Induction of Apoptosis Is Driven by Nuclear Retention of Protein Kinase Cδ*

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Protein kinase Cδ (PKCδ) mediates apoptosis downstream of many apoptotic stimuli. Because of its ubiquitous expression, tight regulation of the proapoptotic function of PKCδ is critical for cell survival. Full-length PKCδ is found in all cells, whereas the catalytic fragment of PKCδ, generated by caspase cleavage, is only present in cells undergoing apoptosis. Here we show that full-length PKCδ transiently accumulates in the nucleus in response to etoposide and that nuclear translocation precedes caspase cleavage of PKCδ. Nuclear PKCδ is either cleaved by caspase 3, resulting in accumulation of the catalytic fragment in the nucleus, or rapidly exported by a Crm1-sensitive pathway, thereby assuring that sustained nuclear accumulation of PKCδ is coupled to caspase activation. Nuclear accumulation of PKCδ is necessary for caspase cleavage, as mutants of PKCδ that do not translocate to the nucleus are not cleaved. However, caspase cleavage of PKCδ per se is not required for apoptosis, as an uncleavable form of PKCδ induces apoptosis when retained in the nucleus by the addition of an SV-40 nuclear localization signal. Finally, we show that kinase negative full-length PKCδ does not translocate to the nucleus in apoptotic cells but instead inhibits apoptosis by blocking nuclear import of endogenous PKCδ. These studies demonstrate that generation of the PKCδ catalytic fragment is a critical step for commitment to apoptosis and that nuclear import and export of PKCδ plays a key role in regulating the survival/death pathway.

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‡ The abbreviations used are: PKC, protein kinase C; δFL, PKCδ full-length; δCF, PKCδ catalytic fragment; δKN, kinase-negative full-length PKCδ; TUNEL, terminal deoxynucleotidyltransferase nick end labeling; zVAD-FMK, Z-Val-Ala-Asp(O-methyl)-CH₂F; NLS, nuclear localization signal; GFP, green fluorescent protein; PBS, phosphate-buffered saline; WT, wild type.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—Culture of parC5 cells has been described previously (3). For immunofluorescence studies, cells were grown on 12-mm coverslips in 12 well polystyrene dishes. Subconfluent parC5 cells were transiently transfected 18–24 h before etoposide treatment using a 3:1 or 6:1 lipid/DNA ratio of FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. Primary mouse parotid cells were prepared under sterile conditions as previously described (7). Primary cells grew to ~80% confluence in 5 days and were used at that time for experiments without further
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passage. Tissue culture reagents were obtained from Invitrogen or BIOSOURCE (Rockville, MD). Leptomycin B and etoposide were purchased from Sigma-Aldrich, and Z-Val-Ala-Asp(O-methyl)-CH₂F (zVAD-FMK) is from Enzyme Systems Products, Livermore CA.

Construction of Enhanced Green Fluorescent Protein Plasmids and Site-directed Mutagenesis—pCAG-casp3 and pcasp3-D175A-GFP were a generous gift of Dr. S. Kamada (Kobe University, Japan) (15). The PKCδ constructs of pGFP-PKCδ, in which the GFP tag was placed on the COOH terminus of the kinase, pGFP-PKCδ<sup>K376R</sup>, pGFP-PKCδ<sup>D327A</sup>, pGFP-NLML-FLδ, and pEFPG (Clontech, Palo Alto CA) were previously described (11). pGFP-NTPKCδ, in which the GFP tag was place on the amino terminus of the kinase, was a generous gift from Dr. Stuart Yuspa (National Institutes of Health). To generate pGFP-NLSPKCD, the SV40-NLS was fused to the NH₂ terminus of pGFP-PKCδ and pGFP-PKCδ<sup>D327A</sup> by PCR using the following primers: 5'-GGCTCGAGATGACACCCCAAGAGAAGAGCGAAAGGTAGAAGATCCCGAAGCACCC-3', which contains an Xhol site, and 5'-GCTC- TAGAGTCGCGGCGCTTACTGT-3', which contains an Xbal site. Xhol and Xbal double restriction digests were performed on the PCR products along with DNA from the backbone pEFGP-N1 vector. pFLAG PKCδ<sup>K376R</sup>, which contains an NLS; however, nuclear localization is also suppressed by mutation of the caspase cleavage site of PKCδ, was a generous gift of Feng Chu, MD Anderson Cancer Center) by PCR site-directed mutagenesis.

