

# Nuclear import of PKC $\delta$ is required for apoptosis: identification of a novel nuclear import sequence

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**We have shown previously that protein kinase C $\delta$  (PKC $\delta$ ) is required for mitochondrial-dependent apoptosis. Here we show that PKC $\delta$  is imported into the nucleus of etoposide-treated cells, that nuclear import is required for apoptosis and that it is mediated by a nuclear localization signal (NLS) in the C-terminus of PKC $\delta$ . Mutation of the caspase cleavage site of PKC $\delta$  inhibits nuclear accumulation in apoptotic cells, indicating that caspase cleavage facilitates this process. Expression of the PKC $\delta$  catalytic fragment (CF $\delta$ ) in transfected cells results in nuclear localization and apoptosis. We show that the PKC $\delta$  NLS is required for nuclear import of both full-length PKC $\delta$  and CF $\delta$ , and drives nuclear localization of a multimeric green fluorescent protein. Mutations within the NLS of CF $\delta$  prevent nuclear accumulation and block apoptosis. Conversely, nuclear expression of a kinase-negative catalytic fragment (KN-CF $\delta$ ) protects cells from etoposide-induced apoptosis. Mutation of the NLS blocks the ability of KN-CF $\delta$  to protect against etoposide-induced apoptosis. These results indicate that PKC $\delta$  regulates an essential nuclear event(s) that is required for initiation of the apoptotic pathway.**

**Keywords:** apoptosis/DNA damage/nuclear localization/protein kinase C $\delta$

## Introduction

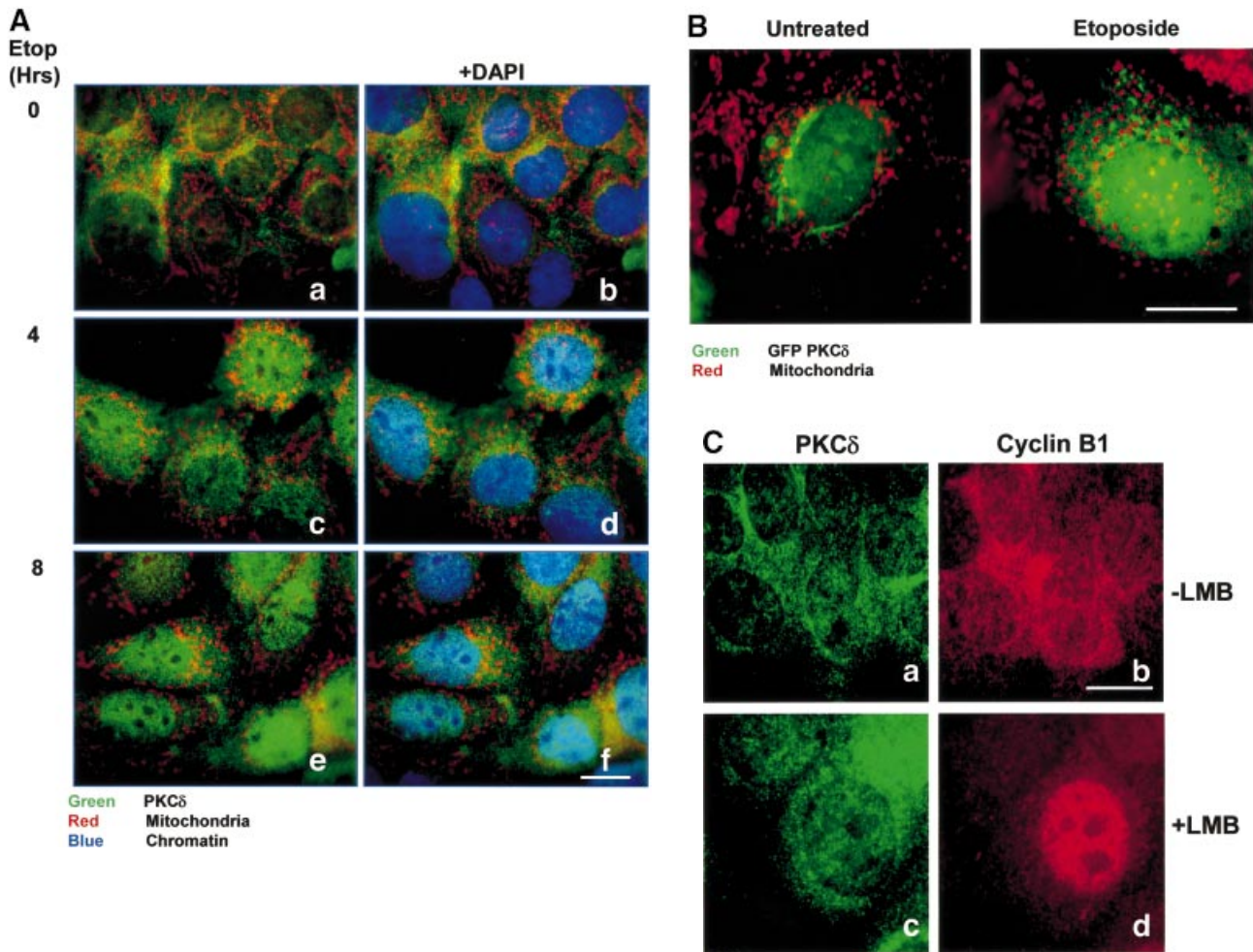
Apoptosis is a genetically programmed form of cell death required for normal development, tissue homeostasis and the elimination of damaged cells (Utz and Anderson, 2000). Initiation of a mitochondrial-dependent pathway of apoptosis occurs in response to genotoxins, organelle toxins, irradiation and other types of cell stress. These agents converge at the mitochondria, resulting in the release of cytochrome *c* and caspase activation (Chinnaiyan, 1999; Wolf and Green, 1999; Antonsson and Martinou, 2000; Kroemer and Reed, 2000). Pro- and anti-apoptotic members of the Bcl-2 family regulate this pathway, as do specific signal transduction cascades, including members of the mitogen-activated protein kinase family, the pro-survival protein kinase, AKT (Datta,S.R. *et al.*, 1997; Widmann *et al.*, 1997; Anderson

*et al.*, 1999) and specific isoforms of protein kinase C (PKC; Ghayur *et al.*, 1996; Datta,R. *et al.*, 1997; Mizuno *et al.*, 1997; Bharti *et al.*, 1998).

PKC $\delta$  is a member of a large superfamily of isoforms that differ based on their requirement for lipid cofactors and Ca<sup>2+</sup> for activation. PKC $\delta$ , a Ca<sup>2+</sup>-independent isoform, has been shown to regulate the mitochondrial-dependent pathway of apoptosis, and in some cell types PKC $\delta$  overexpression can induce apoptosis in the absence of additional stimuli (Emoto *et al.*, 1995, 1996; Ghayur *et al.*, 1996; Denning *et al.*, 1998; Li *et al.*, 1999; Pongracz *et al.*, 1999; Reyland *et al.*, 1999, 2000). In addition, recent studies show that cells derived from PKC $\delta$ -null transgenic mice are defective in mitochondrial-dependent apoptosis (Leitges *et al.*, 2001). Proteolytic activation of PKC $\delta$  by caspases, which results in the generation of an active kinase domain, occurs in response to a variety of stimuli including DNA-damaging agents (Emoto *et al.*, 1995, 1996; Reyland *et al.*, 1999), FAS ligand (Mizuno *et al.*, 1997; Frasch *et al.*, 2000) and mitomycin C (Emoto *et al.*, 1996; Ghayur *et al.*, 1996). Interestingly, when the catalytic domain of PKC $\delta$  is transiently transfected into cultured cells, it rapidly induces apoptosis (Ghayur *et al.*, 1996; Mizuno *et al.*, 1997; Bharti *et al.*, 1998).

Insight into how PKC $\delta$  may regulate apoptosis has been gained from studies that have investigated the subcellular localization of PKC $\delta$  in apoptotic cells. For example, when cells transiently overexpressing PKC $\delta$  were treated with phorbol ester, PKC $\delta$  translocated to the mitochondria, resulting in loss of mitochondrial membrane potential and release of cytochrome *c* (Li *et al.*, 1999; Majumder *et al.*, 2000). However, in T cells induced to undergo apoptosis by cytokine deprivation, and in glioma cells treated with etoposide, PKC $\delta$  localized to the nucleus, consistent with a nuclear function (Scheel-Toellner *et al.*, 1999; Blass *et al.*, 2002). Many proteins that function in the nucleus harbor conserved nuclear localization signals (NLSs) that allow rapid import of proteins via complex formation with the importin protein family members (Nigg, 1997; Gorlich, 1998; Gama-Carvalho and Carmo-Fonseca, 2001; Hodel *et al.*, 2001). Sequences that mediate nuclear import have been identified in PKC $\alpha$  and PKC $\zeta$ , but not in the novel PKC isoform subfamily, which includes PKC $\delta$  (James and Olson, 1992; Perander *et al.*, 2001).

