis is unknown. The present studies demonstrate that full-length PKC δ is cleaved at DMQD₃₃₀N to a catalytically active fragment by the cysteine protease CPP32. The results also demonstrate that overexpression of the catalytic kinase fragment in cells is associated with chromatin condensation, nuclear fragmentation, induction of sub-G1 phase DNA and lethality. By contrast, overexpression of full-length PKC δ or a kinase inactive PKC δ fragment had no detectable effect. The findings suggest that proteolytic activation of PKC δ by a CPP32-like protease contributes to phenotypic changes associated with apoptosis.

The protein kinase C (PKC) family consists of multiple subspecies that possess a conserved catalytic domain. The classic or group A isoforms (α , β , and γ) require Ca^{2+} for activity and contain cysteine-rich motifs that confer phospholipid-dependent binding of diacylglycerol (1). The group A PKCs are cleaved at the third variable region (V_3) by the neutral proteases, calpains I and II, to catalytically active fragments (2). Recent studies have demonstrated that the Ca^{2+} -independent δ isoform, and not the group A PKCs, is selectively cleaved at V_3 to a catalytically active fragment in cells induced to undergo apoptosis (3, 4). Inhibition of apoptosis by overexpression of Bcl-2 or Bcl-x_L is associated with a block of PKC δ cleavage (3, 4). The finding that PKC δ is cleaved at a site (DMQD/N) adjacent to aspartic acid has supported the potential involvement of aspartate-specific cysteine proteases which are known to be activated during apoptosis.

The nematode Ced-3 cysteine protease is related to the mammalian interleukin-1 β converting enzyme (ICE) (5, 6). The demonstration that overexpression of Ced-3 or ICE induces apoptosis has provided support for involvement of these cysteine proteases in cell death pathways (7). ICE/Ced-3 family members include Nedd2/Ich-1, CPP32/YAMA/apopain, Tx/Ich-2/ICE_{rel}II, ICE_{rel}III, Mch2, Mch3/ICE-LAP3/CMH-1 (reviewed in reference 8), ICE-LAP6 (9), FLICE/Mch5 (10, 11), and Mch4 (11). ICE cleaves the precursor of IL-1 β to the active cytokine (6, 12, 13). Other known substrates of the ICE/Ced-3 family include:

(a) poly (ADP-ribose) polymerase (PARP) which is cleaved by CPP32, Mch3 and Ced-3, but not ICE (14-16); and (b) DNA-dependent protein kinase (DNA-PK), the U1 small nuclear ribonucleoprotein and D4-GDP dissociation inhibitor for the Rho family GTPases (D4-GDI), which are cleaved by CPP32 (17, 18). However, the functional role of these cleavage products in the induction of apoptosis is unclear.

The present results demonstrate that PKC δ is cleaved by CPP32 and not certain other ICE/Ced-3 family members. We also demonstrate that overexpression of the PKC δ catalytic fragment is involved in the induction of phenotypic changes that are characteristic of apoptosis.

Materials and Methods

In Vitro Cleavage of PKC δ and PARP. The full-length PKC δ cDNA was cloned into the SpeI and BamHI sites of a modified pSV β plasmid (Clontech, Palo Alto, CA). PKC δ (D327A/D330A) was generated in two steps by overlapping primer extension. PARP cDNA was generated by PCR cloning. The proteins were labeled with [³⁵S]methionine by coupled transcription and translation reactions (Promega, Madison, WI). Labeled proteins were incubated with 5 $\mu\text{g}/\text{ml}$ *Escherichia coli*-derived CPP32 β in 50 mM Hepes (pH 7.5), 10% glycerol, 2.5 mM DTT, and 0.25 mM EDTA at room temperature for 30 min. The reaction products were analyzed by electrophoresis in 10–20% SDS–polyacrylamide gels and then autoradiography. For the kinase assays, full-length PKC δ , PKC δ (D327A/D330A), PKC δ catalytic fragment (CF),

Table 1. CPP32 Proteolysis of Peptides Spanning the PARP, PKC δ , and IL-1 β Cleavage Sites

Substrate	Sequence	Relative V_{max}/K_m
PARP	Ac-WGDEVD ₂₁₆ -GVDEVW-NH ₂	1.00
PKC δ	Ac-GEDMQD ₃₃₀ NSGTYW-NH ₂	0.42
IL-1 β	Ac-NEAYVHD ₁₁₆ APVRSLY-NH ₂	0.00

and PKC δ CF(K-R) were prepared by coupled transcription and translation. PKC δ and PKC δ (D327A/D330A) were incubated with 5 μ g/ml CPP32 β at room temperature for 30 min. Protein kinase assays using MBP as a substrate were performed as described (PKC Assay Kit; GIBCO BRL, Gaithersburg, MD).

