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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 subfamily of protein kinase C (PKC) isozymes, 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 mitochondria-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).

Members of 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 antiviral 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 tumor necrosis factor-α, 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 a 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 Tyr701 and Ser727. Phosphorylation at Tyr701 by the Janus family of tyrosine kinases (JAK) leads to STAT1 dimerization via its Src homology 2 domains, exposure of a dimer-specific nuclear localization signal, and subsequent nuclear translocation (21, 22). Phosphorylation of STAT1 at Ser727 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 cAMP-response element-binding protein, 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 Ser727 appears to be essential, whereas phosphorylation at Tyr701 may be dispensable (18, 26–28). A variety of serine/threonine kinases have been shown to contribute to STAT1 Ser727 phosphorylation, including members of the extracellular signal-regulated kinase family and Akt (29, 30). Recently, STAT1 has been identified as a PKCδ substrate in cells treated with Type I or Type II interferon (31, 32).

The identification of STAT1 Ser727 as a potential regulatory site for STAT1 proapoptotic functions, and the observation that PKCδ can phosphorylate STAT1 at Ser727 in response to some stimuli, suggests a link between these two proapoptotic 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 PKCδ. Our studies indicate that induction of apoptosis requires nuclear translocation of PKCδ and STAT1 as well as STAT1 Ser727 phosphorylation. These studies
demonstrate a novel and functional interaction between PKCδ and STAT1 during DNA damage-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture—**HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The parental human fibroblast cell lines (2fTGH and U3A), and mutant cell lines deficient in JAK and STAT signaling proteins (U3A, STAT1; U1A, TYK2; U4A, JAK1; y2A, JAK2) (33–35) were generously provided by Dr. George R. Stark (The Cleveland Clinic Foundation) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The salivary parotid C5 cell line was cultured on Primaria dishes (Falcon Plastics, Franklin Lakes, NJ) as previously described (36).

**Immunoblot Analysis and Immunoprecipitation—**Cells were harvested for immunoblot analysis using MSLB buffer (15 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 20 μM aprotinin, and 20 μg/ml leupeptin). Nuclear fractions were isolated using a nuclease-free fractionation kit (Biovision) according to the manufacturer's instructions except that 1 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 (Bio-Rad). The following antibodies were used: anti-GFP, Zymed Laboratories Inc. (catalog no. C163); anti-human PKCδ, Santa Cruz Biotechnology, Inc., Santa Cruz, CA (catalog no. C-20, sc-9171S) or Santa Cruz Biotechnology (catalog no. sc-7988); anti-phospho-Ser727 STAT1, Upstate Biotechnology, Inc., Lake Placid, NY (catalog no. 06-802); anti-tubulin, BD Pharmingen (catalog no. 556321); anti-lamin B, Santa Cruz Biotechnology (catalog no. sc-6217). 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 h at 4°C. The beads were washed three times in MSLB buffer, and the precipitated proteins were resolved on a 10% SDS-polyacrylamide gel.

**Plasmid Constructs—**Generation of mouse pGFPFKCδ and pGFP-LMFL5δ has been previously described (12). The plasmids pSTAT1α (STAT1α) and pSTAT1α S27A (STAT1α S27A) were obtained from Dr. James Darnell (Rockefeller University) (23). The plasmids pGFPSTAT-AT1α (STAT1α) and pGFPSTAT L407A (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 oligonucleotide 5'-GATCCCGGACAGGGTCCACGCATGCGAGATGCTGATGTTGACAGCCTTCTTCTTCTTTGAAA-3'.

**Transient Transfection—**For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis, subconfluent 2fTGH and U3A cells were transiently transfected using a 6:1 lipid/DNA ratio of FuGene 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. Etoposide (Sigma) was dissolved in Me2SO and used at a final concentration of 50 or 100 μM as indicated in the figures. Human recombinant interferon-γ (IFNγ) was purchased from Sigma and used at a final concentration of 5 ng/ml 30 min prior to harvesting. HeLa cells were transfected with the pIRES and pGAS cis-reporter plasmids or the pCIS-CK control plasmid using the calcium phosphate method. Cells were co-transfected with the pCMV-β-galactosidase as a control for transfection efficiency. Luciferase and β-galactosidase were assayed as previously described (38). The siRNA vector, pRETRO-SUPER, was introduced into HeLa cells by wave electroporation.

