PKCδ REGULATES APOPTOSIS VIA ACTIVATION OF STAT1

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SUMMARY

PKCδ is required for mitochondrial-dependent apoptosis, however little is known about downstream effectors of PKCδ in apoptotic cells. Here we show that activation of STAT1 is an early response to DNA damage, and that STAT1 activation requires PKCδ. Treatment of HeLa cells with etoposide results in phosphorylation of STAT1 on S727, and the association of STAT1 with PKCδ. Etoposide increases transcription from STAT1 dependent reporter constructs. Increased transcription, as well as STAT1 S727 phosphorylation, can be blocked by inhibition or depletion of PKCδ. To ask if STAT1 is required for PKCδ mediated apoptosis, we utilized U3A STAT1 deficient cells. Induction of apoptosis by PKCδ is suppressed in U3A cells, but can be rescued by co-transfection with STAT1α, but not STAT1 mutated at S727. Nuclear accumulation of STAT1, phospho-S727 STAT1 and PKCδ are detectable 30-60 mins after treatment with etoposide. Nuclear localization is necessary for apoptosis, as a nuclear localization mutant of PKCδ does not induce apoptosis in U3A cells reconstituted with STAT1α, and a nuclear localization mutant of STAT1 does not support PKCδ induced apoptosis in U3A cells. Our data identify STAT1 as a downstream target of PKCδ, and suggest that PKCδ may regulate apoptosis by activation of STAT1 target genes.
INTRODUCTION

Drugs, chemicals, irradiation and cellular stress induce apoptosis via activation of the mitochondria-dependent, or intrinsic, apoptotic pathway (1-3). Apoptotic signals act at the mitochondria to induce the release of cytochrome c, activation of the initiator caspase, caspase-9, and the subsequent activation of effector caspases (2,4). PKCδ, a ubiquitously expressed member of the novel sub-family of protein kinase C (PKC) isoforms, is required for activation of this pathway by diverse agents including etoposide (5), ionizing radiation (6), ara-C (7), FAS ligand (8), brefeldin A, and Taxol (9). Studies from our laboratory and others have shown that PKCδ functions early in the apoptotic pathway and that inhibition of PKCδ suppresses the release of cytochrome c and caspase activation (7-10). PKCδ has been shown to translocate to the nucleus in response to DNA damaging agents including etoposide and γ-irradiation (11-13), and we have previously shown that nuclear localization of PKCδ is both necessary and sufficient for initiation of the mitochondrial-dependent apoptotic pathway (12). Substrates of PKCδ in apoptotic cells appear to be largely nuclear and include lamin B, the DNA repair protein, DNA-PK, the p53 family member p73, and the cell cycle protein Rad9 (14-16).

The signal transducer and activator of transcription (STAT) family of transcription factors are induced by interferon and cytokines, and regulate the expression of a large group of genes involved in inflammation and anti-viral defense (17). Studies in cell lines and transgenic mice suggest that some members of the STAT family also regulate cell death. STAT1 is required for apoptosis induced by ischemia/reperfusion in cardiac myocytes, and by TNFα, oxysterols, and DNA damage (18-20). STAT1 deficient U3A human fibroblasts cells are resistant to apoptosis and this correlates with suppression of caspase and p21WAF1 expression (18,19). Fibroblasts derived from STAT1 -/- mouse embryos show reduced p53 response to
DNA damaging agents and increased expression of the p53 inhibitor, Mdm2, suggesting that STAT1 is required for p53 dependent apoptosis (20).

Activation of STAT1, leading to the transcription of STAT1 regulated genes, is regulated by phosphorylation at Y701 and S727. Phosphorylation at Y701 by the Janus family of tyrosine kinases (JAK)\(^1\) leads to STAT1 dimerization via its SH2 domains, exposure of a dimer specific NLS, and subsequent nuclear translocation (21,22). Phosphorylation of STAT1 at S727 is critical for maximal transcriptional activation and for the interaction of STAT1 with transcriptional co-factors (18,23,24). Activated STATs, together with co-factors such as c-Jun, BRCA1, MCM5, IRF-1, IRF-9 and CREB, regulate transcription by binding to GAS or ISRE elements in the promoters of specific target genes (21,25). In the context of apoptosis, phosphorylation of STAT1 at S727 appears to be essential, while phosphorylation at Y701 may be dispensable (18,26-28). A variety of serine/threonine kinases have been shown to contribute to STAT1 S727 phosphorylation, including members of the ERK family and Akt (29,30). Recently, STAT1 has been identified as a PKC\(\delta\) substrate in cells treated with Type I or Type II interferon (31,32).