Analysis of Peptide Proteolysis. Peptides were synthesized and purified to \sim 95% by standard methods and confirmed by mass spectrometry. Reaction mixtures (810 μ l) contained: 100 mM Hepes (pH 7.5), 20% (vol/vol) glycerol, 5 μ M dithiothreitol, 0.5 mM EDTA, and 380 ng *N*-His CPP32 (19). Peptide substrates were added to final concentrations of 10 μ M. The reaction mixtures were incubated at 30°C. Aliquots were removed at 10 min intervals for 60 min and added to vials containing 3 M HCl to stop the reactions. The amount of substrate remaining at each time was quantitated by reverse phase HPLC. Data were fit to the equation $(S_t/S_0) = e^{-kt}$, where k is the decay rate constant equal to V_{max}/K_m . Observed V_{max}/K_m values were normalized to 1.00 for the PARP peptide.

Cell Transfections. Cells were seeded at a density of 1.7×10^5 in each well of 6-well dishes 24 h before transfection. For each well, 2 μ g DNA construct and 0.5 μ g pSv β plasmid containing β -gal were coprecipitated with calcium phosphate. Cells were incubated with the coprecipitate for 30 h at 37°C and then analyzed by X-gal staining. Cells (1.7×10^5 /well) were also transfected with 2 μ g DNA construct for 30 h at 37°C, fixed with 4% paraformaldehyde, postfixed with 5% acetic acid in ethanol and then stained with 5 μ g/ml Hoechst dye. For sub-G1 DNA content, cells transfected by lipofectamine were stained with propidium iodide and monitored by FACScan[®]. Chromatin condensation was assessed by staining with acridine orange and ethidium bromide (20).

Results and Discussion

To determine whether PKC δ is cleaved by one of the known ICE-like proteases, full-length 78-kD PKC δ labeled with [³⁵S]methionine was incubated with purified recombinant proteases. Cleavage of PKC δ to a 40-kD fragment was observed with purified CPP32 β (14) (Fig. 1 A). In contrast, ICE failed to cleave PKC δ at concentrations up to 600 U/ μ l (3). The related Ich-1, Ich-2, Mch2, Mch3, and ICE_{rel}III proteases also failed to cleave PKC δ (data not shown). Because PKC δ is cleaved at DMQD₃₃₀N in vivo (3, 4), we asked whether this site is responsible for CPP32-mediated cleavage in vitro. CPP32 may prefer peptidic substrates with aspartic acid at the P1 and P4 positions (15). Consequently, we prepared a PKC δ mutant with substitution of D327A and D330A. Incubation with CPP32 resulted in no detectable CPP32-mediated cleavage of this