Immunoprecipitation and Immunoblot Analysis—Immunoprecipitations and immunoblots were preformed as previously described (3). The mouse monoclonal antibody to GFP was obtained from Zymed Laboratories Inc. (C163, San Francisco, CA). Rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (PKCδ and lamin B) or Abcam (GFP).

Isolation of Cytosolic and Nuclear Fractions—For the experiments in Fig. 1, fractionation was performed using a Biovision (Mountain View, CA) nuclear/cytosol fractionation kit according to the manufacturer’s directions. For Fig. 3B, cell pellets were resuspended in 300 μl of nuclei isolation buffer (20 mM HEPES-KOH, 100 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin). The cells were incubated on ice 20 min and then lysed using a Dounce homogenizer (25×). Efficient cell lysis was verified by trypsin blue staining. The cell lysate was centrifuged (1000 × g for 5 min), and the pellet (nuclei) and supernatant (cytosol) were collected. The nuclei were washed once in 200 μl of nuclei isolation buffer and pelleted by centrifugation, and the resulting supernatant was added to the cytosolic extract. For all experiments, Triton X-100 was added to a final concentration of 1.0% to solubilize the proteins. Protein concentration was quantified using the DC Protein Assay kit (Bio-Rad). In some experiments leptomycin B (Sigma-Aldrich) (10 ng/ml) was added during the last hour of etosopide treatment.

Immunofluorescence Microscopy—To detect endogenous PKCδ, cells were washed with PBS and fixed in 2% paraformaldehyde/PBS for 15 min followed by permeabilization with 0.5% Triton-X, PBS for 5 min at room temperature. Cells were incubated for 1 h in 20 mg/ml bovine serum albumin, PBS before incubation with a rabbit polyclonal PKCδ primary antibody (#C-17 Santa Cruz Biotechnology) for 1 h. Cells were washed five times with PBS and then incubated with a donkey anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove PA) for 1 h. Cells were again washed five times with PBS, and coverslips were mounted with o-phenylenediamine dihydrochloride mounting medium (Sigma). Mounted slides of cells transfected with GFP were prepared identically without the antibody staining. Cells were visualized, and images were collected using SlideBook software (Intelligent Imaging Innovations Inc., Denver, CO) on a Nikon Diaphot TMD microscope equipped for fluorescence with a xenon lamp and filter wheels (Sutter Instruments, Novato, CA), fluorescent filters (Chroma, Battleboro, VT), cooled CCD camera (Cooke, Tonawanda, NY), and a stepper motor (Intelligent Imaging Innovations Inc., Denver CO).

Terminal Deoxynucleotidyltransferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) Analysis and Cell Counts—TUNEL analysis was performed using the in situ Cell Death Detection kit TMR Red (Roche Applied Science) according to the manufacturer’s protocol. GFP-positive cells were visualized by immunofluorescent microscopy and counted using a 40× objective. TUNEL-positive cells containing GFP were identified by colocalization with 4',6-diamidino-2-phenylindole dihydrochloride hydrate and by morphology and were quantified as the percent of the total GFP-positive cells per field. Experiments quantifying the percent of cells exhibiting nuclear accumulation of GFP were performed by immunofluorescent microscopy using a 40× objective.

