Phosphorylation of Protein Kinase Cδ on Distinct Tyrosine Residues Induces Sustained Activation of Erk1/2 via Down-regulation of MKP-1

ROLE IN THE APOPTOTIC EFFECT OF ETOPOSIDE*

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The mechanism underlying the important role of protein kinase Cδ (PKCδ) in the apoptotic effect of etoposide in glioma cells is incompletely understood. Here, we examined the role of PKCδ in the activation of Erk1/2 by etoposide. We found that etoposide induced persistent activation of Erk1/2 and nuclear translocation of phospho-Erk1/2. MEK1 inhibitors decreased the apoptotic effect of etoposide, whereas inhibitors of p38 and JNK did not. The activation of Erk1/2 by etoposide was downstream of PKCδ since the phosphorylation of Erk1/2 was inhibited by a PKCδ-KD mutant and PKCδ small interfering RNA. We recently reported that phosphorylation of PKCδ on tyrosines 64 and 187 was essential for the apoptotic effect of etoposide. Using PKCδ tyrosine mutants, we found that the phosphorylation of PKCδ on these tyrosine residues, but not on tyrosine 155, was also essential for the activation of Erk1/2 by etoposide. In contrast, nuclear translocation of PKCδ was independent of its tyrosine phosphorylation and not necessary for the phosphorylation of Erk1/2. Etoposide induced down-regulation of kinase phosphatase-1 (MKP-1), which correlated with persistent phosphorylation of Erk1/2 and was dependent on the tyrosine phosphorylation of PKCδ. Moreover, silencing of MKP-1 increased the phosphorylation of Erk1/2 and the apoptotic effect of etoposide. Etoposide induced polyubiquitylation and degradation of MKP-1 that was dependent on PKCδ and on its tyrosine phosphorylation. These results indicate that distinct phosphorylation of PKCδ on tyrosines 64 and 187 specifically activates the Erk1/2 pathway by the down-regulation of MKP-1, resulting in the persistent phosphorylation of Erk1/2 and cell apoptosis.

PKCδ is a novel PKC isoform that plays a major role in apoptosis in a cell- and stimulus-specific manner (1). PKCδ has been reported to affect both the extrinsic and intrinsic apoptotic pathways and to mediate the apoptotic effect of various stimuli such as etoposide (2), oxidative stress (4), ceramide (5), cisplatin (6), and phosphor 12-myristate 13-acetate (7). Conversely, it has been recently recognized that PKCδ can act as an anti-apoptotic kinase in some cellular systems including Sindbis virus-infected (8) and TRAIL-treated glioma cells (9), nitric oxide-induced macrophage cell death (10), and cells expressing activated p21RAS (11).

Important factors that regulate the apoptotic function of PKCδ are phosphorylation on distinct tyrosine residues and its subcellular localization (1). Tyrosine phosphorylation of PKCδ is now recognized as a critical determinant in the activation, cleavage, localization and substrate affinity of this isoform (12–16). In addition to the tyrosine phosphorylation of PKCδ by phosphor 12-myristate 13-acetate and various growth factors (12, 17, 18), PKCδ undergoes phosphorylation in response to many apoptotic stimuli including etoposide (2), TRAIL (9), oxidative stress (4, 19), γ-irradiation (20), and cisplatin (13). PKCδ has been shown to activate multiple signaling pathways that are associated with cell apoptosis such as extracellular signal-regulated kinases 1/2 (Erk1/2) (21), AKT (22), and p38 (23). However, the role of the tyrosine phosphorylation of PKCδ in the activation of specific downstream signaling pathways is not yet characterized.

The MAP kinase family plays a major role in the regulation of cell proliferation, differentiation, apoptosis, and survival (24). This family is comprised of four members, the Erk1/2, the p38 kinase, the Jun-N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and ERK5/BMK1. Members of this family are dually phosphorylated on threonine and tyrosine residues in the TEY sites in their activation loop by different MEKs (25)