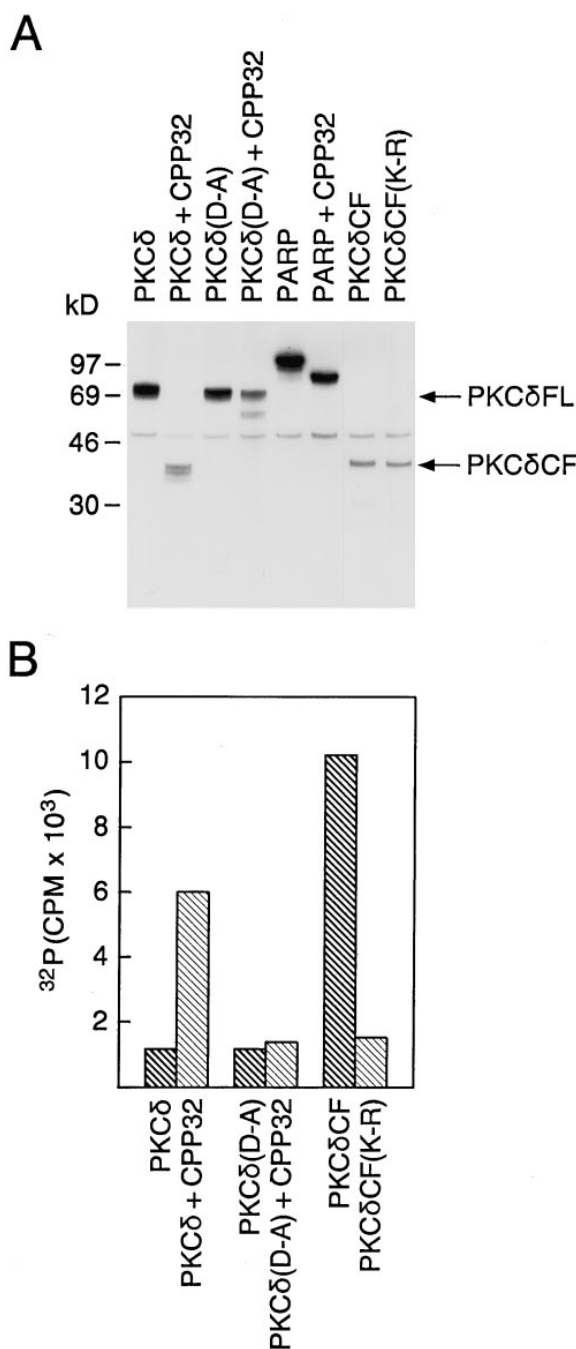


Figure 1. PKC δ is proteolytically activated by CPP32 in vitro. (A) PKC δ (full-length: FL), PKC δ (D327A/D330A) and PARP were labeled with [³⁵S]methionine and incubated with recombinant CPP32 β . The reaction products were analyzed by SDS-PAGE and autoradiography. The kinase active PKC δ catalytic fragment (CF) and the kinase inactive PKC δ CF(K-R) were labeled with [³⁵S]methionine and analyzed under similar conditions. (B) Recombinant PKC δ and PKC δ (D327A/D330A) were incubated with CPP32 and then assayed for protein kinase activity using MBP as substrate.

mutant to the 40-kD catalytic fragment, while there was partial digestion to a species of \sim 55 kD (not observed with wild-type substrate) (Fig. 1 A). Recombinant CPP32 also cleaved the 116-kD full-length PARP to the predicted 85-kD fragment (14, 15) (Fig. 1 A). Using peptides derived