The identification of STAT1 S727 as a potential regulatory site for STAT1 pro-apoptotic functions, and the observation that PKC\(\delta\) can phosphorylate STAT1 at S727 in response to some stimuli, suggests a link between these two pro-apoptotic signaling pathways. Here we show that activation of STAT1 is an early response to DNA damaging agents, and that activation of STAT1 in apoptotic cells requires PKC\(\delta\). Our studies indicate that induction of apoptosis requires nuclear translocation of PKC\(\delta\) and STAT1, as well as STAT1 S727 phosphorylation. These studies demonstrate a novel and functional interaction between PKC\(\delta\) and STAT1 during DNA damage induced apoptosis.
EXPERIMENTAL PROCEDURES

Cells and cell culture. HeLa cells were cultured in Dulbecco's Modified Eagle Media supplemented with 10% fetal calf serum. The parental human fibroblast cells lines (2fTGH and 2C4), and mutant cell lines deficient in JAK and STAT signaling proteins (U3A, STAT1; U1A, TYK2; U4A, JAK1; γ2A, JAK2) (33-35) were generously provided by Dr. George Stark (The Cleveland Clinic Foundation, OH) and were cultured in Dulbecco's Modified Eagle Media supplemented with 10% fetal calf serum. The salivary parotid C5 cell line was cultured on Primaria culture dishes (Falcon Plastics, Franklin Lakes, NJ) as previously described (36).

Immunoblot analysis and immunoprecipitation. Cells were harvested for immunoblot analysis using MSLB buffer (15mM Tris pH 7.4, 150mM NaCl, 1% Triton-X, 1mM EDTA, 1mM DTT, 10mM NaF, 1mM NaVO₄, 1mM PMSF, 20µg/ml Aprotinin, and 20µg/ml Leupeptin). Nuclear fractions were isolated using a Nuclear/Cytosol Fractionation kit (Biovision) according to the manufacture’s instructions except that one mM NaVO₃ was included in all buffers and Triton X-100 was added to the nuclear extraction buffer at a final concentration of 1%. Protein concentrations were determined using the DC Protein Assay Kit (Biorad Laboratories, Hercules CA). The following antibodies were used: anti-GFP, Zymed Laboratories Inc (C163); anti-human PKCδ, Santa Cruz Biotechnology (C-20, sc-937); anti-STAT1 p84/p91, Santa Cruz Biotechnology (E23); anti-phospho-Y701 (Y701) STAT1, Cell Signaling Technology (9171S) or Santa Cruz Biotechnology (sc-7988); anti-phospho-S727 (S727) STAT1, Upstate Biotechnology (06-802); anti-tubulin, BD Pharmingen (#556321); anti-
Immunoblot analysis was done as previously described using 50 or 100 µg of protein (5). For immunoprecipitation, 0.5 or 1 mg of protein was mixed with the appropriate antibody overnight and the antigen-antibody complexes were recovered by incubation with Sepharose-protein A (Sigma) for 1-4 hours at 4ºC. The beads were washed 3 times in MSLB buffer and the precipitated proteins were resolved on a 10% SDS-polyacrylamide gel.

**Plasmid Constructs.** Generation of mouse pGFPPKCδ and pGFPNLFL8δ has been previously described (12). The plasmids pSTAT1α (ST1α) and pSTAT1αS727A (ST1αS727A) were obtained from Dr. James Darnell, Rockefeller University (23). The plasmids pGFP STAT1α (ST1α) and pGFPST1αL407A (L407A) were obtained from Dr. Nancy Reich, SUNY Stony Brook (22). The interferon stimulatory response element (ISRE) and GAS cis-Reporting Systems, and the pCIS-CK negative control plasmid were purchased from Stratagene. To generate the PKCδ specific siRNA, pRETRO-SUPER (37) was digested with BglII/HindIII and ligated to the double stranded oligo 5’ GATCCCCGAACGCTTCAACATCGACATTCAAGAGATGCGATGTGAAGCGTTCTTTTTGGAAA3’.

**Transient transfection.** For TUNEL analysis subconfluent 2fTGH and U3A cells were transiently transfected using a 6:1 lipid /DNA ratio of FuGene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s protocol. Etoposide (Sigma-Aldrich) was dissolved in DMSO and used at a final concentration of 50µM or 100µM as indicated in the figures. Human recombinant Interferon-γ (IFNγ) was purchased from Sigma.
and added at a final concentration of 5 ng/mL 30 minutes prior to harvesting. HeLa cells were transfected with the pISRE and pGAS cis-Reporter plasmids or the pCIS-CK control plasmid using the calcium phosphate method. Cells were co-transfected with pCMV β-galactosidase (β-gal) as a control for transfection efficiency. Luciferase and β-glucosidase were assayed as previously described (38). The siRNA vector, pRETRO-SUPER, was introduced into HeLa cells by square wave electroporation.

**TUNEL analysis and cell counts.** Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) analysis was performed using the In Situ Cell Death Detection Kit TMR Red (Roche Molecular Biochemicals; Indianapolis, IN) according to the manufacturers protocol. GFP positive cells were visualized by immunofluorescence microscopy and TUNEL positive cells containing GFP were quantified as the percent of the total GFP positive cells per field. Greater than 250 cells were counted for each variable per experiment.
RESULTS