Previous studies from our laboratory have demonstrated an essential requirement for PKC $\delta$  in apoptosis induced by agents that target the mitochondrial-dependent pathway (Reyland *et al.*, 1999, 2000; Matassa *et al.*, 2001). Here we show that nuclear localization of PKC $\delta$  is required for apoptosis induced by etoposide, and that caspase cleavage of PKC $\delta$  contributes to its pro-apoptotic function by facilitating nuclear import. Furthermore, we have defined a unique



**Fig. 1.** PKC $\delta$  localizes to the nucleus during etoposide treatment. **(A)** C5 cells were treated with 50  $\mu$ M etoposide for 0, 4 and 8 h. The cells were stained with an FITC-conjugated antibody specific for PKC $\delta$  (green), with DAPI (blue) to identify the nuclei, and with Mitotracker red to identify the mitochondria. The cells were visualized by confocal microscopy (magnification,  $\times 100$ ). The white bar represents 10  $\mu$ m. Panels a, c and e are an overlay of FITC and Mitotracker red. Panels b, d and f are an overlay of FITC, Mitotracker red and DAPI. **(B)** C5 cells were transiently transfected with pGFP-PKC $\delta$ . After 18 h, cells were left untreated or treated with etoposide for an additional 8 h, stained with Mitotracker red and then viewed by confocal microscopy (magnification,  $\times 100$ ). Panels are an overlay of GFP and Mitotracker red. The white bar represents 10  $\mu$ m. **(C)** C5 cells were left untreated or treated with LMB (5 ng/ml) for 6 h, fixed, permeabilized and co-stained with an FITC-conjugated antibody specific for PKC $\delta$  (green, panels a and c), and a Cy3-conjugated antibody specific for cyclin B1 (red, panels b and d). Cells were viewed by confocal microscopy (magnification,  $\times 100$ ). Images were obtained from the same field in untreated and LMB-treated cells in red or green channels.

and functional NLS in the C-terminus of PKC $\delta$  that mediates nuclear import. These studies demonstrate that PKC $\delta$  regulates an essential nuclear event that is required for activation of the apoptotic pathway.

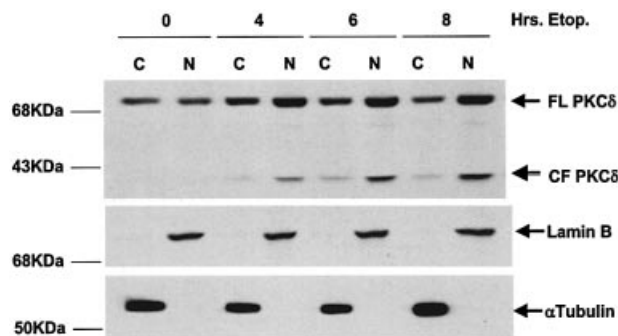
## Results

### *Endogenous PKC $\delta$ translocates to the nucleus after etoposide treatment*

To determine where PKC $\delta$  localizes in apoptotic cells, C5 cells were treated with etoposide, and endogenous PKC $\delta$  was visualized using an antibody that recognizes the C-terminal portion of the protein. As shown in Figure 1A, panels a and b, in untreated cells PKC $\delta$  localized primarily to the cytoplasm, showing a punctate, peri-nuclear distribution. Upon treatment with etoposide, peri-nuclear PKC $\delta$  decreased by 4 h, while the abundance of PKC $\delta$  in the nucleus increased, indicating translocation of PKC $\delta$  into the nucleus (Figure 1A, panels c and d). By 8 h of

etoposide treatment, the majority of PKC $\delta$  appeared to be nuclear (Figure 1A, panels e and f). Staining with Mitotracker red indicated that PKC $\delta$  did not co-localize strongly with the mitochondria in either untreated or etoposide-treated cells.

To understand how PKC $\delta$  is localized to the nucleus, and the contribution of nuclear localization of PKC $\delta$  to apoptosis, we have used a series of C-terminal green fluorescent protein (GFP)-tagged wild-type and mutant PKC $\delta$  proteins, which are illustrated in Figure 4A. As observed in Figure 1B, like endogenous PKC $\delta$ , GFP-PKC $\delta$  localized to the peri-nuclear region in untreated cells and accumulated in the nucleus after treatment of cells with etoposide. Staining with Mitotracker red indicated that GFP-PKC $\delta$  did not co-localize with mitochondria. Transfection with a plasmid that expresses GFP alone resulted in a diffuse distribution throughout the cytoplasm and nucleus in both untreated and etoposide-treated cells (data not shown).



**Fig. 2.** PKC $\delta$  catalytic fragment localizes to the nucleus during etoposide-induced apoptosis. C5 cells were left untreated or treated with etoposide for 4, 6 or 8 h. Nuclear (N) and cytosolic (C) enriched fractions were prepared as described and analyzed by immunoblot for PKC $\delta$  (top panel), the nuclear marker lamin B (middle panel) or the cytosolic marker  $\alpha$ -tubulin. The positions of the full-length (FL PKC $\delta$ ) and the catalytic fragment (CF PKC $\delta$ ) are indicated.

### **Cytoplasmic distribution of PKC $\delta$ in untreated cells is not sensitive to leptomycin B**

Since even in untreated cells a small amount of PKC $\delta$  is seen in the nucleus (Figures 1A, panels a and b), the nuclear accumulation of PKC $\delta$  in response to etoposide could result from either import into the nucleus or the inhibition of nuclear export. To address this latter possibility, cells were treated with an inhibitor of the CRM1-exportin pathway, leptomycin B (LMB), and the subcellular localization of endogenous PKC $\delta$  was assessed. Cyclin B1, which is exported from the nucleus by the CRM1-exportin pathway, was used as a positive control (Yang *et al.*, 1998). In untreated cells, PKC $\delta$  was predominantly cytoplasmic (Figure 1C, panel a), while cyclin B1 was mainly cytoplasmic, with some nuclear localization (Figure 1C, panel b). Treatment with LMB resulted in the nuclear accumulation of cyclin B1 (Figure 1C, panel d), while PKC $\delta$  retained a predominantly cytoplasmic distribution (Figure 1C, panel c). These data suggest that the nuclear accumulation of PKC $\delta$  in response to etoposide does not result from inhibition of nuclear export.

### **The PKC $\delta$ catalytic fragment accumulates in the nucleus during etoposide-induced apoptosis**

Caspase cleavage of PKC $\delta$  occurs in response to a variety of apoptotic agents and results in the production of a 40 kDa constitutively active catalytic fragment (CF $\delta$ ). We have shown previously that the CF $\delta$  protein can be detected in C5 cells as early as 4 h after treatment with etoposide (Reyland *et al.*, 1999). Since the kinetics of PKC $\delta$  nuclear translocation correlated temporally with generation of the CF $\delta$ , we sought to determine whether the CF $\delta$  protein accumulates in the nucleus of cells treated with etoposide. Figure 2 shows an immunoblot of nuclear and cytosolic fractions from untreated cells or cells treated with etoposide. Although a small amount of the CF $\delta$  can be seen in the cytosol at all time points after etoposide treatment, the ratio of nuclear to cytosolic CF $\delta$  protein increases dramatically with time after treatment, suggesting that the caspase-cleaved form of PKC $\delta$  preferentially accumulates in the nucleus during apoptosis. It should be noted that the abundance of full-length PKC $\delta$  in the

nuclear fraction also increases, consistent with the possibility that full-length PKC $\delta$  is also translocated into the nucleus.