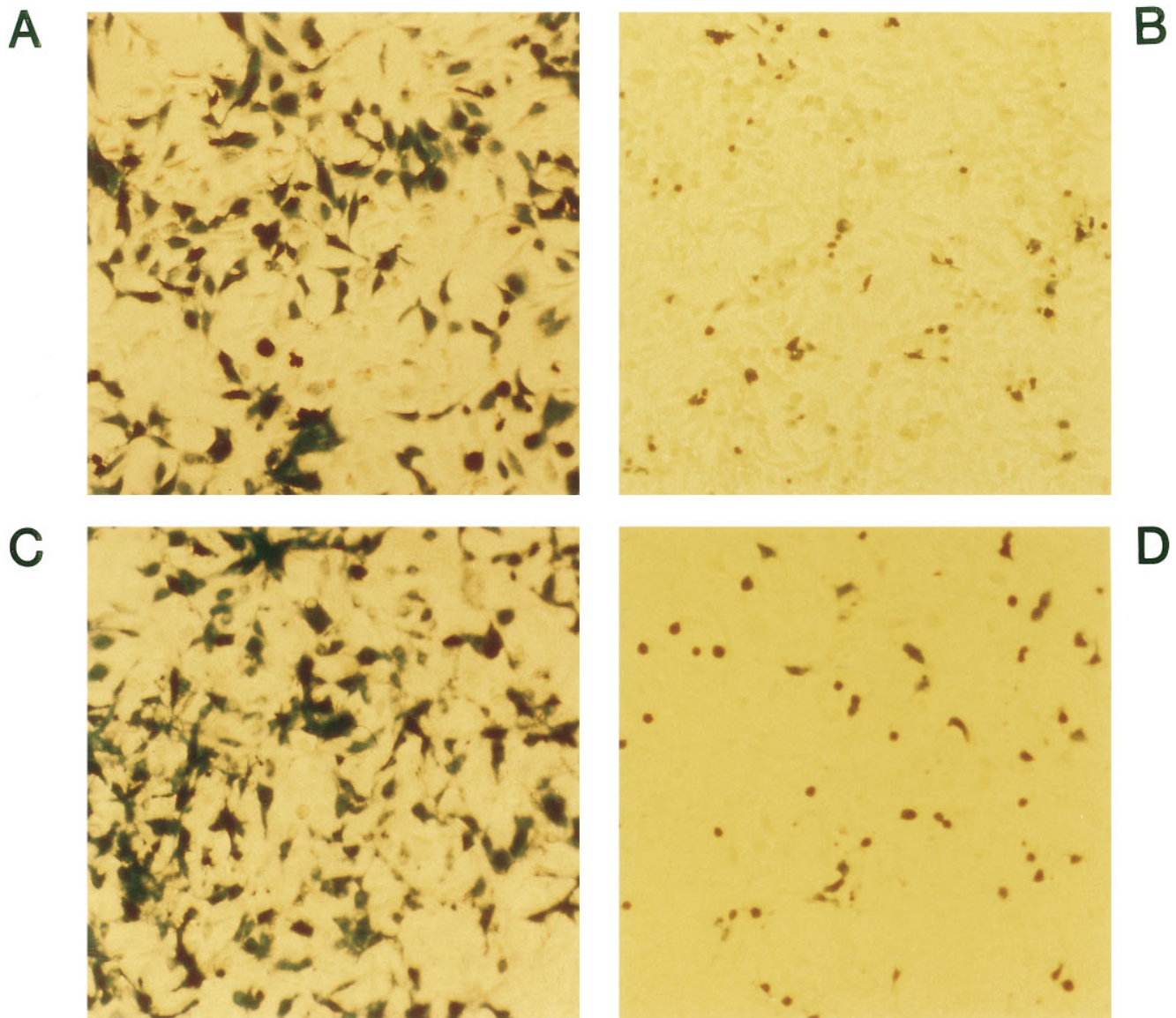


Figure 2. Transfection of the PKC δ catalytic fragment (CF) induces morphologic changes characteristic of apoptosis. HeLa (*upper panels*) and NIH3T3 (*lower panels*) cells were cotransfected with pSv β - β -gal and vectors expressing: (*A* and *C*) kinase inactive PKC δ CF(K-R) and (*B* and *D*) kinase active PKC δ CF. Transfection was determined by X-gal staining and apoptotic cells were identified by their condensed morphology.

from the cleavage sites of PARP and PKC δ in proteolytic assays, we found that CPP32 cleaves both substrates and not a peptide spanning the IL-1 β maturation site (Table 1). These findings confirm that PKC δ , like PARP, is a substrate for CPP32.

We also asked whether cleavage of PKC δ by CPP32 is associated with activation of the kinase function. Full-length PKC δ exhibited a low level of myelin basic protein (MBP) phosphorylation, while incubation with CPP32 resulted in a greater than sixfold increase in kinase activity (Fig. 1 *B*). In contrast, CPP32 had no detectable effect on kinase function of the PKC δ (D327A/D330A) mutant (Fig. 1 *B*). A recombinant 40-kD CF of PKC δ (amino acids 331-676) exhibited constitutive kinase activity, while a mutant of the fragment with K-378 in the ATP binding site mutated to R (K378R; designated K-R) yielded background

levels of MBP phosphorylation found with control bacterial lysates (Figs. 1, *A* and *B*). These findings collectively demonstrate that CPP32-mediated cleavage of the DMQD₃₃₀N site activates PKC δ .