RESULTS

PKCδ Translocates to the Nucleus before Caspase Cleavage—Our laboratory and others have previously shown that both δFL and δCF localize in the nucleus in response to etoposide (10, 11). Nuclear translocation is dependent upon a functional NLS; however, nuclear localization is also suppressed by mutation of the caspase cleavage site of PKCδ, suggesting that caspase cleavage facilitates nuclear accumulation (11). To determine whether nuclear translocation of endogenous PKCδ requires caspase activation, we pretreated parC5 cells with the pan-caspase inhibitor zVAD-FMK before the addition of etoposide. Fig. 1A shows that endogenous PKCδ is found predominately in the perinuclear compartment of untreated parC5 cells and in the nucleus of etoposide-treated cells, consistent with our previous results (11). Pretreatment with zVAD-FMK largely inhibits nuclear accumulation of PKCδ, suggesting that δCF may be the primary form of PKCδ found in the nucleus. To test this, parC5 cells were treated with etoposide, and the accumulation of δFL and δCF in nuclear fractions was analyzed by immunoblot. Surprisingly, Fig. 1B (left side) shows that δFL also accumulates in the nuclear fraction transiently between 30 min and 2 h of etoposide treatment. Pretreatment with zVAD-FMK does not alter the kinetics of nuclear accumulation of δFL. However, nuclear accumulation of δCF, which is apparent by 4–8 h of etoposide treatment, was blocked by zVAD-FMK as expected. These data indicate that nuclear accumulation of δFL occurs before generation of δCF and independent of caspase activity. Fig. 1C shows the kinetics of the reciprocal distribution
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Nuclear accumulation of δFL during apoptosis is transient and caspase-independent. A, ParC5 cells were left untreated or pretreated with 50 μM zVAD-FMK for 30 min and then treated with etoposide for 8 h. Cells were then fixed, permeabilized, and stained with an antibody directed to the COOH terminus of PKCδ, a fluorescein isothiocyanate-conjugated secondary antibody, and visualized by immunofluorescent microscopy. B, ParC5 cells were treated with zVAD-FMK as described above and then followed by treatment with etoposide for the indicated time. Nuclear fractions were prepared and immunoblotted with an antibody directed to the COOH terminus of PKCδ. The top blot shows nuclear translocation of full-length PKCδ (δFL), whereas the middle blot shows nuclear accumulation of the catalytic fragment of PKCδ (δCF). Note that the middle blot was exposed for a longer time than the upper blot to observe the appearance of δCF. The blot was stripped and reprobed with an antibody to lamin B. Note that the initial accumulation of δFL occurred 0.5 h later in cells treated with etoposide plus zVAD-FMK, but this finding was not consistent between experiments. C and D, ParC5 cells were treated with etoposide for the indicated times. Nuclear (N) and cytosolic (C) fractions were prepared and immunoblotted with an antibody directed to PKCδ. δFL in the nuclear fraction was quantified by densitometry and normalized to lamin B. Data in the graph are the average of two similar experiments. E, ParC5 cells were left untreated and treated with 50 μM etoposide for the indicated times (left side). Samples on the right side were treated with 10 ng/ml leptomycin B (Lept B) during the last hour of etoposide treatment. Nuclear fractions were prepared and immunoblotted for PKCδ. The amount of nuclear δFL was quantified by densitometry; data are expressed as fold increase in protein relative to control. Each experiment shown is representative of two or more experiments.

FIGURE 1. Nuclear accumulation of δFL during apoptosis is transient and caspase-independent. A, ParC5 cells were left untreated or pretreated with 50 μM zVAD-FMK for 30 min followed by etoposide for 8 h. Cells were then fixed, permeabilized, and stained with an antibody directed to the COOH terminus of PKCδ, a fluorescein isothiocyanate-conjugated secondary antibody, and visualized by immunofluorescent microscopy. B, ParC5 cells were treated with zVAD-FMK as described above and then followed by treatment with etoposide for the indicated time. Nuclear fractions were prepared and immunoblotted with an antibody directed to the COOH terminus of PKCδ. The top blot shows nuclear translocation of full-length PKCδ (δFL), whereas the middle blot shows nuclear accumulation of the catalytic fragment of PKCδ (δCF). Note that the middle blot was exposed for a longer time than the upper blot to observe the appearance of δCF. The blot was stripped and reprobed with an antibody to lamin B. Note that the initial accumulation of δFL occurred 0.5 h later in cells treated with etoposide plus zVAD-FMK, but this finding was not consistent between experiments. C and D, ParC5 cells were treated with etoposide for the indicated times. Nuclear (N) and cytosolic (C) fractions were prepared and immunoblotted with an antibody directed to PKCδ. δFL in the nuclear fraction was quantified by densitometry and normalized to lamin B. Data in the graph are the average of two similar experiments. E, ParC5 cells were left untreated and treated with 50 μM etoposide for the indicated times (left side). Samples on the right side were treated with 10 ng/ml leptomycin B (Lept B) during the last hour of etoposide treatment. Nuclear fractions were prepared and immunoblotted for PKCδ. The amount of nuclear δFL was quantified by densitometry; data are expressed as fold increase in protein relative to control. Each experiment shown is representative of two or more experiments.
accumulation of GFP-caspase 3 in the nucleus starting between 1 and 2 h, and by 8 h nearly 80% of the transfected cells had nuclear caspase 3 (Fig. 2A). To determine whether active caspase 3 or procaspase 3 accumulates in the nucleus, we repeated the experiment shown in Fig. 2A using a mutated form of caspase 3 that cannot be cleaved and activated (pcaasp3-D175A-GFP) (15). Fig. 2B shows that the active, but not the inactive form of caspase 3, translocates to the nucleus in etoposide. However, this approach does not distinguish if caspase cleavage occurs from 10 fields of view; at least 300 cells were scored per variable. Each experiment shown is representative of two experiments. UT, untransfected.