Etoposide regulates STAT 1 dependent transcription. STAT1 is required for apoptosis induced by doxorubicin and cisplatin, and for the induction of apoptotic genes such as Bax, Fas, and Noxa, suggesting that induction of STAT1 dependent gene transcription is an important component of apoptosis induced by DNA damage (20). STAT1 drives transcription through binding to GAS or ISRE elements in STAT1 responsive genes (21). To determine if the DNA damaging agent, etoposide, can regulate STAT1 dependent transcription, HeLa cells were transfected with promoter-reporter plasmids containing either a GAS or ISRE element and transfected cells were treated with etoposide for 6 hrs. As shown in Figure 1A, both promoters are expressed basally, and the addition of etoposide increases transcription from each promoter >2 fold. We have previously shown that PKCδ is required for etoposide induced apoptosis (12). To determine the contribution of PKCδ to the induction of STAT1 dependent transcription by etoposide, we have used the PKCδ specific inhibitor, rottlerin, as well as depletion of PKCδ by expression of a PKCδ specific siRNA. In the experiment shown in Figure 1B, HeLa cells were transfected with the pGAS or pISRE reporter plasmids and then pretreated with the PKCδ inhibitor, rottlerin, and prior to the addition of etoposide. Rottlerin has previously been shown to block etoposide induced apoptosis (5). As shown in Figure 1B, rottlerin completely suppresses etoposide induction of the pGAS reporter, and suppresses induction of the pISRE reporter by about 80%, indicating that PKCδ activity is required for the induction of STAT1 dependent transcription. In the experiment shown in Figure 1C, HeLa cells were transfected by electroporation with a plasmid which expresses a siRNA directed against PKCδ, or the vector control. After 48 hours PKCδ protein is depleted >90% using this strategy (see Figure 5C).
Cells were then transfected a second time with pGAS luc or pISRE luc using the calcium phosphate method, followed by treatment with etoposide. As shown in Figure 1C, inhibition of PKCδ expression suppressed the etoposide mediated induction of both promoters, while only slightly suppressing basal expression of these promoters. The etoposide induction of pGAS luc was suppressed by 50%, while the induction of pISREluc was reduced by 30%. Taken together, these data suggest that etoposide induces transcriptional activation of STAT1 responsive genes in apoptotic cells, and that this activation requires PKCδ activity. In the case of both rottlerin and the siRNA to PKCδ, etoposide induction of the GAS promoter was suppressed to a greater extent than induction of the ISRE promoter. This differential sensitivity may reflect the fact that the GAS element preferentially binds STAT1 homodimers while ISRE element preferentially binds STAT1:STAT2 heterodimers (21).

**STAT1 is phosphorylated at S727 in response to etoposide.** Activation of STAT1, leading to the transcription of STAT1 regulated genes, is regulated by phosphorylation of STAT1 at Y701 and S727. Phosphorylation at Y701 is important for nuclear translocation and retention (22,39,40), while phosphorylation at S727 is required for maximal transcriptional activation of STAT1 dependent genes, including genes required for the apoptotic response (23,28). To ask if changes in phosphorylation of STAT1 occur in response to etoposide, we utilized antibodies which specifically recognize STAT1 phosphorylated at Y701 or S727. As demonstrated in Figure 2A (top), STAT1 is phosphorylated at S727 in untreated HeLa cells and treatment with etoposide results in an increase in phosphorylation at this site within 60 mins, which is sustained for at least 4 hrs. In contrast, no basal phosphorylation at STAT1 Y701 is detectable in HeLa cells (Figure 2A, middle). Likewise phospho-STAT1 Y701 was not detectable in cells treated
with etoposide, however Y701 phosphorylation was observed in response to IFNγ treatment as has been previously reported. Previous studies from other laboratories suggest that STAT1 phosphorylated at Y701 is dephosphorylated in the nucleus and subsequently exported into the cytoplasm (39-41). To investigate whether the lack of detectable STAT1 phospho-Y701 is due to its rapid dephosphorylation, HeLa cells were pretreated with pervanadate prior to the addition of etoposide. While some accumulation of phospho-Y701 STAT1 was observed in pervanadate pretreated cells, etoposide did not further increase the amount of phospho-Y701 STAT1 (data not shown). Tyrosine phosphorylation of STAT1 is mediated by the JAK tyrosine kinases, JAK1, JAK2 and TYK2. To further address the contribution of Y701 phosphorylation to etoposide induced STAT1 activation; we have utilized three human fibroblast cells lines, each deficient in one of these tyrosine kinases. As shown in Figure 2B, treatment of the control cell lines, 2fTGH and 2C4, with etoposide results in the rapid phosphorylation of STAT1 on S727. A similar time course of STAT1 S727 phosphorylation was observed in U1A (TYK2 deficient) and U4A (JAK1 deficient) cells. Etoposide induced STAT1 S727 phosphorylation was also observed in the γ2A (JAK2 deficient) cells, however in this cell line the time course was slightly delayed, and phosphorylation appeared to be more robust, suggesting that a JAK2 regulated pathway may suppress the etoposide induced phosphorylation of STAT1. Taken together this data indicates that etoposide induced activation of STAT1 does not require the JAK family of tyrosine kinases and suggests that S727 phosphorylation of STAT1 is sufficient for activation of STAT1 in apoptotic cells.