To study the role of PKC δ in apoptosis, we used the transient HeLa cell transfection system previously found to demonstrate induction of apoptosis by ICE-like proteases (7). Cotransfection of the kinase inactive PKC δ CF(K-R) mutant with the β -galactosidase (β -gal) marker gene had little effect on HeLa cell morphology (Fig. 2 *A*). Most of the blue X-gal positive cells remained flat and attached to the dish (Fig. 2 *A*). Cotransfection of the kinase active PKC δ CF and β -gal resulted in condensed, small blue cells (Fig. 2 *B*), consistent with the induction of apoptosis (7). Similar findings were obtained with NIH3T3 cells (Figs. 2, *C* and *D*). Overexpression of PKC δ in both cell types also

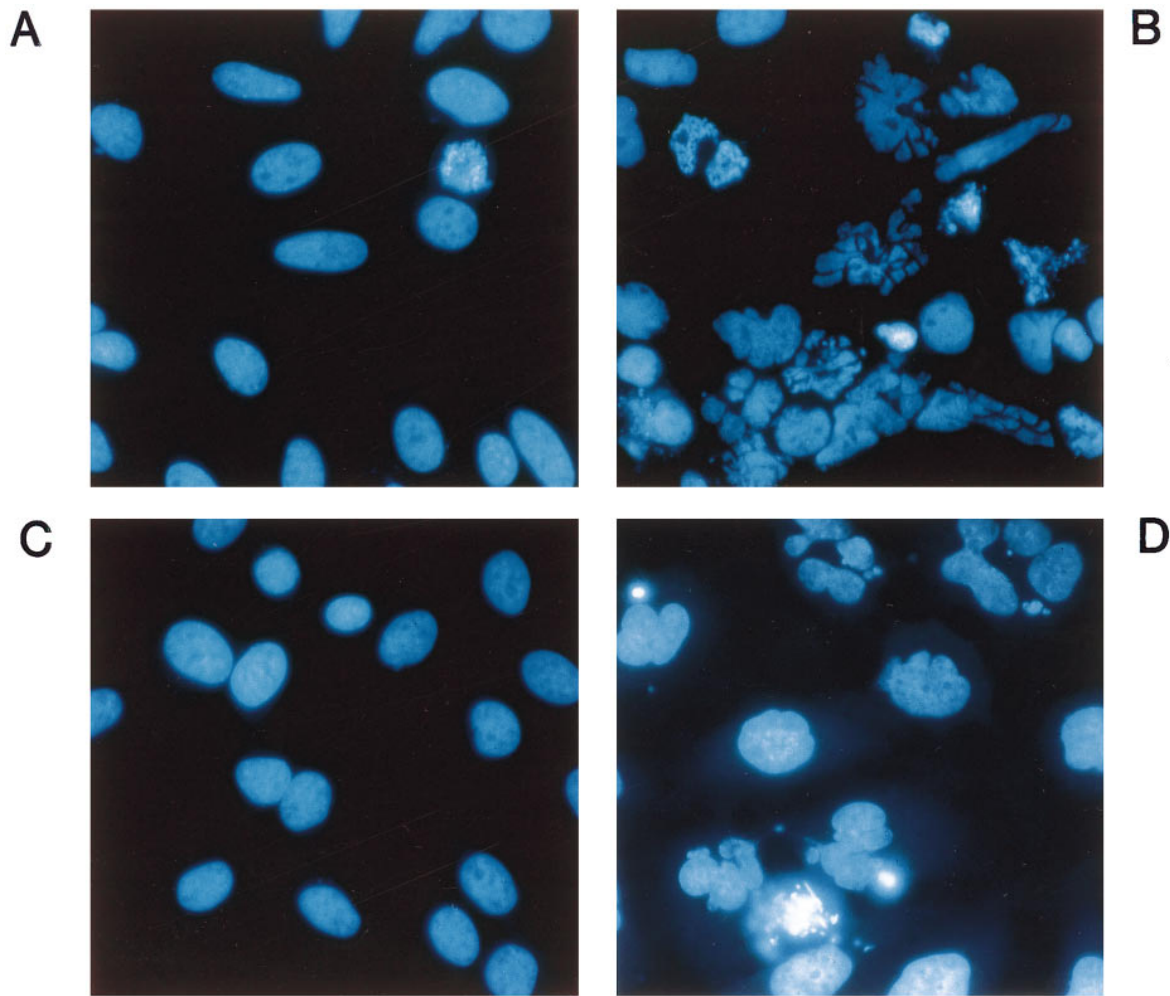
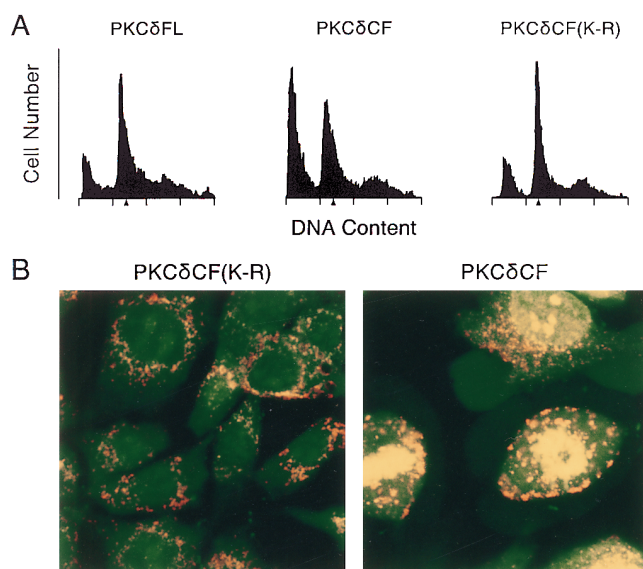