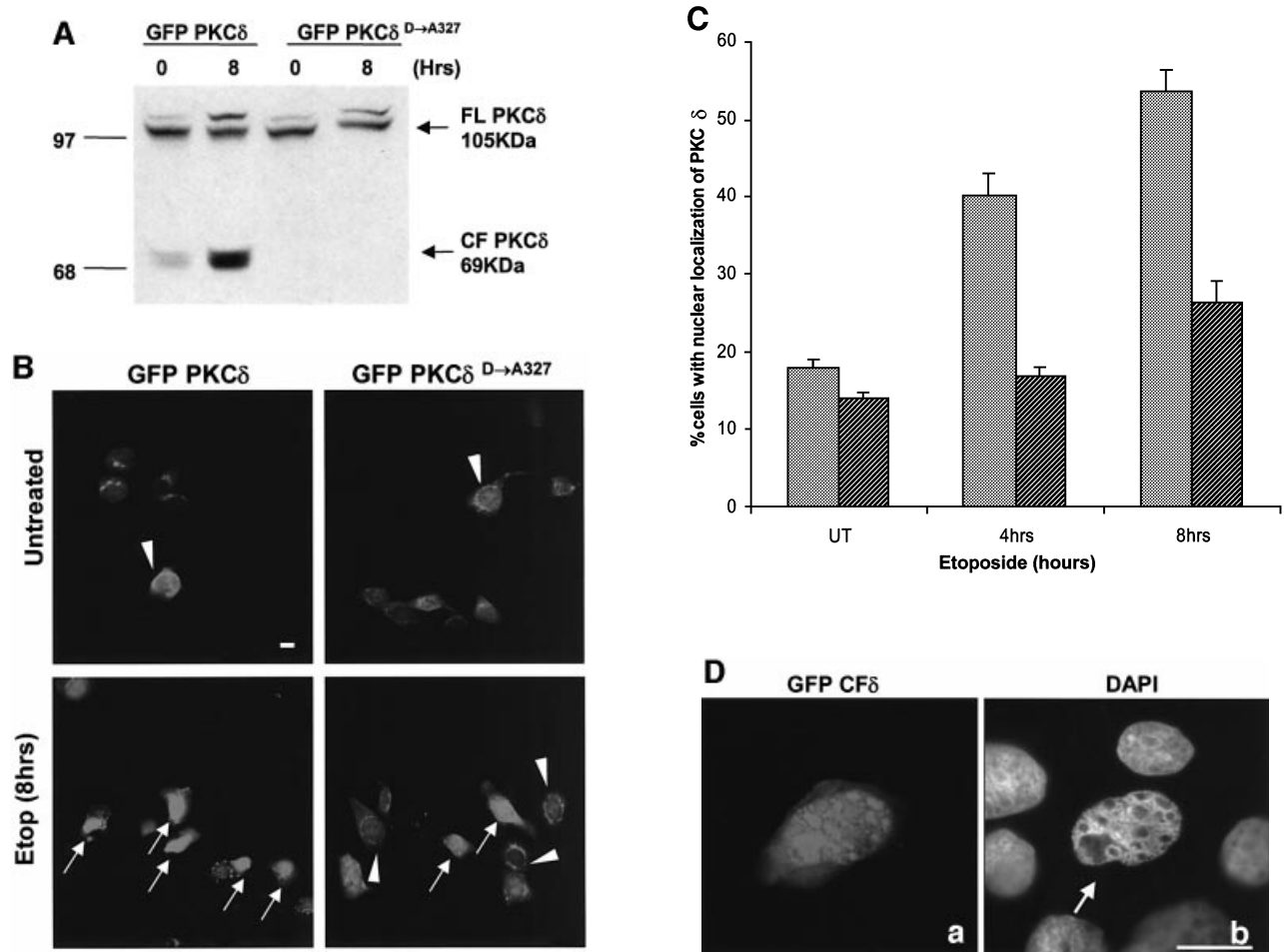
### **Caspase cleavage of PKC $\delta$ promotes its nuclear accumulation in apoptotic cells**

The data in Figure 2 suggest that caspase cleavage of PKC $\delta$  may facilitate its nuclear localization. To test this hypothesis directly, we mutated the caspase-3 cleavage site and examined the effect of this mutation on the nuclear accumulation of PKC $\delta$  in apoptotic cells. In pGFP-PKC $\delta$  (GFP-PKC $\delta^{D \rightarrow A327}$ ), the aspartic acid at the P1 position of the caspase cleavage site (DILD327) was changed to alanine (DILA327), which results in loss of caspase cleavage as shown in Figure 3A. Mutation of this site had no effect on the kinase activity of PKC $\delta$  (data not shown). Figure 3B shows a representative field of cells transfected with pGFP-PKC $\delta$  (left) or pGFP-PKC $\delta^{D \rightarrow A327}$  (right) and treated with etoposide. As indicated by the arrows, nuclear localization of both GFP-PKC $\delta$  and GFP-PKC $\delta^{D \rightarrow A327}$  is observed; however, the percentage of pGFP-PKC $\delta^{D \rightarrow A327}$ -transfected cells that exhibit nuclear localization is significantly reduced. To verify this, we counted the number of cells exhibiting predominantly nuclear localization of PKC $\delta$  and expressed the data as a percentage of the total number of cells expressing GFP (Figure 3C). As shown, GFP-PKC $\delta$  rapidly accumulated in the nucleus of cells treated with etoposide (40% of cells exhibit nuclear localization at 4 h and 54% at 8 h). In contrast, pGFP-PKC $\delta^{D \rightarrow A327}$ -transfected cells displayed significant inhibition of nuclear translocation (17% of cells exhibit nuclear localization at 4 h and 26% at 8 h), indicating that caspase cleavage facilitates nuclear accumulation of PKC $\delta$ .

The reduced nuclear accumulation of GFP-PKC $\delta^{D \rightarrow A327}$  in etoposide-treated cells suggested that CF $\delta$  may be imported more efficiently into the nucleus. To address this possibility, we asked if expression of the CF $\delta$  results in nuclear accumulation in the absence of an apoptotic stimuli. Cells were transiently transfected with GFP-tagged catalytic fragment PKC $\delta$  (pGFP-CF $\delta$ , amino acids 325–673) and visualized by confocal microscopy. Figure 3D demonstrates that in contrast to full-length GFP-PKC $\delta$  (see Figure 1B), GFP-CF $\delta$  co-localizes with the 4',6-diamidino-2-phenylindole (DAPI)-labeled DNA in untreated cells, indicating accumulation within the nucleus.

### **Nuclear accumulation of CF $\delta$ requires a putative nuclear localization signal**

PKC $\delta$  contains a series of basic amino acids in its far C-terminus (amino acids 611–623) that closely resemble the functional bipartite NLS published for Myo-D (Vandromme *et al.*, 1995) (see Figure 4C). This sequence is also conserved between the novel and two conventional PKC isoforms in that each carries at least four of the six basic amino acids (Figure 4C). To determine if this sequence functions in the nuclear import of PKC $\delta$ , pGFP-CF $\delta$  constructs were generated with various mutations in the putative NLS (Figures 4A and B). pGFP-NLM-CF1 $\delta$  contains mutations in which the left motif of the NLS sequence was mutated to alanines (K611A/R612A/K613A), while pGFP-NLM-CF4 $\delta$  contains



**Fig. 3.** Caspase cleavage of PKC $\delta$  facilitates nuclear accumulation in apoptotic cells. **(A)** Parotid C5 cells were transiently transfected with pGFP-PKC $\delta$  or pGFP-PKC $\delta^{D \rightarrow A327}$ . After 18 h, cells were left untreated or treated with 50  $\mu$ M etoposide for 8 h and harvested for immunoblot analysis for GFP expression. Arrows indicate the positions of the full-length and catalytic fragment of GFP-PKC $\delta$ . **(B)** C5 cells were transiently transfected with pGFP-PKC $\delta$  or pGFP-PKC $\delta^{D \rightarrow A327}$ . After 18 h, cells were left untreated or treated with etoposide for an additional 8 h and viewed by confocal microscopy (magnification,  $\times 20$ ). The white bar represents 10  $\mu$ m. Arrows indicate transfected cells in which PKC $\delta$  is localized to the nucleus. Arrowheads indicate cells with predominantly cytoplasmic localization of PKC $\delta$ . **(C)** Cells were counted by fluorescence microscopy and the number of cells exhibiting nuclear localization of PKC $\delta$  after treatment for the indicated time with etoposide was obtained as a percentage of the whole GFP population (~500 cells counted/variable). Gray bars represent cells transfected with pGFP-PKC $\delta$ , while the hatched bars represent cells transfected with pGFP-PKC $\delta^{D \rightarrow A327}$ . Data are the mean  $\pm$  SEM from 10 fields of view. The graph represents one of three independent experiments that gave similar results. **(D)** C5 cells were transiently transfected with pGFP-CF $\delta$ . After 15 h, cells were fixed, counterstained with DAPI (right panels) and viewed by confocal microscopy (magnification,  $\times 100$ ). The white bar represents 10  $\mu$ m.

mutations in which the right motif of the NLS sequence was mutated to alanines (K619A/K621A/K623A). pGFP-NLM-CF $\delta$  harbors mutations in all six amino acids. C5 cells were transiently transfected with pGFP-CF $\delta$  or the pGFP-NLM-CF $\delta$  mutants and visualized by confocal microscopy. As shown in Figure 5A (panels a and b), while GFP-CF $\delta$  localized predominantly to the nucleus, mutation of either the left (NLM-CF1 $\delta$ ; panels c and d) or right motif (NLM-CF4 $\delta$ ; panels e and f) or both (NLM-CF8 $\delta$ ; panels g and h) severely inhibited nuclear accumulation. As seen in Figure 5B, 91% of cells transfected with pGFP-CF $\delta$  showed nuclear localization of PKC $\delta$ , while nuclear localization was observed in only 14% of cells transfected with pGFP-NLM-CF $\delta$ .