**TUNEL Analysis and Cell Counts—**TUNEL analysis was performed using the in situ cell death detection kit TMR Red (Roche Applied Science) according to the manufacturer's protocol. GFP-positive cells were visualized by immunofluorescence microscopy, and TUNEL-positive cells containing GFP were quantified as the percentage of the total GFP-positive cells per field. Greater than 250 cells were counted for each variable per experiment.

**RESULTS**

**Etoposide Regulates STAT1-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 whether 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 h. As shown in Fig. 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 Fig. 1B, HeLa cells were transfected with the pGAS or pISRE reporter plasmids and then pretreated with the PKCδ inhibitor, rottlerin, prior to the addition of etoposide. Rottlerin has previously been shown to block etoposide-induced apoptosis (5). As shown in Fig. 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 Fig. 1C, HeLa cells were transfected by electroporation with a plasmid that expresses an siRNA directed against PKCδ or the vector control. After 48 h, PKCδ protein is depleted >90% using this strategy (see Fig. 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 Fig. 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%, whereas 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, whereas ISRE element preferentially binds STAT1/STAT2 heterodimers (21).

**STAT1 Is Phosphorylated at Ser727 in Response to Etoposide—**Activation of STAT1, leading to the transcription of STAT1-regulated genes, is regulated by phosphorylation of STAT1 at Tyr701 and Ser727. Phosphorylation at Tyr701 is important for nuclear translocation and retention (22, 39, 40), whereas phosphorylation at Ser727 is required for maximal transcriptional activation of STAT1-dependent genes, including genes required for the apoptotic response (23, 28). To ask whether changes in phosphorylation of STAT1 occur in response to etoposide, we utilized antibodies that specifically recognize STAT1 phosphorylated at Tyr701 or Ser727. As demonstrated in Fig. 2A (top), STAT1 is phosphorylated at Ser727 in untreated HeLa cells, and treatment with etoposide results in an increase in phosphorylation at this site within 60 min, which is sustained for at least 4 h. In contrast, no basal phosphorylation at STAT1 Tyr701 is detectable in HeLa cells (Fig. 2A, middle). Likewise, phospho-STAT1 Tyr701 was not detectable in cells treated with etoposide; however, Tyr701 phosphorylation was observed in response to IFNγ treatment as has been previously reported. Previous studies from other labora-
tories suggest that STAT1 phosphorylated at Tyr701 is dephosphorylated in the nucleus and subsequently exported into the cytoplasm (39–41). To investigate whether the lack of detectable STAT1 phospho-Tyr701 is due to its rapid dephosphorylation, HeLa cells were pretreated with pervanadate 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). C, HeLa cells were transfected with a vector expressing an siRNA to PKCδ (δ) or the control vector (C) by electroporation. Electroporated cells were allowed to grow for 48 h and then transfected again with pISREluc or pGASluc by the calcium phosphate method. Etoposide (100 μM) was added for 6 h 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-glucuronidase activity ± S.D. Representative experiments are shown; each experiment was repeated two or more times. Etoposide and etoposide plus PKCδ siRNA values were significantly different for both the pGAS (*) and pISRE (**) reporter plasmids (p < 0.05).
utilized three human fibroblast cell lines, each deficient in one of these tyrosine kinases. As shown in Fig. 2B, treatment of the control cell lines, 2fTGH and 2C4, with etoposide resulted in the rapid phosphorylation of STAT1 on Ser727. A similar time course of STAT1 Ser727 phosphorylation was observed in U1A (TYK2-deficient) and U4A (JAK1-deficient) cells. Etoposide-induced STAT1 Ser727 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, these data indicate that etoposide-induced activation of STAT1 does not require the JAK family of tyrosine kinases and suggests that Ser727 phosphorylation of STAT1 is sufficient for activation of STAT1 in apoptotic cells.

Association of STAT1 and PKCα in Etoposide-treated Cells—Recent studies suggest that PKCα is required for phosphorylation of STAT1 at Ser727 in response to IFNα, IFNγ, and interleukin-6 (31, 42). To determine the contribution of PKCα to etoposide-induced phosphorylation of STAT1, cell lysates were immunoprecipitated with an antibody to STAT1 and immunoblotted for PKCα or phospho-Ser727 STAT1 (Fig. 3A). An association of STAT1 with full-length PKCα was observed as early as 30 min after etoposide treatment and was concomitant with phosphorylation of STAT1 at Ser727 (Fig. 3A, top and bottom). In the experiment shown in Fig. 3B, lysates from etoposide-treated cells were immunoprecipitated with anti-PKCα and immunoblotted for STAT1. Immunoprecipitation of PKCα likewise demonstrated an etoposide-dependent association with STAT1, which was detectable within 30 min of etoposide stimulation. Since in HeLa cells the cleavage product of PKCα does not accumulate until 12–24 h after etoposide treatment (data not shown), whereas PKCα and STAT1 associate within 30 min, our data suggest that STAT1 interacts with PKCα prior to its caspase cleavage. Furthermore, as shown in Fig. 3C, pretreatment of HeLa cells with the broad spectrum caspase inhibitor benzyl oxy carbonyl-VAD-fluoromethyl ketone does not inhibit the association of PKCα and STAT1 or the phosphorylation of STAT1 Ser727 in etoposide-treated cells. Thus, caspase activation is not required for interaction of PKCα with STAT1 or for phosphorylation of STAT1 at Ser727.