Figure 3. Expression of PKC δ CF results in nuclear fragmentation. HeLa cells were transfected with vectors that express: (A) full-length PKC δ ; (B) kinase active PKC δ CF; and (C) kinase inactive PKC δ CF(K-R). (D) Cells were exposed to 2 μ M ara-C. The cells were fixed with paraformaldehyde and then stained with Hoechst dye. Cotransfection of PKC δ FL, PKC δ CF and PKC δ CF(K-R) with pSv β - β -gal demonstrated transfection efficiencies of 50, 42, and 43%, respectively. Percentage of apoptotic cells for the PKC δ FL, PKC δ CF, and PKC δ CF(K-R) transfected populations was 2, 26, and 4%, respectively. Bar, 15 μ M.



resulted in detachment of non-viable cells into the culture medium.

Hoechst staining of HeLa cells transfected with a vector that expresses full-length PKC δ had no detectable changes in nuclear morphology (Fig. 3 A), but overexpression of PKC δ CF resulted in fragmented nuclei (Fig. 3 B). Transfection of kinase inactive PKC δ CF(K-R) was associated with a normal nuclear morphology (Fig. 3 C). The changes observed with expression of the PKC δ CF were also compared to those found upon exposure to 1- β -D-arabinofura-

Figure 4. Overexpression of PKC δ CF induces sub-G1 DNA and chromatin condensation. (A) HeLa cells were transfected with PKC δ FL, PKC δ CF, or PKC δ CF(K-R). Cells were assessed for DNA content by flow cytometry at 48 h after transfection. The small triangle denotes G₀/G₁ DNA. (B) HeLa cells transfected with PKC δ CF(K-R) (left) and PKC δ CF (right) were assessed for chromatin condensation after staining with acridine orange and ethidium bromide.

nosylcytosine (ara-C), a DNA-damaging agent that induces proteolytic cleavage of PKC δ and apoptosis (4). Treatment of HeLa cells with ara-C resulted in a similar pattern of nuclear fragmentation (Fig. 3 D).

To confirm that the nuclear changes induced by PKC δ CF are associated with induction of apoptosis, we assessed the effects of transfection on the appearance of HeLa cells with sub-G1 DNA content. Transfection of the empty vector, full-length PKC δ or PKC δ CF(K-R) resulted in 10–15% of cells with sub-G1 DNA (Fig. 4 A and data not shown). By contrast, transfection of PKC δ CF was associated with 30–35% of cells with sub-G1 DNA (Fig. 4 A). Cells were also stained with acridine orange and ethidium bromide to assess chromatin condensation (20). Transfection of PKC δ CF, but not PKC δ CF(K-R), resulted in the appearance of bright yellow-green nuclear staining of condensed chromatin (Fig. 4 B).