To determine if the putative NLS also directs nuclear import of the full-length protein, we generated a pGFP-PKC $\delta$  construct in which all six basic amino acids were mutated to alanines. Cells were transiently

transfected with pGFP-PKC $\delta$  or pGFP-NLM-FL8 $\delta$ , treated with etoposide and visualized by confocal microscopy. Figure 5C shows the cellular distribution of both constructs in untreated and etoposide-treated cells, while Figure 5D shows the percentage of cells transfected with pGFP-PKC $\delta$  or pGFP-NLM-FL8 $\delta$  in which PKC $\delta$  is localized predominantly to the nucleus. As shown here, 55% of etoposide-treated cells showed nuclear accumulation of GFP-PKC $\delta$  at 8 h, while the nuclear accumulation of GFP-NLM-FL8 $\delta$  was completely inhibited in both untreated and etoposide-treated cells. These studies show that the PKC $\delta$  NLS is essential for nuclear import of both the full-length and the catalytic fragment of PKC $\delta$ . Our observation that the GFP-NLM-FL8 $\delta$  protein does not accumulate in the nucleus of untreated cells suggests that this sequence is also required for the nuclear accumulation of PKC $\delta$  in response to non-apoptotic stimuli.

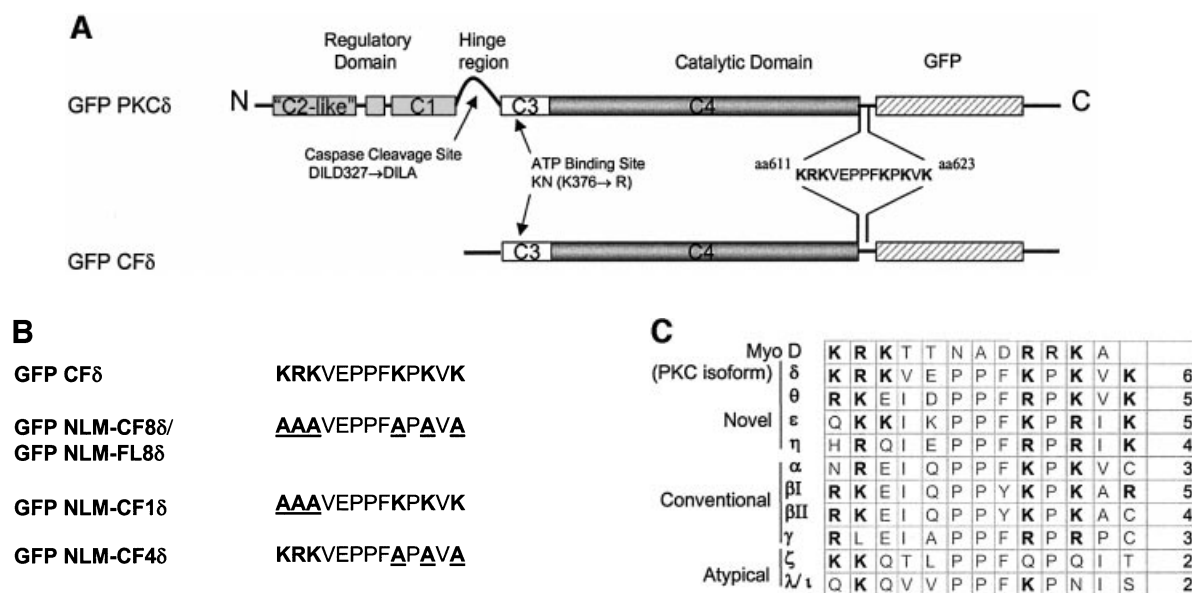
**The PKC $\delta$  NLS is sufficient to drive nuclear import**

To determine if the PKC $\delta$  NLS is sufficient in driving nuclear import of a heterologous protein, we generated a construct in which the 13 amino acids of the PKC $\delta$  NLS were cloned downstream of two copies of GFP in tandem (pGFP<sub>2</sub>- $\delta$ NLS). As seen in Figure 5E, panel a, in transfected cells the GFP<sub>2</sub> protein alone distributed diffusely throughout the cytosol and nucleus. However, as seen in panel b, fusion of the PKC $\delta$  NLS to GFP<sub>2</sub> resulted in an almost exclusively nuclear pattern of accumulation of GFP, identical to that observed when the SV40 T-antigen NLS (Nigg, 1997) was fused to GFP<sub>2</sub> (GFP<sub>2</sub>-SV40) (panel c). These studies indicate that the PKC $\delta$  NLS alone is sufficient to drive nuclear import.

**Nuclear localization of CF $\delta$  is necessary and sufficient for the induction of apoptosis**

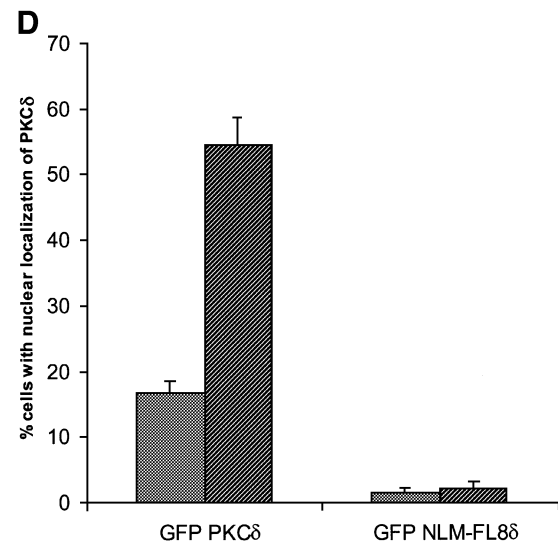
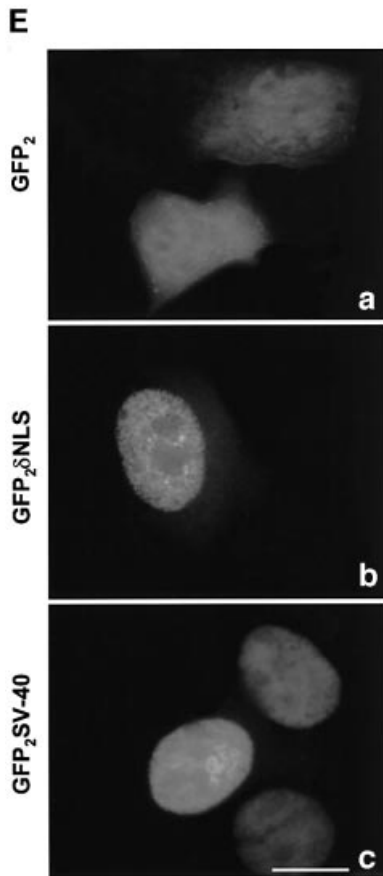
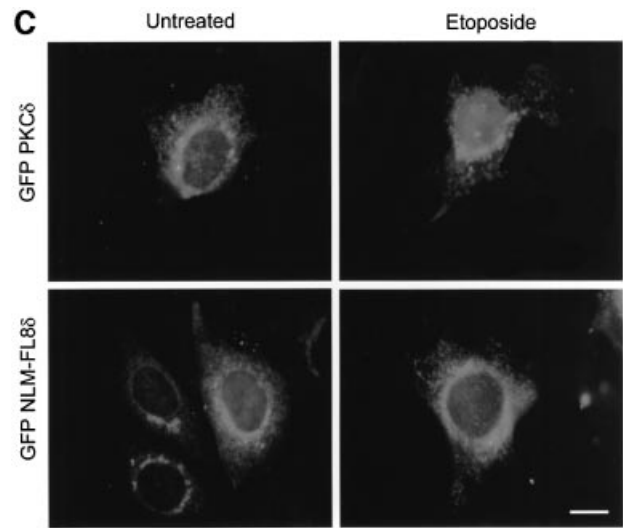
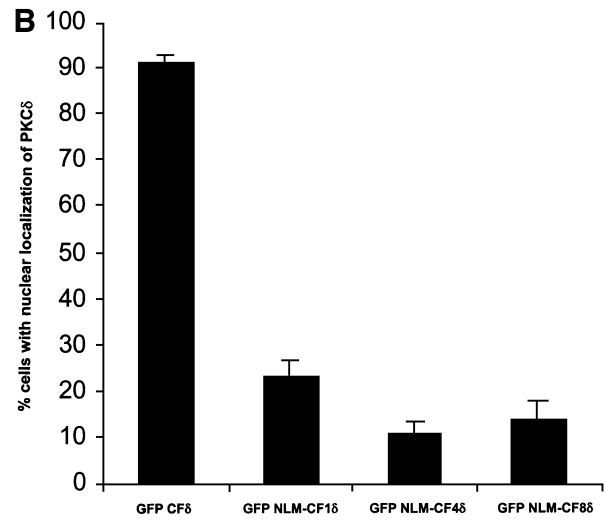
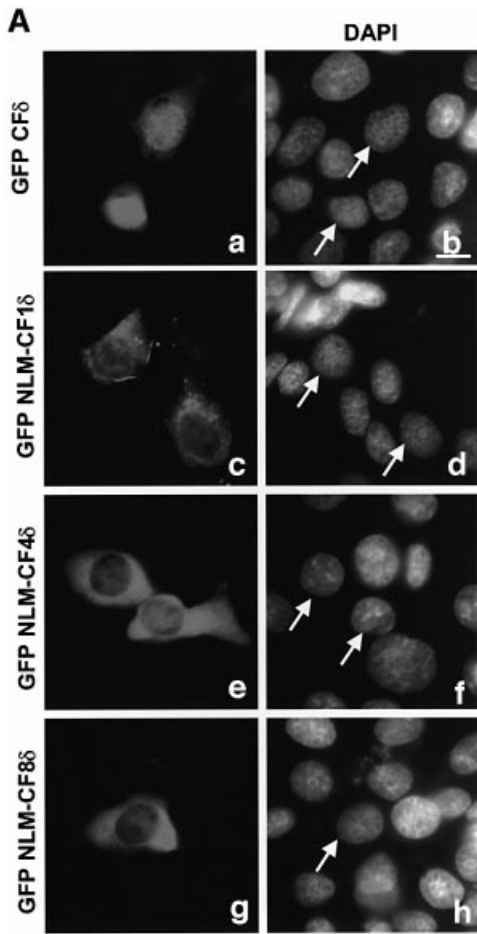
Our studies suggest that nuclear localization of the catalytic fragment of PKC $\delta$  contributes to the apoptotic pathway. To determine if nuclear localization of CF $\delta$  is required for apoptosis, we asked if GFP-NLM-CF1 $\delta$ ,