**Association of STAT1 and PKCδ in etoposide treated cells.** Recent studies suggest that PKCδ is required for phosphorylation of STAT1 at S727 in response to IFNα, IFNγ and IL-6
To determine the contribution of PKCδ to etoposide-induced phosphorylation of STAT1, cell lysates were immunoprecipitated with an antibody to STAT1 and immunoblotted for PKCδ or phospho-S727 STAT1 (Figure 3A). An association of STAT1 with full-length PKCδ was observed as early as 30 minutes after etoposide treatment and was concomitant with phosphorylation of STAT1 at S727 (Figure 3A top and bottom). In the experiment shown in Figure 3B, lysates from etoposide treated cells were immunoprecipitated with anti-PKCδ and immunoblotted for STAT1. Immunoprecipitation of PKCδ likewise demonstrated an etoposide dependent association with STAT1 which was detectable within 30 mins of etoposide stimulation. Since in HeLa cells the cleavage product of PKCδ does not accumulate until 12-24 hours after etoposide treatment (data not shown), while PKCδ and STAT1 associate within 30 minutes, our data suggests that STAT1 interacts with PKCδ prior to its caspase cleavage. Furthermore, as shown in Figure 3C, pretreatment of HeLa cells with the broad spectrum caspase inhibitor ZVAD-fmk does not inhibit the association of PKCδ and STAT1, or the phosphorylation of STAT1 S727 in etoposide treated cells. Thus caspase activation is not required for interaction of PKCδ with STAT1, or for phosphorylation of STAT1 at S727.

Our studies indicate that in HeLa cells the full length PKCδ protein associates with STAT1 prior to caspase cleavage of PKCδ. To address this further we have utilized parotid C5 cells, which undergo early and robust cleavage of PKCδ in response to etoposide (5). Figure 3D shows that in parotid C5 cells, etoposide induces the association of PKCδ and STAT1 by 2 hours, and this association is greatly diminished by 4 hours. Loss of the association of PKCδ and STAT1 parallels the initiation of cleavage of PKCδ at 4 hours as shown in Figure 3E. Taken together these studies demonstrate that the etoposide induced association of PKCδ and
STAT1 is an early event in the apoptotic pathway and that loss of this association is co-incident with caspase cleavage of PKCδ. Additionally, the observed co-association between PKCδ and STAT1 in response to etoposide in both HeLa and salivary epithelial cells suggests that this interaction in not cell type dependent and likely represents a general occurrence in response to DNA damaging agents.

Previous studies from our laboratory and others suggest a role for PKCδ and STAT1 in the nucleus of apoptotic cells. To determine if nuclear translocation of STAT1 and PKCδ accompanies etoposide induced apoptosis in HeLa cells, we prepared nuclear extracts from etoposide treated cells and analyzed PKCδ, total STAT1 and phospho-S727 STAT1 by immunoblot. As shown in Figure 4, the accumulation of total STAT1 and phospho-S727 STAT1 can be seen in the nucleus 15-30 min after the addition of etoposide, with maximal nuclear accumulation by 30-60 min. Nuclear accumulation of PKCδ follows a similar time course, with peak accumulation seen 30-60 min after addition of etoposide. These results suggest that PKCδ dependent activation of STAT1 occurs in the nucleus of apoptotic cells.

**PKCδ is required for etoposide induced phosphorylation of STAT1 at S727.** To determine if PKCδ is required for S727 phosphorylation of STAT1 in response to etoposide, HeLa cells were pre-treated with the PKC inhibitor Go6976, which inhibits PKCα, β, and γ, or Go6983 which inhibits PKCα, β, γ, δ and ζ, or rottlerin which inhibits PKCδ. As demonstrated in Figure 5A, top panel, pretreatment with Go6983 or rottlerin, both of which inhibit PKCδ, suppresses etoposide induced S727 phosphorylation of STAT1, while pretreatment with Go6976 which inhibits only the conventional PKC isoforms, does not. To further investigate the contribution of PKCδ to STAT1 S727 phosphorylation, we utilized siRNA to deplete HeLa cells
of endogenous PKCδ. As shown in Figure 5C, expression of siRNA to PKCδ results in depletion of >90% of the PKCδ protein. The addition of etoposide to HeLa cells transfected with the vector control results in phosphorylation of STAT1 at S727, while this is suppressed in cells transfected with the siRNA to PKCδ (Figure 5B). When the abundance of STAT1 phospho-S727 is normalized to total STAT1, the average of three experiments shows that etoposide increases the abundance of phospho-S727 by >3 fold in vector transfected cells, while in cells depleted of PKCδ, the increase in phospho-S727 is reduced to about 1.6 fold. These studies indicate that etoposide induced phosphorylation of STAT1 at S727 is a PKCδ dependent event. The experiments in Figure 1 and Figure 5 show a more robust suppression of both STAT1 dependent transcription and STAT1 S727 phosphorylation by rottlerin as compared to the PKCδ siRNA. This may be due to incomplete depletion of PKCδ by the PKCδ siRNA, or may indicate contributions from additional signaling pathways.