The data shown above suggest that caspase cleavage of δFL occurs in the nucleus of apoptotic cells. Previous studies by Blass et al. (10) used antibodies that recognize distinct NH2 and COOH epitopes in PKCδ and showed by immunohistochemistry that both domains of PKCδ were present in the nucleus. However, this approach does not distinguish δFL from δCF. To more definitively address if δFL is cleaved by caspase 3 in the nucleus, we cotransfected parC5 cells with plasmids that express a PKCδ protein in which the GFP tag is located on either the COOH or NH2 terminus. If caspase cleavage occurs in the nucleus, we predicted that both the regulatory domain fragment (δNF) and δCF should be present in the nucleus. Fig. 3A is a schematic showing the sizes of the fragments generated upon cotransfection and subsequent caspase-mediated cleavage by etoposide. The 70-kDa GFP-tagged COOH-terminal PKCδ fragment is observed predominately in the nuclear fraction, with some in the cytosolic fraction at 4 and 8 h of etoposide treatment (Fig. 3B). In contrast, the 63-kDa GFP-tagged NH2-terminal PKCδ fragment is only detected in the nuclear fraction, with some in the cytosolic fraction at 4 and 8 h of etoposide treatment (Fig. 3B).

If caspase cleavage of PKCδ occurs in the nucleus of apoptotic cells, we predicted that preventing nuclear localization of PKCδ should prevent its caspase cleavage. For these experiments we took advantage of our previous finding that GFP-tagged PKCδ can be targeted to the cytoplasm by a mutation of the PKCδ NLS (11). In the experiment shown in Fig. 3A, left, parC5 cells were transfected with pGFP, pGFP-PKδ (GFP-δWT), or pGFP-NLS-PKδ (GFP-δNLSFL8), in which the PKCδ NLS has been mutated (11). Expression of GFP-
δNLMFL8 is exclusively cytoplasmic accumulation in >99% of transfected parC5 cells (11). Treatment of the transfected cells with etoposide resulted in caspase cleavage of GFP-δWT as indicated by the presence of δCF; however, GFP-δNLMFL8 was not cleaved by caspase, indicating that nuclear translocation of δFL is a prerequisite for its caspase cleavage. Lack of cleavage of GFP-δNLMFL8 is not due to inhibition of caspase activation, as endogenous PKCδ was cleaved in pGFP-NLMFL8-transfected cells (Fig. 3D). To determine whether nuclear retention of PKCδ is sufficient to cause caspase cleavage, we fused an SV40 NLS to the NH₂ terminus of PKCδ (NLS δWT) to drive nuclear import. ParC5 cells were transfected with pGFP-PKCD, pGFP-NLS PKCD, or pGFP-NLS PKCD D327A, a control in which the SV40 NLS is fused to the NH₂ terminus of PKCδ mutated at the caspase cleavage site. Fusion of the SV40 NLS to PKCδ resulted in targeting of the protein to the nucleus in >99% of transfected cells (data not shown). As seen in Fig. 3E, transient expression of δWT resulted in the generation of a small amount of GFP-δCF, consistent with its ability to induce apoptosis (3, 17). However, retention of PKCδ in the nucleus by the addition of an SV40 NLS resulted in a much higher basal level of GFP-δCF. As expected, constitutive nuclear localization of the caspase cleavage mutant did not result in caspase cleavage. Thus, the nuclear targeting of PKCδ is sufficient to drive caspase cleavage of PKCδ in the absence of another apoptotic inducer.