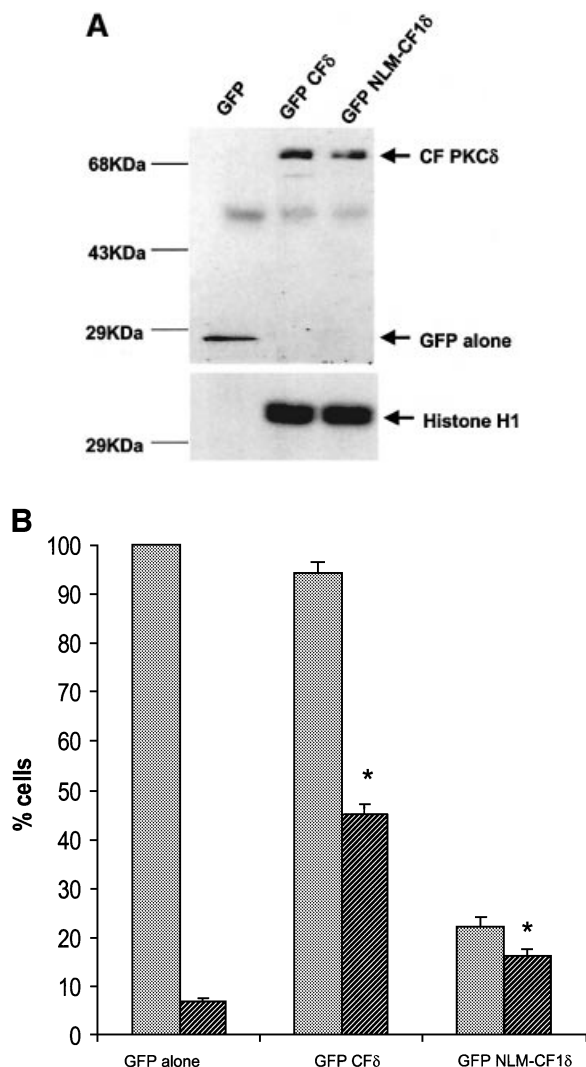
which is excluded from the nucleus, can induce apoptosis. Figure 6A shows that this mutation does not inhibit PKC $\delta$  kinase activity. Cells were transfected with pGFP-CF $\delta$ , pGFP-NLM-CF1 $\delta$  or pGFP control vector and apoptosis was assayed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis. The number of TUNEL-positive cells was expressed as a percentage of the total number of GFP-positive cells. A parallel experiment was carried out on transfected cells, and cell counts were performed to determine the percentage of cells containing GFP within the nucleus. As seen in Figure 6B, while GFP alone readily accumulates in the nucleus due to its small size, only 7% of cells transfected with pGFP were TUNEL positive. In contrast, 94% of cells transfected with pGFP-CF $\delta$  demonstrated nuclear localization of PKC $\delta$  and nearly 50% showed positive TUNEL staining, indicating that expression of GFP-CF $\delta$  results in nuclear localization and induction of apoptosis. However, only 22% of cells transfected with pGFP-NLM-CF1 $\delta$  showed nuclear localization, and 16% were TUNEL positive, indicating that nuclear localization of the PKC $\delta$



**Fig. 4.** Conservation of basic amino acids in the putative PKC $\delta$  nuclear localization motif is found in other PKC isoforms. (A) A schematic representation of mutations generated in the PKC $\delta$  full-length and catalytic fragment GFP fusion constructs used in subsequent experiments. (B) A schematic representation of mutations generated in the putative NLS of the GFP-PKC $\delta$  catalytic fragment fusion constructs used in subsequent experiments. (C) Alignment of amino acid sequences in the NLS domain of Myo-D (Vandromme *et al.*, 1995), and the putative NLS domains of PKC (DDBJ/EMBL/GenBank accession Nos: 125554, PKC $\delta$  rat; 423039, PKC $\theta$  human; 6755084, PKC $\epsilon$  mouse; 1346393, PKC $\eta$  human; 6755078, PKC $\alpha$  mouse; 125540, PKC $\beta$ I rat; 125546, PKC $\beta$ II mouse; 6981400, PKC $\gamma$  rat; 400137, PKC $\zeta$  mouse; and 4506071, PKC $\iota$  human). The far right column indicates the number of basic amino acids in this region that are conserved between the PKC sequences and PKC $\delta$ .

**Fig. 5.** A bipartite NLS is required for nuclear accumulation of PKC $\delta$ . (A) C5 cells were transiently transfected with pGFP-CF $\delta$  (panels a and b), pGFP-NLM-CF1 $\delta$  (panels c and d), pGFP-NLM-CF4 $\delta$  (panels e and f) or pGFP-NLM-CF8 $\delta$  (panels g and h). After 15 h, the cells were fixed, counterstained with DAPI and viewed by confocal microscopy for GFP (left panels) or DAPI (right panels) (magnification,  $\times 100$ ). The white bar represents 10  $\mu$ m. Arrows indicate nuclei of transfected cells. (B) To determine the rate of nuclear accumulation of GFP-CF $\delta$ , GFP-NLM-CF1 $\delta$ , GFP-NLM-CF4 $\delta$  and GFP-NLM-CF8 $\delta$ , transfected C5 cells were counted by fluorescence microscopy and the number of cells exhibiting nuclear localization of PKC $\delta$  was obtained as a percentage of the whole GFP population ( $\sim 100$  cells counted/vector). Data are the mean  $\pm$  SEM from 10 fields of view. The graph represents one of three independent experiments that gave similar results. (C) Cells were transiently transfected with pGFP-PKC $\delta$  or pGFP-NLM-FL8 $\delta$ . After 18 h, cells were left untreated or treated with etoposide for an additional 8 h and viewed by confocal microscopy (magnification,  $\times 100$ ). The white bar represents 10  $\mu$ m. (D) To determine the rate of nuclear accumulation of PKC $\delta$ , transfected cells were counted by fluorescence microscopy and the number of cells exhibiting nuclear localization of PKC $\delta$  after treatment with etoposide was obtained as a percentage of the whole GFP population. Gray bars represent untreated cells, while the hatched bars represent etoposide-treated cells (8 h). Data are the mean  $\pm$  SEM from 10 fields of view and represent one of three independent experiments that gave similar results;  $>100$  cells were counted per variable for each experiment. (E) Cells were transiently transfected with pGFP<sub>2</sub> (panel a), pGFP<sub>2</sub>  $\delta$ NLS (panel b) or pGFP<sub>2</sub> SV-40 (panel c). After 18 h, the cells were analyzed by fluorescence microscopy (magnification,  $\times 100$ ). The white bar represents 10  $\mu$ m.





**Fig. 6.** Nuclear localization of PKC $\delta$  CF is necessary and sufficient for the induction of apoptosis. **(A)** Enzymatic activity of the GFP-CF $\delta$  and GFP-NLM-CF1 $\delta$  proteins and a GFP alone negative control was determined by an immunoprecipitation kinase assay. Top panel: an immunoblot for GFP of the immunoprecipitated GFP fusion proteins. Bottom panel: phosphorylated histone H1 substrate. **(B)** C5 cells were transfected with pGFP, pGFP-CF $\delta$  or pGFP-NLM-CF1 $\delta$  and after 15 h were prepared for TUNEL analysis. TUNEL-positive cells containing GFP were visualized by immunofluorescence microscopy and counted using a 20 $\times$  objective. TUNEL-positive, GFP-positive cells were quantitated as the percentage of the total number of GFP-positive cells per field. Parallel experiments were carried out to determine the percentage of cells containing GFP in the nucleus as previously described. Gray bars represent the percentage of GFP-positive cells exhibiting nuclear accumulation of GFP. Hatched bars represent the percentage of GFP-transfected cells that are TUNEL positive. The graph represents one of three independent experiments, which all produced similar results. At least 100 cells were counted for each variable per experiment. Data are the mean  $\pm$  SEM from 10 fields of view.