**Induction of apoptosis by overexpression of PKCδ requires both nuclear localization and S727 phosphorylation of STAT1.** The data shown above suggests PKCδ may regulate apoptosis via STAT1 dependent transcription and that nuclear accumulation of these signaling proteins may be required for their interaction. Transient overexpression of PKCδ is a potent inducer of apoptosis in a variety of cell types (5-7,10,43-47). To ask if PKCδ induces apoptosis via a STAT1 dependent mechanism, we have utilized the STAT1 deficient human fibroblast cell line, U3A (18,33). U3A cells, or the parental 2fTGH cell line, were transiently transfected with pGFPPKCδ or pGFPNLML8δ, a PKCδ nuclear localization mutant which does not induce apoptosis (12). As seen in Figure 6A, pGFPPKCδ induced apoptosis in 12% of transfected 2fTGH cells as determined by TUNEL staining, while only 4% of cells transfected with
pGFPNL8δ were TUNEL positive, verifying our previous results that nuclear localization of PKCδ is required for induction of apoptosis (12). In contrast, when U3A cells were transfected with pGFPPKCδ, there was no increase in GFP/TUNEL positive cells over that seen in cells transfected with pGFPNL8δ, demonstrating that STAT1 is required for PKCδ induced apoptosis. A requirement for STAT1 was verified by reconstituting U3A cells by transfection with pSTAT1α together with PKCδ. As seen in Figure 6A, in STAT1 reconstituted U3A cells, pGFPPKCδ induced apoptosis is rescued and actually enhanced. However, no induction of apoptosis was seen when U3A cells were re-constituted with STAT1 and transfected with pGFPNL8δ, indicating that PKCδ must translocate to the nucleus to induce apoptosis via STAT1.

The data shown in Figure 5 suggests that PKCδ is required for STAT1 S727 phosphorylation in etoposide treated cells. To determine the contribution of STAT1 phosphorylation to PKCδ induced apoptosis, U3A cells were reconstituted with STAT1 in which the serine at position 727 was mutated to alanine (pST1αS727A). Unlike wild type STAT1, expression of pST1αS727A was unable to restore apoptosis in pGFPPKCδ co-transfected cells, indicating that S727 phosphorylation of STAT1 is required for apoptosis induced by PKCδ.

The data in Figure 6A demonstrates that PKCδ must have access to the nucleus to induce apoptosis via a STAT1 dependent pathway, suggesting that STAT1 is a downstream target of nuclear PKCδ in apoptotic cells. To determine if nuclear translocation of STAT1 is also required for apoptosis induced by PKCδ, 2fTGH and U3A cells were transiently transfected with pGFP PKCδ together with pGFSTAT1α or pGFST1L407Aα which is mutated in a nuclear localization sequence and does not localize to the nucleus (22). As shown previously,
expression of pGFPPKCδ alone does not induce apoptosis in U3A cells while, co-transfection of pGFPPKCδ together with pGFPSTAT1α results in a complete rescue of pGFPPKCδ induced apoptosis (Figures 6A and B). In contrast, reconstitution of U3A cells with the STAT1 nuclear localization mutant, pGFPL407Aα, does not rescue PKCδ induced apoptosis in the U3A cells. Analysis of the localization of STAT1 in GFP transfected cells is shown in Figure 6C. In U3A cells reconstituted with pGFPSTAT1 alone, STAT1 is localized to the nucleus in >40% of the transfected cells. However, co-transfection of pGFPSTAT1 together with PKCδ results in accumulation of STAT1 in the nucleus of 85% of the transfected cells, correlating with the induction of apoptosis seen in Figure 6B. In contrast, nuclear translocation of STAT1 was not observed in U3A cells co-transfected with pGFPPKCδ together with pST1L407A. These studies suggest that activation of an apoptotic program by PKCδ induces the nuclear translocation of STAT1, and implies a functional role for nuclear STAT1 in the apoptotic pathway downstream of nuclear PKCδ signaling.
DISCUSSION

PKC\(\delta\) is required for apoptosis induced by a wide variety of stimuli in many cell types. While a limited number of substrates for PKC\(\delta\) have been described in apoptotic cells, the functional consequences of phosphorylation of these substrates is in large part unknown. Here we have explored the hypothesis that PKC\(\delta\) communicates with the cellular apoptotic machinery via activation of the transcription factor STAT1. Our studies show that etoposide induces a STAT1 transcriptional program that requires PKC\(\delta\) activity, and demonstrates that these two important pro-apoptotic signaling pathways are functionally linked in cells undergoing apoptosis. We propose that in response to DNA damaging agents, an apoptotic program is induced which includes PKC\(\delta\) mediated activation of STAT1 regulated gene expression.