To quantify the effects of PKC δ CF expression on cell viability, we cotransfected PKC δ CF or PKC δ CF(K-R) and the green fluorescence gene (Clontech) into HeLa cells. Positive transfectants were selected by flow cytometry, reseeded in culture medium and assayed at 24 h for viability by trypan blue exclusion. Less than 5% of the PKC δ CF transfectants were viable, while over 90% of the kinase inactive PKC δ CF(K-R) transfectants were viable and attached to the dish. Viability of 90–95% was observed after transfection of the null vector and sorting. We conclude

that the kinase active catalytic domain of PKC δ induces characteristics typical of cells undergoing apoptosis: (a) size reduction and round morphology; (b) nuclear fragmentation; (c) chromatin condensation; (d) sub-G1 DNA content; and (e) detachment and loss of viability.

Multiple events that lead to destruction of nuclear and cytoplasmic integrity are probably required for apoptosis. Activation of ICE-family proteases may be a central trigger, resulting in the cleavage of substrates such as PARP (21), lamin B1 (22, 23), topoisomerase 1 (23), D4-GDI (18), DNA-PK, and the U1 small nuclear ribonucleoprotein (17). PKC δ , but not PKC α , β , ϵ , or ζ , is also cleaved at the onset of apoptosis (3, 4). Little is known about the physiological function of PKC δ (24, 25). We demonstrate that PKC δ is cleaved by CPP32 and not other ICE/Ced-3 family members in vitro. The results also demonstrate that expression of the PKC δ catalytic fragment induces morphologic changes characteristic of apoptosis. We propose that the proteolytic cleavage of PKC δ is a key mediator of nuclear fragmentation and cell death, and not a bystander effect of protease activation. Moreover, the finding that proteolytic activation of PKC δ is blocked by Bcl-2 and Bcl-x_L suggests that these anti-apoptotic proteins act upstream to this event (3). Elucidation of the substrates phosphorylated as a consequence of PKC δ cleavage should provide insights into the pathways activated by the catalytic fragment.

This investigation was supported by Public Health Service grants CA66996, CA55241, and CA29431 awarded by the National Cancer Institute, DHHS.

Address correspondence to Donald W. Kufe, Division of Cancer Pharmacology, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

Received for publication 13 August 1996 and in revised form 8 October 1996.

References

1. Nishizuka, Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Wash. DC)*. 258:607–614.
2. Kishimoto, A. 1990. Limited proteolysis of protein kinase C by calpain, its possible implication. In *The Biology and Medicine of Signal Transduction*. Y. Nishizuka, M. Endo, and C. Tanaka, editors. Raven Press, New York. 472–477.
3. Emoto, Y., G. Manome, G. Meinhardt, H. Kasaki, S. Kharbanda, M. Robertson, T. Ghayur, W.W. Wong, R. Kamen, R. Weichselbaum, and D. Kufe. 1995. Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:6148–6156.
4. Emoto, Y., H. Kasaki, Y. Manome, S. Kharbanda, and D. Kufe. 1996. Activation of protein kinase C δ in human myeloid leukemia cells treated with 1- β -D-arabinofuranosylcytosine. *Blood*. 87:1990–1996.
5. Hugunin, M., L.J. Quintal, J.A. Mankovich, and T. Ghayur. 1996. Protease activity of in vitro transcribed and translated *Caenorhabditis elegans* cell death gene (ced-3) product. *J. Biol. Chem.* 271:3517–3522.
6. Yuan, J., S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz. 1993. The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 β -converting enzyme. *Cell*. 75:641–752.
7. Miura, M., H. Zhu, R. Rotello, E.A. Hartweig, and J. Yuan. 1993. Induction of apoptosis in fibroblasts by IL-1 β -converting enzyme, a mammalian homolog of the *C. elegans* cell death gene ced-3. *Cell*. 75:653–660.
8. Nicholson, D.W. 1996. ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. *Nat. Biotechnol.* 14:297–301.
9. Duan, H., K. Orth, A.M. Chinnaiyan, G.G. Poirier, C.J. Froelich, W.-W. He, and V.M. Dixit. 1996. ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J. Biol. Chem.* 271:16720–16724.
10. Muzio, M., A.M. Chinnaiyan, F.C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J.D. Bretz, M. Zhang, R. Gentz et al. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell*. 85:817–827.
11. Fernandes-Alnemri, T., R.C. Armstrong, J. Krebs, S.M.