CF is necessary for the induction of apoptosis. The difference in the ability of GFP-CF $\delta$  and GFP-NLM-CF1 $\delta$  to induce cell death was also verified by cell counts, which showed a 43% reduction in GFP-positive cells in the pGFP-CF $\delta$ -transfected group, indicating a significant loss of cells, and a 17% reduction in the pGFP-NLM-CF1 $\delta$ -transfected group relative to cells transfected with the

vector alone (data not shown). Taken together, our data demonstrates that nuclear localization of PKC $\delta$  is both necessary and sufficient to induce apoptosis.

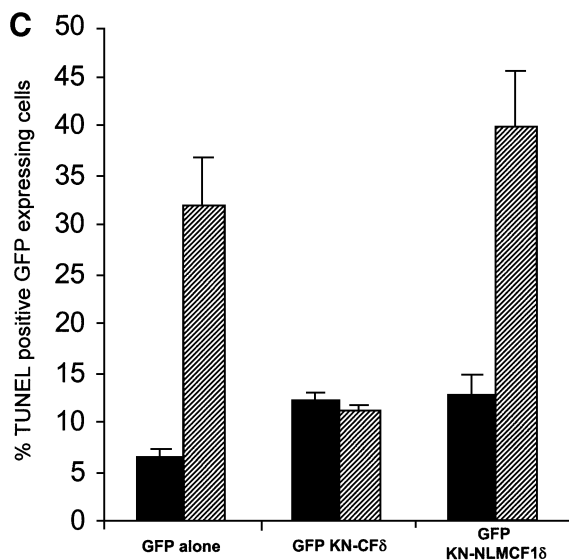
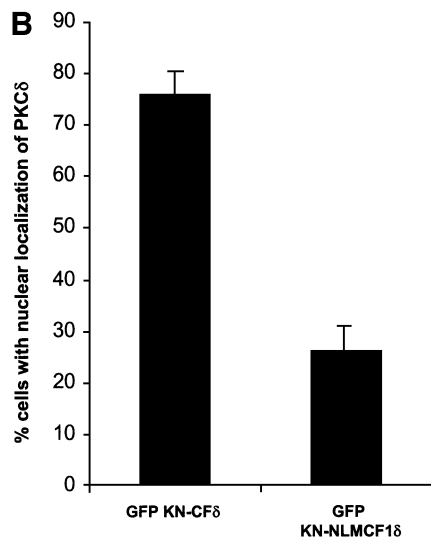
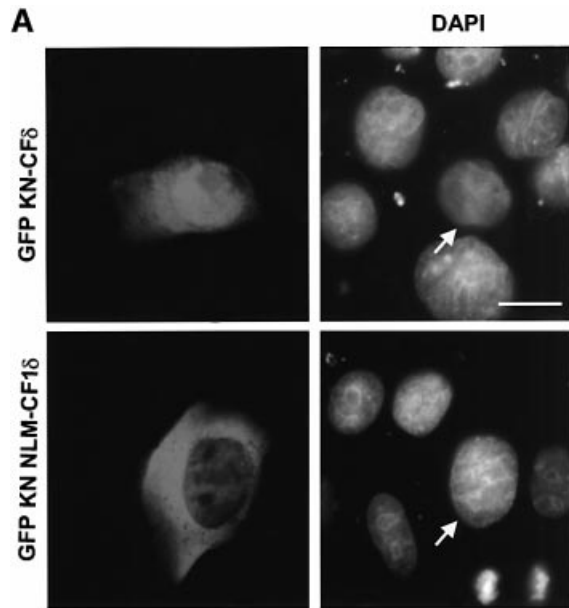
#### **Nuclear, but not cytoplasmic, expression of KN-CF $\delta$ inhibits etoposide-induced apoptosis**

The data presented above indicate that GFP-CF $\delta$  preferentially accumulates in the nucleus in the absence of an apoptotic stimulus and that exclusion of PKC $\delta$  from the nucleus blocks its ability to initiate apoptosis. To determine whether nuclear PKC $\delta$  is also required for apoptosis induced by DNA-damaging agents, we generated a kinase-negative construct of the catalytic fragment of PKC $\delta$  fused to GFP (pGFP-KN-CF $\delta$ ) and the same construct in which the NLS was abolished (pGFP-KN NLM-CF1 $\delta$ ). We have shown previously that a full-length KN PKC $\delta$  can suppress apoptosis in response to etoposide (Li *et al.*, 1995; Matassa *et al.*, 2001). Shown in Figure 7A, in the absence of etoposide stimulation, GFP-KN-CF $\delta$ , like GFP-CF $\delta$ , localized primarily to the nuclei, indicating that kinase activity is not required for nuclear translocation. As expected, mutation of the putative NLS inhibited nuclear accumulation of the catalytic fragment (GFP-KN NLM-CF1 $\delta$ ) (Figure 7A and B). We took advantage of the unique subcellular localization of GFP-KN-CF $\delta$  and GFP-KN NLM-CF1 $\delta$  to ask if expression of KN-CF $\delta$  can inhibit etoposide-induced apoptosis, and if nuclear localization of KN-CF $\delta$  is required for inhibition. As seen in Figure 7C, expression of GFP-KN-CF $\delta$  protected cells from etoposide-induced apoptosis as seen by an almost 65% reduction in TUNEL-positive cells as compared with cells transfected with GFP alone and treated with etoposide. However, in cells transfected with pGFP-KN NLM-CF1 $\delta$ , no suppression of etoposide-induced apoptosis was observed. These results demonstrate that GFP-KN-CF $\delta$  must be imported into the nucleus in order to inhibit etoposide-induced apoptosis, and thus provide conclusive evidence that active nuclear PKC $\delta$  is required to initiate the apoptotic process.

#### **Discussion**

Work from our laboratory and others demonstrates an essential role for PKC $\delta$  as a regulator of mitochondrial-dependent apoptosis (Ghayur *et al.*, 1996; Mizuno *et al.*, 1997; Bharti *et al.*, 1998). In the present studies, we have shown that nuclear accumulation of PKC $\delta$  is both necessary and sufficient for the initiation of apoptosis and have defined a novel and functional NLS sequence in the C-terminus of PKC $\delta$ . We show that nuclear PKC $\delta$  activity is also required for etoposide-induced apoptosis. Taken together, these findings suggest that PKC $\delta$  acts on specific targets within the nucleus that are required directly or indirectly for the induction of apoptosis. Furthermore, they imply that nuclear PKC $\delta$  regulates the cytosolic apoptotic machinery, perhaps by controlling expression or activity of a key apoptotic molecule(s).

Endogenous PKC $\delta$  is located primarily in the perinuclear region in salivary epithelial cells, although diffuse staining is also seen within the cytosol, and in some cells within the nucleus. Our studies clearly demonstrate that cytosolic PKC $\delta$  translocates into the nucleus upon treatment of cells with etoposide, and support previous



observations that PKC $\delta$  localizes to the nucleus during Fas ligand- and cytokine deprivation-induced apoptosis in T cells (Scheel-Toellner *et al.*, 1999), during irradiation-induced apoptosis in MCF-7 cells (Yuan *et al.*, 1998) and in response to etoposide treatment in C6 glioma cells (Blass *et al.*, 2002). It is worth noting that several nuclear proteins have been shown to be substrates of PKC $\delta$  *in vitro*, such as lamin B, DNA-PK and c-Abl tyrosine kinase (Bharti *et al.*, 1998; Cross *et al.*, 2000; Sun *et al.*, 2000). While others have reported that PKC $\delta$  translocates to the mitochondria in response to some apoptotic stimuli (Li *et al.*, 1999; Majumder *et al.*, 2000), we find no evidence of PKC $\delta$  association with the mitochondria in untreated or in apoptotic salivary epithelial cells.

Our studies demonstrate that the kinetics of nuclear translocation parallel the caspase cleavage of PKC $\delta$ , suggesting that these events are linked. Mutation of the caspase cleavage site severely inhibited the ability of PKC $\delta$  to translocate to the nucleus following etoposide treatment; however, nuclear translocation was not completely blocked. Likewise, pre-treatment of cells with the caspase inhibitor ZVAD(Ome)-FMK also inhibits etoposide-induced nuclear translocation of PKC $\delta$  (T.A.DeVries and M.E.Reyland, unpublished data). This suggests that caspase cleavage facilitates translocation of PKC $\delta$  to the nucleus, but is not required for nuclear accumulation *per se*. This is supported further by our observation that in transfected cells GFP-PKC $\delta$  required an apoptotic stimulus to translocate to the nucleus, while GFP-CF $\delta$  localized to the nucleus in the absence of such a stimulus. Taken together, our studies indicate that caspase cleavage of PKC $\delta$  is an important component of the apoptotic response, since it allows for the nuclear accumulation of an activated form of PKC $\delta$ . Notably, apoptotic agents such as 12-*o*-tetradecanoylphorbol-13-acetate (TPA), which do not induce PKC $\delta$  cleavage, only weakly induce apoptosis in C5 cells (Reyland *et al.*, 2000).