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‡ The abbreviations used are: PKC, protein kinase C; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MAP, mitogen-activated protein; Erk, extracellular signal-regulated kinases; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; JNK, Jun-N-terminal kinase; siRNA, small interfering RNA; MKP, MAP kinase phosphatase; CV, control vectors; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein.
and are dephosphorylated by threonine or tyrosine phosphatases and by dual specificity MAP kinase phosphatases (i.e. MKPs 26). The MKP family is constituted of 11 members that differ in their specificities toward various MAP kinase substrates, in their subcellular localization, and in regulation by extracellular stimuli (27). MKP-1 belongs to the type I MKPs that localize in the nucleus, and it has been shown to dephosphorylate JNK, p38, and Erk1/2 in various cellular systems (26, 27). MKP-1 is rapidly induced by growth factors, oxidative stress, and the Erk1/2 cascade (28, 29), and it has been shown to undergo down-regulation by degradation via the ubiquitin-proteasome pathway, which is associated with the sustained phosphorylation of ERK1/2 (30).

In this study we explored the role of PKCδ and its phosphorylation at distinct tyrosine residues on the activation of Erk1/2 by etoposide and in the apoptotic effect of this drug. We found that the activation of PKCδ but not its tyrosine phosphorylation was essential for its nuclear translocation and that the effect of PKCδ on the activation of the Erk1/2 pathway was not dependent on the nuclear translocation of PKCδ. Moreover, the phosphorylation of PKCδ on tyrosines 64 and 187, but not on tyrosine 155, induced sustained phosphorylation of Erk1/2 by the ubiquitinylation and degradation of MKP-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—An affinity-purified polyclonal anti-PKCδ (C-17), anti-MKP-1, MKP-2, and MKP-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-active caspase 3, anti-p38, phospho-p38, JNK, phospho-Erk1/2, phospho-JNK, Erk5, phospho-Erk5, anti-MEK1, and phospho-MEK1 were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Etoposide, leupeptin, aprotinin, phenylmethanesulfonyl fluoride, sodium vanadate, anti-FLAG, and anti-Erk1/2 antibodies were obtained from Sigma.

**Glioma Cells and Cell Transfection**—The C6 glioma cells were grown in medium consisting of Dulbecco’s Modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (0.05 mg/ml).

Cells were transfected either with the control vectors (CV) or with the different PKCδ expression vectors by electroporation using the Nucleofector device and protocol number A29 (Amaxa Biosystems). Transfection efficiency using nucleofection was about 80–90%.

**Site-directed Mutagenesis of PKCδ**—cDNAs encoding PKCδ and the PKCδ mutants were fused into the N-terminal-enhanced GFP vector pEGFP-N1 (Clontech, Palo Alto, CA) (2). The PKCδY64E, PKCδY187F, PKCδY155F, and PKCδ-KD mutants were prepared and characterized as previously described (2, 12). For the construction of the PKCδ Y→E (tyrosine to glutamic acid) mutants, PKCδ cloned into the enhanced green fluorescent protein plasmid was used as a template vector for the site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). The following primers were employed: PKCδY64E forward, CG TTC GAC GCC CAC ATC GAG GAA GCC GTT ATC CAG; PKCδ Y64E reverse, CTG GAT AAC AGC GCC TTC CTC GAT GTG GCC GTG CTA CG; PKCδY187YE forward, CTC AAC AAG CAA GGC gag AAA TGC AGG CAA TGC; PKCδY187YE reverse, GCA TTG CCT GCA TTT CTC GCC TTG CTT GGT GAG; PKCδY155F forward, T AAA CAG GCC AGG ATC CAC GAA ATC AAG AAC CAC GAG TTTATC; PKCδY155F reverse, GAT AAA CTC GTG GGT CTT GAT TTT GAT GAT GCT GTG CTA TTT A. The mutations were confirmed by DNA sequencing, and the mutants were fused into the N-terminal enhanced GFP vector pEGFP-N1.

**siRNA Transfection**—PKCδ and MKP-1 siRNA duplexes (siRNAs) were obtained from Dharmacon (Lafayette, CO), and another MKP-1 siRNA duplex was obtained from Santa-Cruz Biotechnology (Santa Cruz, CA). A scrambled sequence was used as a negative control. Silencing of PKCδ was also performed using a pSuper PKCδ RNA-mediated interference vector. This expression construct was obtained from Alex Toker (Addgene). Transfection of siRNAs was performed using 100 nM PKCδ, MKP-1, or scrambled siRNAs and Oligofectamine (Invitrogen) according to the manufacturer’s instructions. Transfection of the pSuper vectors was performed by electroporation using the Nucleofector device (Amaxa) and protocol number A29.