Nuclear Retention of PKCδ Is Required for Apoptosis—The data presented above demonstrate that nuclear retention of δFL is both necessary and sufficient for caspase cleavage, indicating a role for both δFL and δCF in genotoxin-induced apoptosis. However, it has been difficult to decipher specific functions for these two forms of PKCδ because under conditions where caspase is activated, both δFL and δCF are present in the cell. Reconstitution of Pkcd⁻/⁻ epithelial cells with wild type PKCδ or PKCδ mutants is a powerful model for exploring the function of different domains of this kinase. To address the contribution of caspase cleavage to the proapoptotic function of PKCδ, Pkcd⁻/⁻ cells were reconstituted with wild type PKCδ or an uncleavable form of PKCδ, δCM (GFP-PKCD D327A). As we have previously reported, expression of δWT in Pkcd⁻/⁻ cells completely reconstitutes etoposide-induced apoptosis (Fig. 4A) (7). In contrast, expression of δCM is not sufficient to reconstitute apoptosis, indicating that generation of δCF is necessary for the apoptotic response. In the experiment shown in Fig. 4B, we asked if expression of δCM could induce apoptosis in the context of endogenous PKCδ, since under these conditions δCF could be generated from endogenous PKCδ. As seen in Fig. 4B, although expression of δWT induces apoptosis, δCM is a very weak inducer of apoptosis (1% TUNEL-positive cells compared with 27% of cells transfected with δWT) and is not significantly different from expression of GFP alone (9% TUNEL positive). These data indicate that expression of a caspase cleavage-resistant mutant of PKCδ is insufficient to induce apoptosis even when expressed in the context of endogenous PKCδ. We have previously reported that the δCM has reduced accumulation in the nucleus when compared with wild type PKCδ and attributed this to a lack of δCF production (11). To determine whether nuclear retention of full-length δCM is sufficient for induction of apoptosis in the absence of etoposide, we generated δCM fused to an SV40 NLS (GFP-NLS-δCM). In contrast to δCM, NLS-δCM is retained exclusively in the nucleus (data not shown). Shown in Fig. 4C, NLS-δCM and GFP-δWT, but not δCM, are equally efficient at inducing apoptosis. These results indicate that sustained retention of full-length PKCδ in

FIGURE 4. Nuclear retention of PKCδ is necessary for apoptosis. A, primary parotid epithelial cells were prepared from Pkcd⁻/⁻ mice or their WT littermates as described under “Experimental Procedures.” Cells were transduced with the adenoviral constructs Ad-pGFP, Ad-GFP PKCD (δWT), or Ad-GFP PKCD D327A (δCM) for 24 h, and left untreated (gray bars) or treated with 200 μM etoposide (black bars) for an additional 8 h and were analyzed using TUNEL to measure apoptosis (described below). B, ParC5 cells were transiently transfected with pGFP alone, pGFP PKCD (δWT), or pGFP PKCD D327A (δCM) and after 48 h were analyzed using TUNEL (black bars). Parallel experiments were carried out to obtain the percent of cells containing GFP in the nucleus as described in Fig. 2 (gray bars). C, ParC5 cells were transiently transfected with pGFP PKCD (δWT), pGFP PKCD D327A (δCM), or pGFP NLS-PKCD D327A (NLS-δCM). After 18 h the cells were analyzed by TUNEL. A–C, for apoptosis analysis, TUNEL-positive cells containing GFP were visualized by immunofluorescent microscopy and counted using a 20× objective. TUNEL/GFP-positive cells were quantified as the percent of the total number of GFP-positive cells per field. The graphs represent the average of three independent experiments. At least 100 cells were counted for each variable per experiment. Data are the means ± S.E. from 10 fields of view. Each experiment shown is representative of two or more experiments.
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FIGURE 5. δKN does not translocate to the nucleus and is not cleaved by caspase. A, ParC5 cells were transiently transfected with pGFP alone, pGFP PKCδ (δWT), or pGFP-PKCδCFK376R (δKN). After 18 h cells were then left untreated or treated with etoposide for an additional 8 h and viewed by confocal microscopy (magnification, ×100). The number of cells exhibiting nuclear localization of PKCδ in response to etoposide was obtained as a percent of the whole GFP population (greater than 100 cells counted/variable). Data are the means ± S.E. mean from at least 10 fields of view. B and C, ParC5 cells were transiently transfected with pGFP PKCδ, or pGFP-PKCδCFK376R (δKN), or pGFP-NLS-PKCδCFK376R (NLSδKN) (C). After 18 h cells were then treated with etoposide for an additional 0, 4, or 8 h (E) or for 8 h (C). Cells were harvested and subjected to immunoblot analysis with an antibody directed to GFP. Gray lines represent different areas of the same blot that were spliced together. Each experiment shown is representative of two or more experiments.