Our studies indicate that in HeLa cells the full-length PKCα protein associates with STAT1 prior to caspase cleavage of PKCα. To address this further, we have utilized parotid C5 cells, which undergo early and robust cleavage of PKCα in response to etoposide (5). Fig. 3D shows that in parotid C5 cells, etoposide induces the association of PKCα and STAT1 by 2 h, and this association is greatly diminished by 4 h. Loss of the association of PKCα and STAT1 parallels the initiation of cleavage of PKCα at 4 h as shown in Fig. 3E. Taken together, these studies demonstrate that the etoposide-induced association of PKCα and STAT1 is an early event in the apoptotic pathway.
and that loss of this association is coincident with caspase cleavage of PKCδ. Additionally, the observed co-association between PKCδ/H9254 and STAT1 in response to etoposide in both HeLa and salivary epithelial cells suggests that this interaction is not cell type-dependent and probably 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 whether 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-Ser727 STAT1 by immunoblot. As shown in Fig. 4, the accumulation of total STAT1 and phospho-Ser727 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 the 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 Ser727—To determine whether PKCδ is required for Ser727 phosphorylation of STAT1 in response to etoposide, HeLa cells were pretreated with the PKC inhibitor Go6976, which inhibits PKCα,-β, and -γ; Go6983, which inhibits PKCα-, -β, -γ, -δ, and -ζ; or rottlerin, which inhibits PKCδ. As demonstrated in Fig. 5A, top, pretreatment with Go6983 or rottlerin, both of which inhibit PKCδ, suppresses etoposide-induced Ser727 phosphorylation of STAT1, whereas pretreatment with Go6976, which inhibits only the conventional PKC isoforms, does not. To further investigate the contribution of PKCδ to STAT1 Ser727 phosphorylation, we utilized siRNA to deplete HeLa cells of endogenous PKCδ. As shown in Fig. 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.

**Fig. 3.** Etoposide induces the association of PKCδ with STAT1. A, HeLa cells were treated for the indicated times with 100 μM etoposide, and whole cell lysates were prepared as described under “Experimental Procedures.” Lysates were immunoprecipitated (IP) with an antibody to STAT1 and immunoblotted for PKCδ (top), total STAT1 protein (middle), or phospho-Ser727 STAT1 (P-S727; bottom). B, cell lysates were immunoprecipitated with an antibody to PKCδ and immunoblotted for STAT1 (top) or PKCδ (bottom). C, HeLa cells were incubated with or without 100 μM benzoxycarbonyl-VAD-fluoromethyl ketone (ZVAD) for 1 h, followed by treatment for an additional 1 h with 100 μM etoposide (E). Lysates were immunoprecipitated with an antibody to STAT1 or Protein A beads alone. Proteins were separated by SDS-PAGE and immunoblotted for phospho-Ser727 STAT1 (top), total STAT1 protein (middle), or PKCδ (bottom). WCL, whole cell lysates. UT, untreated. D, parotid C5 cells were treated with 50 μM etoposide for the indicated times, and whole cell lysates were immunoprecipitated with an antibody to STAT1 and immunoblotted for PKCδ (bottom). E, whole cell lysates from the experiment shown in B were immunoblotted with an antibody directed to the C terminus of human PKCδ. Representative experiments are shown; each experiment was repeated three or more times.
STAT1 at Ser^{727}, whereas this is suppressed in cells transfected with the siRNA to PKC\(\delta\) (Fig. 5B). When the abundance of STAT1 phospho-Ser^{727} is normalized to total STAT1, the average of three experiments shows that etoposide increases the abundance of phospho-Ser^{727} by >3-fold in vector-transfected cells, whereas in cells depleted of PKC\(\delta\), the increase in phospho-Ser^{727} is reduced to about 1.6-fold. These studies indicate that etoposide-induced phosphorylation of STAT1 at Ser^{727} is a PKC\(\delta\)-dependent event. The experiments in Figs. 1 and 5 show a more robust suppression of both STAT1-dependent transcription and STAT1 Ser^{727} phosphorylation by rotterin as compared with the PKC\(\delta\) siRNA. This may be due to incomplete depletion of PKC\(\delta\) by the PKC\(\delta\) siRNA or may indicate contributions from additional signaling pathways.