- Srinivasula, L. Wang, F. Bullrich, L. Fritz, J.A. Trapani, K.J. Tomaselli, G. Litwack, and E.S. Alnemri. 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. USA*. 93:7464–7469.
12. Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, K.V. Ness, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L.A. Canizzaro et al. 1992. Molecular cloning of the interleukin-1 β -converting enzyme. *Science (Wash. DC)*. 256: 97–100.
 13. Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, D.K. Miller, S.M. Molineaux, J.R. Weidner, J. Aunins et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes. *Nature (Lond.)*. 356:768–774.
 14. Tewari, M., L.T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D.R. Beidler, G.G. Poirier, G.S. Salvesen, and V.M. Dixit. 1995. Yama/ CPP32 β , a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell*. 81:801–809.
 15. Nicholson, D.W., A. Ali, N.A. Thornberry, J.P. Vaillancourt, C.K. Ding, M. Gallant, Y. Gareau, P.R. Griffin, M. Labelle, Y.A. Lazebnik et al. 1995. Identification and apoptosis. *Nature (Lond.)*. 376:37–43.
 16. Fernandes-Alnemri, T., A. Takahashi, R. Armstrong, J. Krebs, L. Fritz, K.J. Tomaselli, L. Wang, Z. Yu, C.M. Croce, G. Salveson, W.C. Earnshaw, G. Litwack, and E.S. Alnemri. 1995. Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res*. 55:6045–6052.
 17. Casciola-Rosen, L., D.W. Nicholson, T. Chong, K.R. Rowan, N.A. Thornberry, D.K. Miller, and A. Rosen. 1996. Apopain/ CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. *J. Exp. Med*. 183:1957–1964.
 18. Na, S., T.-H. Chuang, A. Cunningham, T.G. Turi, J.H. Hanke, G.M. Bokoch, and D.E. Danley. 1996. D4-GDI, a substrate of CPP32, is proteolyzed during Fas-induced apoptosis. *J. Biol. Chem*. 271:11209–11213.
 19. Bump, N.J., M. Hackett, M. Hugunin, S. Seshagiri, K. Brady, P. Chen, C. Ferenz, S. Franklin, T. Ghayur, P. Li et al. 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. *Science (Wash. DC)*. 269:1885–1888.
 20. McGahon, A.J., S.J. Martin, R.P. Bissonnette, A. Mahboubi, Y. Shi, R.J. Mogli, W.K. Nishioka, and D.R. Green. 1995. The end of the (cell) line: methods for the study of apoptosis in vitro. *Methods Cell Biol*. 46:153–185.
 21. Kaufmann, S.H., S. Desnoyers, Y. Ottaviano, N.E. Davidson, and G.G. Poirier. 1993. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res*. 53:3976–3985.
 22. Neamati, N., A. Fernandez, S. Wright, J. Kiefer, and D.J. McConkey. 1995. Degradation of lamin B1 precedes oligonucleosomal DNA fragmentation in apoptotic thymocytes and isolated thymocyte nuclei. *J. Immunol*. 154:3788–3795.
 23. Voelkel-Johnson, C., A.J. Entingh, W.S.M. Wold, L.R. Gooding, and S.M. Laster. 1995. Activation of intracellular proteases is an early event in TNF-induced apoptosis. *J. Immunol*. 154:1707–1716.
 24. Watanabe, T., Y. Ono, Y. Taniyama, K. Hazama, K. Igarashi, K. Ogita, U. Kikkawa, and Y. Nishizuka. 1992. Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C- δ subspecies. *Proc. Natl. Acad. Sci. USA*. 89:10159–10163.
 25. Mischak, H., J. Goodnight, W. Kolch, G. Martiny-Baron, C. Schaechtle, M.G. Kazanietz, P.M. Blumberg, J.H. Pierce, and J.F. Mushinski. 1993. Overexpression of protein kinase c- δ and - ϵ in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. *J. Biol. Chem*. 268:6090–6096.