the nucleus can initiate the apoptotic program and that caspase cleavage of PKCδ functions primarily to facilitate nuclear retention of the active kinase.

Kinase-negative Mutants of PKCδ Define Multiple Points of Regulation—Our laboratory and others have shown that expression of kinase-negative full-length PKCδ (δKN) protects cells from apoptosis induced from a variety of agents including etoposide (2, 10). Likewise, we have shown that a kinase-negative mutant of δCF (δKN-CF) is a potent inhibitor of apoptosis and that inhibition of apoptosis by δKN-CF depends upon nuclear localization (11). Although the subcellular localization of δKN in etoposide-treated cells has not been explored, it might be expected to localize to the nucleus based on our previous findings (11). To address this question, parC5 cells were transfected with pGFP-PKCδ (δWT) or pGFP-PKCδCFK376R (δKN) and treated with etoposide, and localization of GFP-PKCδ was analyzed by confocal microscopy. Shown in Fig. 5A, δKN localizes to the perinuclear region in untreated cells similar to that observed for both δWT and endogenous PKCδ (11). Surprisingly, after etoposide treatment, δWT accumulates in the nucleus, whereas δKN remains localized to the perinuclear region (Fig. 5A). As expected, cells transfected with pGFP showed a diffuse distribution of the GFP protein throughout the cells both before and after etoposide treatment. Consistent with a requirement for nuclear localization, the data in Fig. 5B indicate that δKN is not cleaved by caspase in etoposide-treated cells as no δCF is detectable. However, targeting δKN to the nucleus with the addition of a SV-40 NLS allows δKN to be cleaved by caspase after etoposide treatment, indicating that lack of caspase cleavage is due to its retention in the cytoplasm (Fig. 5C).

Our data suggest that δKN and δKN-CF inhibit etoposide-induced apoptosis by different mechanisms. Although nuclear localization of δKN-CF is necessary to inhibit apoptosis, δKN inhibits apoptosis even though it is localized exclusively to the cytoplasm (2). To determine whether suppression of apoptosis by δKN and δKN-CF are additive, parC5 cells were transiently transfected with pGFP, pGFP-PKCδCFK376R (δKN-CF), pGFP-PKCδCFK376R (δKN), or pGFP-PKCδCFK376R plus pGFP-PKCδCFK376R, and after 18 h the cells were treated with etoposide. Expression of δKN suppressed DNA fragmentation in response to etoposide by ~60%, in agreement with previous reports from our laboratory (2). Expression of δKN-CF resulted in a slightly more robust protection from etoposide-induced apoptosis as seen by an almost 75% reduction in TUNEL-positive cells (Fig. 6A).

However, co-transfection with both constructs together did not lead to enhanced inhibition when compared with δKN-CF alone, consistent with the possibility that these forms of PKCδ inhibit distinct steps in the apoptotic pathway.