We demonstrate that STAT1 is phosphorylated on S727 in HeLa cells treated with etoposide and that this correlates with the co-association of STAT1 with full-length PKC\(\delta\). Using two inhibitor strategies, we show that STAT1 S727 phosphorylation in apoptotic cells is dependent at least in part on PKC\(\delta\). This is in agreement with published studies which show that in cells treated with Type I and Type II interferons, and IL-6, PKC\(\delta\) is required for the induction of STAT1 transcriptional activity and for S727 STAT1 phosphorylation. PKC\(\delta\) has also been shown to directly phosphorylate STAT1 at S727 in-vitro (31,32). We find no evidence that etoposide induces phosphorylation of PKC\(\delta\) at Y701, or that etoposide activation of STAT1 requires the JAK family of tyrosine kinases. The obligatory role for Y701 phosphorylation of STAT1 in the context of apoptosis is somewhat controversial. Induction of apoptosis by TNF\(\alpha\) and actinomycin D, or oxysterol, requires S727 phosphorylation but not Y701 phosphorylation.
of STAT1 (18,19). However Y701 phosphorylation is required to enhance cell death during induction of apoptosis by heat shock or ischemia (48). Additionally, studies have found that STAT1 can regulate transcription of genes in both tyrosine phosphorylation dependent and independent contexts (28,49).

Our current studies demonstrate that both PKCδ and STAT1 are translocated to the nucleus within 30 min of etoposide treatment and that this is concurrent with the accumulation of phosphor-S727 STAT1 in the nucleus. While etoposide does not increase phosphorylation of STAT1 at Y701, we were able to detect a small amount of Y701 phosphorylated STAT1 in both untreated and etoposide treated cells (data not shown), suggesting that basal phosphorylation of STAT1 at tyrosine 701 may contribute to its nuclear accumulation in apoptotic cells. However, it should be noted that even in etoposide treated cells, the majority of total STAT1 and phospho-S727 STAT1 is found in the cytosol (data not shown). Since Y701 phosphorylation of STAT1 has recently been shown to be necessary for its retention in the nucleus (40), STAT1 may be exported more readily from the nucleus in etoposide treated cells where Y701 phosphorylation is minimal. Several recent studies suggest that in resting cells unphosphorylated STAT1 shuttles between the nucleus and cytosol (27,40,50). We propose that the addition of etoposide induces an increase in S727 phosphorylation and this may increase the abundance of STAT1 in the nucleus by increasing the binding affinity of STAT1 for DNA.

To examine more directly the relationship between PKCδ and STAT1 in apoptosis we have utilized a PKCδ overexpression model for apoptosis. Our studies clearly indicate that in the absence of STAT1, overexpression of PKCδ does not induce apoptosis. Furthermore we demonstrate that both PKCδ and STAT1 must have access to the nucleus for apoptosis to occur. Nuclear accumulation of STAT1 and PKCδ occurs both in etoposide treated HeLa cells and in...
STAT1 reconstituted U3A cells transiently transfected with PKCδ. Our studies demonstrate that the nuclear localization of both STAT1 and PKCδ are required for apoptosis induced by full-length PKCδ. Since the NLS mutant of full-length PKCδ did not induce apoptosis, this finding supports our previous results that nuclear localization of PKCδ is required for the induction of apoptosis (12). Recently it has been shown that STAT1 L407 is essential for the interaction of STAT1 with Importinα and subsequent nuclear translocation (22). The STAT1 L407 mutant however retains the ability to become Tyr phosphorylated, to dimerize, and bind DNA in-vitro (22). Transiently transfected STAT1 L407A did not localize to the nucleus and was not able to rescue PKCδ induced apoptosis in U3A cells indicating that nuclear localization of STAT1 is required for induction of apoptosis by PKCδ. In accordance with these results, recent data from another lab has demonstrated that a constitutively active dimerized form of STAT1, that is retained in the nucleus, sensitizes cells to IFNγ induced apoptosis by inducing activation of caspases 2, 3, and 7 (51).

A model of apoptosis has been proposed in which apoptotic agents activate PKCδ, resulting in the activation of caspase-3 and the subsequent cleavage of PKCδ to generate a constitutively active fragment of this kinase. Our laboratory and others have proposed that this constitutively active fragment of PKCδ is rapidly translocated into the nucleus and functions to amplify the apoptotic pathway (12,52,53). In this regard, PKCδ has been shown to have a functional role in the nucleus of apoptotic cells and several nuclear binding partners for the caspase cleavage fragment of PKCδ have been identified such as p73β, DNA-PK, Lamin B, c-Abl, SHPTP1 and Rad9 (13,15,54-56). We have previously demonstrated that nuclear translocation of PKCδ in etoposide treated cells also occurs independently of caspase cleavage, albeit less efficiently, and have suggested a role for full-length PKCδ in the apoptotic pathway.
prior to caspase cleavage (12). Our current data shows that STAT1 and PKCδ associate prior to caspase cleavage of PKCδ, providing further support for a role for the full length form of PKCδ in apoptotic cells.