**Induction of Apoptosis by Overexpression of PKC\(\delta\) Requires both Nuclear Localization and Ser^{727} Phosphorylation of STAT1**—The data shown above suggest that PKC\(\delta\) may regulate apoptosis via STAT1-dependent transcription and that nuclear accumulation of these signaling proteins may be required for their interaction. Transient overexpression of PKC\(\delta\) is a potent inducer of apoptosis in a variety of cell types (5–7, 10, 43–47). To ask if PKC\(\delta\) 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 2TGH cell line were transiently transfected with pGFPPKC\(\delta\) or pGFPNLMPF8\(\delta\), a PKC\(\delta\) nuclear localization mutant that does not induce apoptosis (12). As seen in Fig. 6A, pGFPPKC\(\delta\) induced apoptosis in 12% of transfected 2TGH cells as determined by TUNEL staining, whereas only 4% of cells transfected with pGFPNLMPF8\(\delta\) were TUNEL-positive, verifying our previous findings that nuclear localization of PKC\(\delta\) is required for induction of apoptosis (12). In contrast, when U3A cells were transfected with pGFPPLC\(\delta\), there was no increase in GFP/TUNEL-positive cells over that seen in cells transfected with pGFPNLMPF8\(\delta\) (Fig. 4A), demonstrating that STAT1 is required for PKC\(\delta\)-induced apoptosis. A requirement for STAT1 was verified by reconstituting U3A cells by transfection with PLAS/STAT1 together with PKC\(\delta\). As seen in Fig. 6A, in STAT1-reconstituted U3A cells, gFPPPKC\(\delta\)-induced apoptosis is rescued and actually enhanced. However, no induction of apoptosis was seen when U3A cells were reconstituted with STAT1 and transfected with pGFPNLMPF8\(\delta\), indicating that PKC\(\delta\) must translocate to the nucleus to induce apoptosis via STAT1.

The data shown in Fig. 5 suggest that PKC\(\delta\) is required for STAT1 Ser^{727} phosphorylation in etoposide-treated cells. To determine the contribution of STAT1 phosphorylation to PKC\(\delta\)-induced apoptosis, U3A cells were reconstituted with STAT1 in which the serine at position 727 was mutated to alanine (pST1\(\alpha\) S727A). Unlike wild type STAT1, expression of pST1\(\alpha\) S727A was unable to restore apoptosis in pGFPPKC\(\delta\) co-transfected cells, indicating that Ser^{727} phosphorylation of STAT1 is required for apoptosis induced by PKC\(\delta\).

The data in Fig. 6A demonstrate that PKC\(\delta\) must have access to the nucleus to induce apoptosis via a STAT1-dependent pathway, suggesting that STAT1 is a downstream target of nuclear PKC\(\delta\) in apoptotic cells. To determine whether nuclear translocation of STAT1 is also required for apoptosis induced by PKC\(\delta\), 2TGH and U3A cells were transiently transfected with pGFP PKC\(\delta\) together with pGFPSTAT1\(\alpha\) or pGFPLL407A, which is mutated in a nuclear localization sequence and does not localize to the nucleus (22). As shown previously, expression of pGFPPKC\(\delta\) alone does not induce apoptosis in U3A cells, whereas co-transfection of pGFPPKC\(\delta\) together with pGFPSTAT1\(\alpha\) results in a complete rescue of pGFPPKC\(\delta\)-induced apoptosis (Fig. 6, A and B). In contrast, reconstitution of U3A cells with the STAT1 nuclear localization mutant, pGFPLL407A, does not rescue PKC\(\delta\)-induced apoptosis in the U3A cells. Analysis of the localization of STAT1 in GFP-transfected cells is shown in Fig. 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 PKC\(\delta\) results in accumulation of STAT1 in the nucleus of 85% of the transfected cells, correlating with the induction of apoptosis seen in Fig. 6B. In contrast, nuclear translocation of STAT1 was not observed in U3A cells co-transfected with pGFPPKC\(\delta\) together with pST1L407A. These studies suggest that activation of an apoptotic program by PKC\(\delta\) induces the nuclear translocation of STAT1 and implies a functional role for nuclear STAT1 in the apoptotic pathway downstream of nuclear PKC\(\delta\) signaling.