Based on our finding that δFL translocates into the nucleus before caspase activation, we predicted that δKN may inhibit apoptosis by preventing translocation of PKCδ into the nucleus. To test this, parC5 cells were transfected with pGFP-PKCδ or cotransfected with pGFP-PKCδ (δWT) and pFLAG-PKCδCFK376R (δKN). The FLAG-δKN localized exclusively to the cytoplasm as previously demonstrated for GFP-δKN (data not shown and Fig. 5A). As shown in Fig. 6B, δWT translocated to the nucleus in 46% of cells treated with etoposide. However, coexpression of δKN and δWT significantly suppressed nuclear translocation of δWT (28 and 18% when cotransfected with δKN1 and δKN2, respectively). These studies indicate that in contrast to δKN-CF, which inhibits apoptosis only when it is localized to the nucleus, expression of δKN suppresses apoptosis by inhibiting nuclear translocation of PKCδ. These data demonstrate that nuclear translocation of PKCδ is essential for transduction of the apoptotic signal during genotoxin-induced stress.

DISCUSSION

Molecular mechanisms that regulate the proapoptotic function of PKCδ are not well understood but are clearly important for cell survival. Furthermore, the respective contribution of PKCδ and its constitutively activated caspase cleavage product,
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requires the COOH-terminal NLS, our current studies indicate that in the absence of an apoptotic signal PKCδ is retained in the cytoplasm by a mechanism that is dependent upon the regulatory domain of the protein. Thus, in addition to the NLS, posttranslational modifications in the PKCδ regulatory domain, such as tyrosine phosphorylation, may control nuclear accumulation of δFL. An early role for δFL in apoptotic signaling is supported by its rapid, caspase-independent accumulation in the nucleus. Although necessary, this step is not sufficient for inducing apoptosis because PKCδ is rapidly exported. Moreover, δFL nuclear accumulation precedes activation of any of the known components of the apoptotic pathway in etoposide-treated parC5 cells, including cytochrome c release and activation of the initiator caspase, caspase 9 (2).

We show that the regulatory domain (δNF), δCF, and active caspase 3 all accumulate in the nucleus during apoptosis, in agreement with previous studies (10, 15). Nuclear accumulation of caspase 3 follows accumulation of δFL and is coincident with generation of the cleavage product of PKCδ. This sequential regulation may assure that sustained nuclear accumulation of nuclear PKCδ occurs only when caspase 3 is activated. Studies from our laboratory and others also show that overexpression of δFL is sufficient to induce its caspase cleavage and apoptosis. This may be due to the high basal expression of caspase 3 in the nucleus that can cleave PKCδ when it is overexpressed (Fig. 2), or alternatively, high levels of nuclear δFL may activate caspase 3 directly or indirectly. However, because nuclear retention of a caspase cleavage mutant of δFL is required for apoptosis, these results indicate that the initiating signal by which δFL activates the apoptotic pathway arises in the nucleus. In this regard our data suggest that rapid export of uncleaved δFL out of the nucleus is likely to be critical for cell survival. Although we were unable to find a leucine-rich nuclear export sequence in PKCδ, δFL could be exported during apoptosis through binding molecules that contain nuclear export sequences. For example, two nuclear export sequences containing proteins c-Abl and Stat1 have each been shown to bind PKCδ during apoptosis (18–20).

D’Costa et al. (21) have previously shown that a caspase cleavage-resistant mutant of δFL can act as a dominant negative to block UV-induced apoptosis. Likewise, various reports have shown that PKCδ-mediated apoptosis can be inhibited by overexpression of a kinase-negative form of PKCδ (2, 10, 22, 23). Previously, we showed that a mutation of the NLS within δKN-CF results in its retention in the cytoplasm and renders it unable to inhibit apoptosis (11). In this study we show that δKN, which is localized exclusively in the cytoplasm, inhibits apoptosis by blocking nuclear translocation of δFL. These combined data suggest that δKN and δKN-CF regulate distinct steps in transduction of the PKCδ proapoptotic signal that are dependent upon where they are localized in the cell. Because of the differential localization of these dominant-inhibitory PKCδ mutants, our studies suggest that care should be taken when using these or similar tools to study PKCδ-regulated signal transduction.