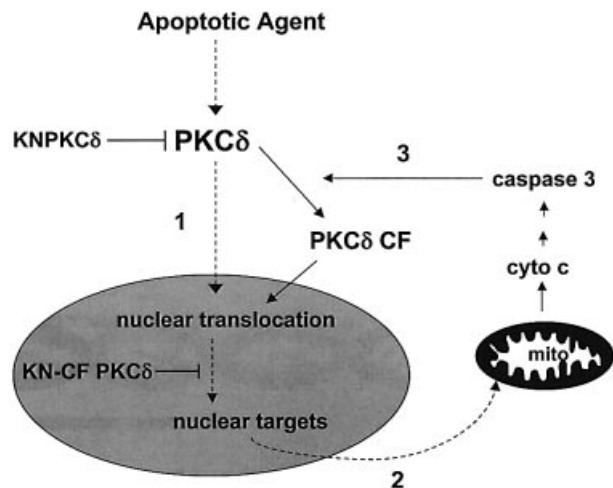
We show that CF $\delta$  is capable of localizing to the nucleus, suggesting that the structural requirement for nuclear localization of PKC $\delta$  resides in the catalytic domain and not the regulatory domain. Analysis of the protein sequence of PKC $\delta$  demonstrates a putative NLS

**Fig. 7.** Nuclear, but not cytoplasmic, expression of KN-CF $\delta$  inhibits etoposide-induced apoptosis. (A) C5 cells were transiently transfected with pGFP-KN-CF $\delta$  (top panels) or pGFP-KN NLM-CF1 $\delta$  (bottom panels). After 15 h, the cells were fixed, counterstained with DAPI and viewed by confocal microscopy for GFP (left panels) or DAPI (right panels) (magnification,  $\times 100$ ). The white bar represents 10  $\mu$ m. Arrows indicate nuclei of transfected cells. (B) To determine the rate of nuclear accumulation of GFP-KN-CF $\delta$  and GFP-KN NLM-CF1 $\delta$ , transfected C5 cells were counted by fluorescence microscopy and the number of cells exhibiting nuclear localization of PKC $\delta$  was obtained as a percentage of the whole GFP population ( $\sim 100$  cells counted/vector). Data are the mean  $\pm$  SEM from 10 fields of view per experiment and represent the average of three independent experiments. (C) TUNEL analysis was performed on untreated (black bars) and etoposide- (8 h, hatched bars) treated cells transfected with pGFP, pGFP-KN-CF $\delta$  or pGFP-KN NLM-CF1 $\delta$ . After 18 h, TUNEL positive GFP-positive cells were visualized by immunofluorescence microscopy and counted using a 40 $\times$  objective as described above. Data are the mean  $\pm$  SEM from at least 10 fields of view per experiment and represent the average of three independent experiments. More than 100 cells were counted per variable for each experiment.

between amino acids 611 and 623 of PKC $\delta$  that resembles the functional NLS published for Myo-D (Vandromme *et al.*, 1995). When mutations were made within this sequence, nuclear localization was significantly inhibited, demonstrating that these basic amino acids are required for efficient import of PKC $\delta$  (Figure 5). Additionally, both KRK (611–613) and KKK (619, 621 and 623) are required for nuclear import as neither half can drive nuclear translocation independently. We show that the PKC $\delta$  NLS is required for nuclear translocation of the PKC $\delta$  catalytic fragment as well as full-length PKC $\delta$ . In fact, mutation of this sequence results in near total exclusion of full-length PKC $\delta$  from the nucleus of untreated cells, suggesting that this sequence regulates nuclear entry of PKC $\delta$  in response to non-apoptotic stimuli. The 13 amino acid PKC $\delta$  NLS is also sufficient for driving nuclear import of a tandem GFP protein, indicating that it functions as a canonical NLS. Sequence alignment of the novel, conventional and atypical PKC isoforms shows that the PKC $\delta$  NLS is highly conserved in this kinase family (Figure 4C). Interestingly, PKC $\theta$  contains five of the six conserved basic residues of the putative PKC $\delta$  NLS and has also been shown to translocate to the nucleus in A549 lung carcinoma cells in response to staurosporine (Jones *et al.*, 1997). PKC $\beta$ II, which translocates to the nucleus during cell proliferation, shares four of the six basic amino acids (Walker *et al.*, 1995; Gokmen-Polar and Fields, 1998). Recently, PKC $\lambda$ I and  $\zeta$  have been shown to contain a functional NLS within the N-terminal regulatory region of the kinase (Perander *et al.*, 2001).

Our studies indicate that even in the absence of caspase cleavage, some PKC $\delta$  can localize to the nucleus. This is an important observation since our previous studies, as well as recent studies on cells from PKC $\delta$ -null mice (Leitges *et al.*, 2001), indicate that PKC $\delta$  is required for early events in the apoptotic pathway which occur at, or prior to, the mitochondria. Since caspase cleavage of PKC $\delta$  is a post-mitochondrial event (Emoto *et al.*, 1995), the implication is that the full-length PKC $\delta$  protein can also function as a regulator of apoptosis. This is supported by recent studies that showed that tyrosine phosphorylation of the regulatory domain of full-length PKC $\delta$  was required for caspase-3 activation and induction of apoptosis. Interestingly, when the tyrosine phosphorylation sites were mutated, they found that full-length PKC $\delta$  failed to induce apoptosis, but did, however, translocate to the nucleus (Blass *et al.*, 2002).

A model presented in Figure 8 suggests that full-length PKC $\delta$  may be translocated initially into the nucleus where it regulates an early event required for initiation of apoptosis and the subsequent activation of caspase. Activation of caspase resulting in caspase cleavage of PKC $\delta$  increases the rate of PKC $\delta$  nuclear translocation, and results in amplification of the apoptotic signal. Our studies indicate that a PKC $\delta$ -dependent nuclear event regulates initiation of the apoptotic pathway. This scenario presented in Figure 8 predicts that full-length PKC $\delta$  and the CF $\delta$  may have the same nuclear target(s). Furthermore, since the apoptotic machinery resides in the cytosol, our results imply that PKC $\delta$  functions in the nucleus to regulate the cytosolic apoptotic machinery.



**Fig. 8.** Steps in the regulation of apoptosis by PKC $\delta$ . (1) PKC $\delta$  is translocated to the nucleus. We have shown previously that expression of KN PKC $\delta$  inhibits an early event in the apoptotic pathway that occurs at, or prior to, the mitochondria (Matassa *et al.*, 2001). Our current studies suggest that this PKC $\delta$ -dependent event occurs in the nucleus, and that it may regulate entry into the apoptotic pathway. We show that PKC $\delta$  is translocated into the nucleus during apoptosis, and that nuclear PKC $\delta$  activity is required for apoptosis. (2) PKC $\delta$  functions in the nucleus to regulate apoptosis. Expression of GFP-CF $\delta$ , which localizes to the nucleus, induces apoptosis, whereas nuclear expression of GFP-KN-CF $\delta$  blocks apoptosis. These studies suggest that nuclear PKC $\delta$  can regulate the cytosolic apoptotic machinery, resulting in caspase activation and DNA fragmentation. One possibility is that nuclear PKC $\delta$  may regulate the expression or activity of key apoptotic molecules which act at, or upstream of, the mitochondria. (3) Caspase cleavage of PKC $\delta$  facilitates nuclear import, hence caspase cleavage of PKC $\delta$  may function to amplify the apoptotic response by enabling rapid import of PKC $\delta$  into the nucleus of apoptotic cells.

## Materials and methods

### Cells and cell culture

The isolation of C5 rat parotid salivary acinar cells was described previously (Quissell *et al.*, 1998). Cells were cultured on Primaria 100 mm culture dishes (Falcon Plastics, Franklin Lakes, NJ) in a 1:1 mixture of Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented with 2.5% fetal calf serum, 5  $\mu$ g/ml transferrin, 1.1  $\mu$ M hydrocortisone, 0.1  $\mu$ M retinoic acid, 2.0 nM T3, 5  $\mu$ g/ml insulin, 80 ng/ml epidermal growth factor (Collaborative Biomedical Products, Bedford, MA), 5 mM L-glutamine, 50  $\mu$ g/ml gentamicin sulfate and a trace element mixture (Biofluids, Rockville, MD). Tissue culture reagents were obtained from Gibco-BRL (Gaithersburg, MD). Etoposide (Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) and used at a final concentration of 50  $\mu$ M in all experiments. LMB (Sigma-Aldrich) was used at a final concentration of 5 ng/ml for 6 h in all experiments.

For immunofluorescence studies, cells were grown on 12 mm coverslips in 12-well polystyrene dishes. For immunoblot analysis, cells were grown on Primaria 100 mm culture dishes. Subconfluent C5 cells were transiently transfected 15–18 h prior to etoposide treatment using a 6:1 lipid:DNA ratio of FuGene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol.