How full length PKCδ is activated by DNA damaging agents, and how this activation leads to caspase-3 activation, is largely unknown. Tyrosine phosphorylation of PKCδ occurs very rapidly upon exposure of cells to DNA damaging agents, and is likely to be one mechanism by which PKCδ is “activated” in apoptotic cells. Although numerous studies have attempted to demonstrate a direct effect of tyrosine phosphorylation on the catalytic activity of PKCδ with variable results, mutation of specific tyrosine residues has been shown to suppress the ability of PKCδ to induce apoptosis in transfected cells (Humphries and Reyland, unpublished observations and (11). We propose that tyrosine phosphorylation links PKCδ to downstream signaling pathways by creating docking sites for SH2 domain proteins such as STAT1. PKCδ mediated activation of these signaling pathways may be required for transcriptional reprogramming of the cell to facilitate apoptosis. This may include induction of STAT1 dependent pro-apoptotic genes, such as caspases, Bcl-2 family members and STAT1 dependent regulation of p53 dependent genes such as Bax and NOXA (51). In addition, PKCδ dependent activation of other signal transduction pathways such as the c-Jun N-terminal kinase pathway has been reported, and may lead to transcriptional regulation of a distinct subset of pro or anti apoptotic genes (57). The summation of these multiple transcriptional endpoints may serve to activate the apoptotic pathway directly or to “prime” the pathway for activation by additional apoptotic signals.
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FOOTNOTES

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1The abbreviations used are: STAT, signal transducer and activator of transcription; PKC, protein kinase C; IFNγ, interferon-γ; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; ISRE, interferon stimulatory response element; JAK, Janus kinase.
FIGURE LEGENDS

Figure 1. Induction of STAT1 dependent gene transcription by etoposide requires PKCδ.

Panel A: HeLa cells were transfected with either pISREluc or pGASluc, or the pCIS-CK negative control plasmid, and co-transfected with pCMV β-galactosidase. Eighteen hours after transfection, 100 uM etoposide was added for an additional 6 hours as indicated. Panel B: HeLa cells were transfected as described for panel A and pre-treated with 1 uM rottlerin for 30 mins prior to the addition of etoposide. Etoposide and etoposide plus rottlerin values were significantly different from each other for both the pGAS (*) and pISRE (**) reporter plasmids (p<0.005). Panel C: HeLa cells were transfected with a vector expressing a siRNA to PKCδ (δ), or the control vector (C) by electroporation. Electroporated cells were allowed to grow for 48 hours and then transfected again with pISREluc or pGASluc by the calcium phosphate method. Etoposide (100uM) was added for 6 hours as indicated. The values shown in all panels are the average of triplicate measurements in a single experiment and are expressed as relative light units (RLU) normalized to β-glucosidase activity +/- the S.D. Representative experiments are shown; each experiment was repeated 2 or more times. Etoposide and etoposide plus PKCδ siRNA values were significantly different for both the pGAS (*) and pISRE (**) reporter plasmids (p<0.05).

Figure 2: Etoposide induces phosphorylation of STAT1 at S727, but not Y701. Panel A: HeLa cells were treated with 100 uM etoposide for the indicated time. As a control, cells were treated with 5ng/ml IFNγ for 30 minutes. Cell lysates were prepared and immunoblotted as
described in Materials and Methods. Immunoblots were probed for phospho-S727 STAT1 (top panel), phospho-Y701 STAT1 (middle panel) or total STAT1 (bottom panel).  Panel B: The parental human fibroblast cell lines (2fTGH and 2C4), and mutant cell lines deficient in JAK signaling proteins (U1A, TYK2; U4A, JAK1; γ2A, JAK2) were treated with etoposide for the indicated time. Cell lysates were prepared and immunoblotted for phospho-S727 STAT1 (top panel) or total STAT1 (bottom panel). Representative experiments are shown; each experiment was repeated 3 or more times.

Figure 3. Etoposide induces the association of PKCδ with STAT1. Panel A: HeLa cells were treated for the indicated time with 100µM etoposide and whole cell lysates were prepared as described in Materials and Methods. Lysates were immunoprecipitated with an antibody to STAT1 and immunoblotted for PKCδ (top panel), total STAT1 protein (middle panel), or P-S727 STAT1 (lower panel).  Panel B: Cell lysates were immunoprecipitated with an antibody to PKCδ and immunoblotted for STAT1 (top panel), or PKCδ (lower panel).  Panel C: HeLa cells were incubated with or without 100µM ZVAD-fmk for one hour, followed by treatment for an additional hour with 100µM etoposide. Lysates were immunoprecipitated with an antibody to STAT1 or Protein A beads alone. Proteins were separated by SDS-PAGE and immunoblotted for P-S727 STAT1 (top panel), total STAT1 protein (middle panel), or PKCδ (lower panel). Panel D: Parotid C5 cells were treated with 50 µM etoposide for the indicated time and whole cell lysates were immunoprecipitated with an antibody to the C-terminus of PKCδ and immunoblotted for STAT1.  Panel E: Whole cell lysates from the experiment shown in panel B were immunoblotted with an antibody directed to the C-terminus of human PKCδ. Representative experiments are shown; each experiment was repeated 3 or more times.
Figure 4. Nuclear translocation of PKCδ and STAT1 in etoposide treated cells. HeLa cells were treated with 100 uM etoposide for the time indicated and nuclear extracts were prepared as described in Materials and Methods. The abundance of STAT1, phospho-S727 STAT1, PKCδ and lamin B were analyzed by immunoblot as described in Materials and Methods.