Our data suggest that under normal growth conditions, tight regulation of nuclear import and export of δFL is required for cell survival. Moreover, a recent study has shown that the B cell

δCF, to apoptotic signaling have not been elucidated. Here we show that the proapoptotic function of PKCδ is regulated by its nuclear retention. δFL accumulates rapidly but transiently in the nucleus in response to apoptotic signals. Transient nuclear accumulation of δFL is not sufficient to induce apoptosis; rather, caspase cleavage of PKCδ facilitates the sustained nuclear accumulation of PKCδ that is necessary for apoptosis. Targeting of a caspase cleavage mutant of δFL to the nucleus is sufficient to induce apoptosis, indicating that it is the sustained accumulation of PKCδ in the nucleus that is essential for apoptotic signaling and not caspase cleavage per se. Hence, our studies demonstrate that nuclear retention of PKCδ is a critical step for commitment to apoptosis and suggest that nuclear transport of PKCδ regulates the survival/death pathway.

We show that apoptotic signaling in etoposide-treated cells is coordinately regulated by δFL and δCF, with the overall outcome being a sustained increase in nuclear PKCδ. We propose that δFL may act as an apoptotic sensor, transmitting cell damage signals to the nucleus. Although nuclear import of PKCδ

![FIGURE 6. δKN protects cells from apoptosis by blocking nuclear translocation of endogenous PKCδ. A, ParC5 cells were transfected with pGFP, pGFP-PKCCδK376R (δKN), pGFP-PKCCδ-CF K376R (δKNCF), or cotransfected with pGFP-PKCCδK376R and pGFP-PKCCδCF K376R. After 18 h the cells were treated with etoposide for 8 h and analyzed using TUNEL as described in Fig. 4. The graph represents the average of two independent experiments. At least 200 cells were counted for each variable. Data are the means ± S.E. mean from at least 20 fields of view. B, ParC5 cells were transfected with pGFP-PKCCδ (δWT) or cotransfected with pGFP-PKCCδ (δWT) plus pFLAG-PKCCδK376R (δKN). δKN1 and δKN2 are distinct pFLAG-PKCCδ K376R clones. Transfected cells were left untreated or treated for 8 h with etoposide. The number of cells exhibiting nuclear localization of PKCδ in response to etoposide was obtained as a percent of the whole GFP population (greater than 100 cells counted/variable). Data are the means ± S.E. mean from at least 10 fields of view. The graph represents one of three independent experiments that produced similar results.](image-url)
Nuclear Localization of PKCζ and Apoptosis

growth factor, BAFF, contributes to cell survival at least in part through sequestration of PKCζ in the cytoplasm (24). We show that sustained nuclear retention occurs only under conditions where caspase is activated, resulting in removal of the regulatory domain and nuclear retention. Because both δFL and δCF can induce apoptosis under conditions of nuclear retention, δFL and δCF likely share the same nuclear targets. PKCζ has been shown to have a multitude of proapoptotic nuclear targets including STAT1, Rad 9, topoisomerase IIα, DNA-dependent protein kinase, and lamin B (9, 25–30). Moreover, DNA-dependent protein kinase and topoisomerase II protein kinase, and lamin B (9, 25–30). Moreover, DNA-dependent protein kinase and topoisomerase IIα have been shown to have a multitude of proapoptotic nuclear targets including STAT1, Rad 9, topoisomerase IIα, DNA-dependent protein kinase, and lamin B (9, 25–30). Moreover, DNA-dependent protein kinase and topoisomerase IIα, DNA-dependent protein kinase and topoisomerase IIα have been shown to have a multitude of proapoptotic nuclear targets including STAT1, Rad 9, topoisomerase IIα, DNA-dependent protein kinase, and lamin B (9, 25–30).

Although δCF is generated in the nucleus, our studies show that it can also be found in the cytoplasm of apoptotic cells, indicating that δCF also exits the nucleus. This observation may explain other published studies reporting that PKCζ can localize to various cellular compartments in response to many stimuli (4, 5, 32, 33). Because nuclear δCF is a potent inducer of caspase activation and apoptosis, cytoplasmic δCF may function to amplify apoptosis through direct or indirect modification of the cytoplasmic apoptotic machinery. Recently Sitaio et al. (34) have shown that δCF may directly regulate the apoptotic pathway by targeting the antiapoptotic protein, Mcl-1, for degradation. Taken together, our studies suggest that tight regulation of nuclear import and export of δFL is critical for cell survival and that caspase cleavage of δFL in the nucleus signals an irreversible commitment of cells to apoptosis.

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