### Construction of EGFP plasmids, and site-directed mutagenesis

Mouse wild-type PKC $\delta$  and the dominant-negative PKC $\delta$ <sup>K376R</sup> were cloned into pEGFP-N1. The constructs, pPKC $\delta$ -EGFP (pGFP-PKC $\delta$ ) and pPKC $\delta$ <sup>K376R</sup>-EGFP, along with pEGFP (pGFP alone; Clontech, Palo Alto, CA) were obtained as a generous gift from Dr Stuart Yuspa (National Institutes of Health) (Li *et al.*, 1999). The pPKC $\delta$ -EGFP and pPKC $\delta$ <sup>K376R</sup>-EGFP constructs express a protein in which enhanced GFP is fused to the C-terminus of the PKC $\delta$  protein. The K376 $\rightarrow$ R mutation in kinase-negative PKC $\delta$  resides in the ATP-binding site and the protein

has been shown to function as an isoform-specific dominant inhibitory kinase (Li *et al.*, 1995). A mutation in the caspase cleavage site (pGFP-PKC $\delta^{D\rightarrow A327}$ ) was generated from pPKC $\delta$ -EGFP by PCR site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA) according to the manufacturer's protocol (Clontech, Palo Alto, CA). The primers used were: primer 1, 5'-GTGACATCCTAGCCAACAACGGGACC-3'; and primer 2, 5'-GGTCCCCTTGGCTAGGATGTCAC-3'.

Catalytic fragments of wild-type and kinase-negative PKC $\delta$  were generated by PCR using full-length pPKC $\delta$ -EGFP and pPKC $\delta^{K376R}$ -EGFP as a template starting at the caspase cleavage site (amino acid 325) and finishing at the end of GFP. The primers used were: primer 1, 5'-GATGGAATTCGCCACCATGAACAACGGGACCTATGGCAAG-3'; and primer 2, 5'-GGCTGATTATGATCTAGAGTCGCG-3'. Primer 1 incorporated an *EcoRI* site, Kozak sequence and a start codon. Primer 2 contained an endogenous *XbaI* site found downstream of the stop codon in the pPKC $\delta$ -EGFP plasmid. The PCR product was double digested with *EcoRI* and *XbaI*, then inserted and ligated into a pEGFP-N1 vector backbone lacking the GFP gene. The resulting constructs were named pPKC $\delta$  CF-EGFP (pGFP-CF $\delta$ ) and pPKC $\delta$  CF $^{K376R}$ -EGFP (pGFP-KN-CF $\delta$ ).

The pPKC $\delta$  CF $^{K611A/R612A/K613A}$ -EGFP (pGFP-NLM-CF1 $\delta$ ) and pPKC $\delta$  CF $^{K376R/K611A/R612A/K613A}$ -EGFP (pGFP-KN NLM-CF1 $\delta$ ) were generated by site-directed mutagenesis according to the manual for the Quick-Change Site-directed Mutagenesis Kit (Stratagene) using pGFP-CF $\delta$  or pGFP-KN-CF $\delta$ , respectively, as a template with the primers: primer 1, 5'-GGTCCCTCCTGGAGGCGCGCGGTGGAGCCGC-3'; and primer 2, 5'-GCGGCTCCACCGCCGCCCTCCAGGAGGG-ACC-3'.

The pPKC $\delta$  CF $^{K619A/K621A/K623A}$ -EGFP (pGFP-NLM-CF4 $\delta$ ) and pPKC $\delta$  CF $^{K611A/R612A/K613A/K619A/K621A/K623A}$ -EGFP (pGFP-NLM-CF8 $\delta$ ) were generated as described above using pGFP-CF $\delta$  or pGFP-NLM-CF1 $\delta$ , respectively, as a template with the primers: primer 1, 5'-GAGCCGCTTTGGCGCCCGCAGTGGCATCCCTTCAGAC-3'; and primer 2, 5'-GTCTGAAGGGGATGCCACTGCGGGCGCAAAGGCGGCTC-3'.

The pPKC $\delta$  FL $^{K611A/R612A/K613A/K619A/K621A/K623A}$ -EGFP (pGFP-NLM-FL8 $\delta$ ) was generated as described above using pGFP-NLM-FL1 $\delta$  as a template with the above primers.

The pGFP $_2$  and pGFP $_2$  SV40 vectors were obtained as a generous gift from Drs Yihong Wan and Steve Nordeen (University of Colorado Health Sciences Center). pGFP $_2$   $\delta$ NLS was generated by inserting the following annealed primer pair into a *Bam*HI site downstream of GFP $_2$ : primer 1, 5'-GATCCTGGAAAAGCGGAAGGTGGAGCCGCTTTAAGCCAAAGTGAAAAG-3'; and primer 2, 5'-GATCCTTTCACCTTTGGGCTTAAAGGCGGCTCCACCTCCGCTTTCCAG-3'.

Automated sequencing and immunoblot analysis were used to verify the identity and size of all constructs. PCR primers were purchased from either Gibco-BRL (La Jolla, CA) or Macromolecular Resources (Fort Collins, CO).

### Immunoblot analysis

Cells were harvested for immunoblots as described previously (Reyland *et al.*, 1999). Enhanced chemiluminescence (NEN) was used to detect the signal. The mouse monoclonal antibody to GFP was obtained from Zymed Laboratories Inc. (San Francisco, CA). Rabbit polyclonal antibodies to PKC $\delta$  and lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal  $\alpha$ -tubulin from NeoMarkers (Fremont, CA).

### Isolation of cytosolic and nuclear enriched fractions

Adherent cells were scraped onto phosphate-buffered saline (PBS) and collected by centrifugation (1000 *g* for 5 min). The cells were washed in 1 ml of PBS, centrifuged and the cell pellet resuspended in 300  $\mu$ l of nuclei isolation buffer [20 mM HEPES-KOH, 100 mM KCl, 1.5 mM MgCl $_2$ , 1 mM EGTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 10  $\mu$ g/ml each of aprotinin, leupeptin and pepstatin]. The cells were incubated on ice for 20 min, and then lysed using a Dounce homogenizer (25 $\times$ ). Efficient cell lysis was verified by Trypan 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  $\mu$ l of nuclei isolation buffer, pelleted by centrifugation and the resulting supernatant was added to the cytosolic extract. To solubilize the proteins in both cytosolic and nuclear enriched fractions, Triton X-100 was added to a final concentration of 1.0%. Protein concentration was quantified using the DC Protein Assay Kit (Bio-Rad).

### Immunofluorescence microscopy

Cells were washed with PBS and fixed in 2% paraformaldehyde/PBS for 15 min, followed by permeabilization with 0.5% Triton X-100/PBS for 5 min at room temperature. Cells were washed with PBS and blocked for 1 h in 20 mg/ml of bovine serum albumin/PBS prior to incubation with a rabbit polyclonal PKC $\delta$  primary antibody (no. 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 (FITC)-conjugated secondary antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) containing 5  $\mu$ g/ml DAPI (Sigma, St Louis, MO) for 1 h. Cells were washed again five times with PBS and coverslips were mounted with *o*-phenylenediamine dihydrochloride (OPDA) mounting medium (Sigma, St Louis, MO). Mounted slides of cells transfected with GFP were prepared in the same way without the antibody staining. In some experiments, cells were incubated with 300 nM Mitotracker red (Molecular Probes, Eugene, OR) for 15 min prior to fixation. In LMB experiments, cells were co-incubated with a mouse cyclin B1 primary antibody (no. sc-245, Santa Cruz Biotechnology) and a donkey anti-mouse Cy3-conjugated secondary antibody as described above. Cells were visualized and images 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). Multi-fluor images were merged, deconvolved and normalized using SlideBook software.

### TUNEL analysis and cell counts

TUNEL analysis was performed using the *in situ* Cell Death Detection Kit TMR Red (Roche Molecular Biochemicals; Indianapolis, IN) according to the manufacturer's protocol. GFP-positive cells were visualized by immunofluorescent microscopy and counted using a 20 $\times$  objective. TUNEL-positive cells containing GFP were identified by co-localization with DAPI and by morphology, and were quantitated as the percentage of the total GFP-positive cells per field. More than 100 cells were counted for each variable per experiment. Experiments quantitating the percentage of cells exhibiting nuclear accumulation of GFP were performed by immunofluorescence microscopy using a 40 $\times$  objective.

### Immunoprecipitation kinase assay

The kinase activity of the PKC $\delta$ -GFP fusion proteins was assayed by an immunoprecipitation kinase assay using histone H1 as substrate as previously described. Antibody used in immunoprecipitation was the rabbit polyclonal anti-GFP-ab290 (Abcam) (Reyland *et al.*, 1999).

## Acknowledgements

The contributions of Kathy Barzen, Linda Sanders, Dr Shinobu Umemura and Linda Hanson are gratefully acknowledged. We thank Drs James DeGregori and Peter Parker for comments on the manuscript. This work is supported by Public Health Service grant PO1DE12798-02 from the National Institute of Dental and Craniofacial Research to M.E.R.

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Received March 26, 2002; revised August 6, 2002;  
accepted September 23, 2002