**DISCUSSION**

PKC\(\delta\) is required for apoptosis induced by a wide variety of stimuli in many cell types. Whereas 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 demonstrate that these two important proapoptotic 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 Ser^{727} 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 Ser^{727} phosphorylation in apoptotic cells is dependent at least in part on PKC\(\delta\). This is in agreement with published studies that show that cells treated with Type I and Type II interferons and interleukin-6, PKC\(\delta\) is required for the induction of STAT1 transcriptional activity and for Ser^{727} STAT1 phosphorylation. PKC\(\delta\) has also been shown to directly phosphorylate STAT1 at Ser^{727} in vitro (31, 32). We find no evidence that etoposide induces phosphorylation of PKC\(\delta\) at Tyr^{701} or that etoposide activation of STAT1 requires the JAK family of tyrosine kinases. The obligatory role for Tyr^{701} phosphorylation of STAT1 in the context of apoptosis is somewhat controversial. Induction of apoptosis by tumor necrosis factor-\(\alpha\) and actinomycin D or oxysterol requires Ser^{727} phosphorylation but not Tyr^{701} phosphorylation of STAT1 (18, 19). However, Tyr^{701} phosphorylation is required to enhance cell death during induction of apo-
ptosis 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 PKC and STAT1 are translocated to the nucleus within 30 min of etoposide treatment and that this is concurrent with the accumulation of phospho-Ser727 STAT1 in the nucleus. Whereas etoposide does not increase phosphorylation of STAT1 at Tyr701, we were able to detect a small amount of Tyr701-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-Ser727 STAT1 is found in the cytosol (data not shown). Since Tyr701 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 Tyr701 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 Ser727 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 PKC and STAT1 in apoptosis, we have utilized a PKC overexpression model for apoptosis. Our studies clearly indicate that in the absence of STAT1, overexpression of PKC does not induce apoptosis. Furthermore, we demonstrate that both PKC and STAT1 must have access to the nucleus for apoptosis to occur. Nuclear accumulation of STAT1 and PKC occurs both in etoposide-treated HeLa cells and in STAT1 reconstituted U3A cells transiently transfected with PKC. Our studies demonstrate that the nuclear localization of both STAT1 and PKC are required for apoptosis induced by full-length PKC. Since the nuclear localization signal mutant of full-length PKC did not induce apoptosis, this finding supports our previous results that nuclear localization of PKC is required for the induction of apoptosis (12).

Recently, it has been shown that STAT1 Leu407 is essential for the interaction of STAT1 with importin α and subsequent nuclear translocation (22). The STAT1 Leu407 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 PKC-induced apoptosis in U3A cells, indicating that nuclear localization of STAT1 is required for induction of apoptosis by PKC. In accordance with these results, recent data from another laboratory has demonstrated that a constitutively active dimerized form of STAT1 that is retained in the nucleus sensitizes cells to IFN-induced apoptosis by inducing activation of caspase-2, -3, and -7 (51).

A model of apoptosis has been proposed in which apoptotic agents activate PKC, resulting in the activation of caspase-3 and the subsequent cleavage of PKC to generate a constitu-
STAT1 and PKCδ in Apoptosis

Fig. 6. STAT1 is required for apoptosis induced by PKCδ. A, the parental cell line 2fTGH or the STAT1-deficient cell line U3A was transfected with pGFP-PKCδ (WTδ) or pGFP-NLS δ (NLS) and cotransfected with pGFP alone (GFP), pST1α (ST1), or pST1α S727A (S727A). After 24 h, lysates were prepared and analyzed by immunoblot for total STAT1 (top) or for apoptosis by TUNEL (bottom). 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 ± S.E. At least 250 cells were counted for each variable/experiment. 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 pGFP-St1α L407A (L407A). Inset, 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. The graph represents the average of three independent experiments that produced similar results. Data are the mean ± S.E. from 10 fields of view/experiment. At least 250 cells were counted for each variable in each experiment. The data are representative of three or more independent experiments.
tively 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, SHPTF1, 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 show 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 (11),2 We propose that tyrosine phosphorylation links PKCδ to downstream signaling pathways by creating docking sites for Src family kinases. This may include induction of STAT1-dependent proapoptotic pathways, such as caspases and 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 antiapoptotic genes (57). The summation of these multiple transcriptional end points may serve to activate the apoptotic pathway directly or to “prime” the pathway for activation by additional apoptotic signals.

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