Figure 5. Inhibition of PKCδ diminishes S727 phosphorylation of STAT1. Panel A: HeLa cells were incubated with DMSO alone, 100nM Go6976, 160nM Go6983, or 1uM Rottlerin for 1 hour and then treated for the indicated times with 100µM etoposide. Lysates were prepared, immunoprecipitated with an antibody to STAT1, resolved by SDS-PAGE, and immunoblotted for P-S727 STAT1 (top panel), total STAT1 protein (lower panel). Panel B: HeLa cells were transfected with pRETROSUPER alone (siRNAcontrol), or pRETROSUPER with siRNA to PKCδ, (siRNAδ) for 48 hours and then treated for the indicated times with 50µM etoposide. Whole cell lysates were immunoblotted for P-S727 STAT1 (top) or total STAT1 (lower). Duplicate samples are shown for each time point. Panel C: Lysates from the experiment in Panel A were immunoblotted with an antibody directed to the C-terminus of human PKCδ.

Figure 6. STAT1 is required for apoptosis induced by PKCδ. Panel A: The parental cell line 2fTGH, or the STAT1 deficient cell line U3A, were transfected with pGFPPKCδ (WTδ) or pGFPNLML8δ (NLS) and cotransfected with pGFP alone (GFP), pST1α (ST1) or pST1αS727A (S727A). After 24 hours lysates were prepared and analyzed by immunoblot for total STAT1 (upper panel) or for apoptosis by TUNEL (lower graph). TUNEL positive cells containing GFP were visualized by fluorescent microscopy and quantified as the percentage of
the total number of GFP positive cells per field. The graph represents the average of three independent experiments ± the S.E.M. At least 250 cells were counted for each variable/experiment. Panel B: The parental cells line 2fTGH or the STAT1 null cell line U3A were transfected with pGFP PKCδ (δ) together with pGFP alone (GFP), pGFP STAT1α (ST1) or pGFPSt1αL407A(L407A). Inset: Inset shows an immunoblot for total GFP STAT1α in the STAT1 reconstituted cells. TUNEL positive/GFP positive cells were visualized by fluorescent microscopy and the percentage of the total number of GFP positive cells per field was quantified. Graph represents the average of three independent experiments which produced similar results. Data are the mean ± SEM from 10 fields of view/experiment. At least 250 cells were counted for each variable in each experiment. The experiments shown are representative of 3 or more independent experiments. Panel C: The STAT1 null cell line U3A were transfected with pGFpSTAT1α (ST1), or pGFpPKCδ (δ) together with pGFpSTAT1α (ST1), pGFpSt1αL407A (L407A), or pGFpPKCδ (δ) together with (L407A). Cells were counted by fluorescent microscopy and the number of cells exhibiting nuclear localization of STAT1 was obtained as a percent of the whole transfected population. Data are expressed as the mean ± S.E.M. from 10 fields of view/experiment. At least 100 cells were counted for each variable in each experiment. The data are representative of 3 or more independent experiments.
<table>
<thead>
<tr>
<th>Hrs. Etop</th>
<th>2ftgh</th>
<th>U1A</th>
<th>U4A</th>
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<tbody>
<tr>
<td>U</td>
<td>.5</td>
<td>1</td>
<td>2</td>
</tr>
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**FIG 2B**

![Image of gel with bands labeled P-S727 STAT1α, STAT 1α, and STAT1β.](http://www.jbc.org/)
A

<table>
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- PKCδ
- STAT1α
- STAT1β
- P-S727 STAT1α

IP STAT1

B

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<th>2</th>
<th>4</th>
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- STAT1
- PKCδ

IP PKCδ
FIG 3C

WCL + beads
UT
E
E+ZVAD

100
75

P-S727 STAT1 α

100
75

STAT1 α
STAT1 β

75

PKC δ (74kDa)

IP STAT1
FIG 3D, 3E

D

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</table>

- STAT1α

IP: PKCδ

E

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- PKCδ (74kDa)
- CFδ (40kDa)
FIG 4

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<th>2</th>
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- STAT1α
- P-S727 STAT1α
- PKCδ
- Lamin B
Etop (hrs): 0, 0.50, 1, 4, 0.50, 1, 4

+Go6976
+Go6983
+rottlerin

FIG 5A
**FIG 5B, 5C**

**B**

<table>
<thead>
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<th>0</th>
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<tr>
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<tr>
<td>siRNAδ</td>
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![Western blot images with labeled bands](image)

- **P-S727 STAT1**
- **STAT1α**
- **STAT1β**
- **PKCδ**

**C**

<table>
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<th>Etop (hrs)</th>
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<tr>
<td>C</td>
<td>δ</td>
<td>C</td>
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![Western blot images with labeled bands](image)
FIG 6B

% TUNEL positive

<table>
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<td>WTδ+GFP</td>
<td>16</td>
</tr>
<tr>
<td>WTδ+GFP</td>
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<td>WTδ+ST1</td>
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<tr>
<td>WTδ+ST1L407A</td>
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2fTGH

Untransfected WT

δ

+GFP

WT

δ

+ST1

WT

δ

+ST1L407A

STAT1α

STAT1L407A
% Cells with nuclear Stat1

FIG 6C
PKCδ regulates apoptosis via activation of STAT1
Tracie A. DeVries, Rachelle L. Kalkofen, Angela A. Matassa and Mary E. Reyland

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