**Confocal Microscopy**—For analysis of the localization and translocation of GFP-PKCδ and the GFP-PKCδ mutants, control and etoposide-treated cells were fixed in 4% paraformaldehyde for 10 min. Subsequently, cells were mounted in Fluoroguard antifade reagent and viewed using confocal microscopy with 63× magnification at an excitation wavelength of 488 nm as previously described (12).

**Fluorescence Recovery after Photobleaching (FRAP) Analysis**—C6 cells were transfected with PKCδ-GFP, PKCδY64F-GFP, or PKCδY187F-GFP and treated with 50 μM etoposide for 24 h. Before photobleaching, the medium was replaced with phenol-red free Leibovitz’s L15 medium containing 10% fetal bovine serum, and the cells were examined under a microscope at 37 °C using a live cell chamber system. Images were taken using a Zeiss LSM 510 Meta laser scanning confocal microscope equipped with a Plan-Apochromat 63×, 1.4 NA oil objective (Jena, Germany). Cells were scanned using multi-scan with a 488-nm laser for the detection of PKCδ-GFP, PKCδY64F, and PKCδY187F at the nuclear locus. Pre-bleach images were acquired, and then a specific region of interest was bleached at high laser intensity for 198.5 ms. Post-bleach recovery images were acquired at 86.1-ms intervals at zoom factor 6 and a resolution of 168 × 168 pixels. Fluorescence in the bleached region was measured as a function of time using the LSM software. For each analysis eight independent cells were chosen. Data were corrected for background intensity and for the overall loss in total intensity as a result of the bleach pulse and the imaging scans. First, the background intensity was subtracted from each pixel. Then the bleach correction was carried out by taking the pre-bleach average intensity of the whole cell divided by the whole cell average intensity at each time point in the post-bleach period. This bleach correction factor was
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FIGURE 1. Phosphorylation of Erk1/2 contributes to etoposide-induced apoptosis. C6 glioma cells were treated with etoposide (50 μM) for different periods of time, and the expression of phospho (p)-Erk1/2 and Erk1/2 was determined using Western blot analysis (A). The subcellular localization of phospho-Erk1/2 was determined using immunofluorescence staining and confocal microscopy (B). The phosphorylation of p38, JNK, and Erk5 by etoposide was determined as detailed for Erk1/2 (C). The contribution of the different MAP kinases to the apoptotic effect of etoposide was examined using the specific inhibitors SB239063, SP600125, PD98059, and U0126. Cell apoptosis was determined using propidium iodide staining and fluorescence-activated cell sorter analysis (D). The results are from one representative experiment of four similar experiments (A–C) or represent the means ± S.E. of triplicate measurements in each of three experiments (D). *, p < 0.001.

Results

Etoposide Induces Sustained Activation of Erk1/2 Which Contributes to Its Apoptotic Effect—Erk1/2 has been reported to play a role in cell apoptosis and survival (31, 32). We first examined the effect of etoposide on the activation of Erk1/2 and the role of Erk1/2 in the apoptotic effect of etoposide. Treatment of C6 cells with etoposide induced an increase in the phosphorylation of Erk1/2. An initial effect was observed after 1 h, and it persisted up to 24 h thereafter (Fig. 1A). In parallel, we followed the subcellular localization of the phosphorylated Erk1/2 in the cells by immunofluorescence staining using anti-phospho-Erk1/2 antibody. As demonstrated in Fig. 1B, control cells exhibited a weak staining of phospho-Erk1/2. At 1 h after etoposide treatment the expression of phospho-Erk1/2 was increased, and it was mainly localized to the cytosol. After 4 h of treatment, translocation of phospho-Erk1/2 to the nucleus was observed, and it persisted at least up to 24 h post-treatment.

We then examined the effect of etoposide on the activation of the other members of the MAP kinase family. Treatment of C6 cells with etoposide induced an increase in the phosphorylation of p38, JNK, and Erk5 by etoposide was determined as detailed for Erk1/2 (C). The contribution of the different MAP kinases to the apoptotic effect of etoposide was examined using the specific inhibitors SB239063, SP600125, PD98059, and U0126. Cell apoptosis was determined using propidium iodide staining and fluorescence-activated cell sorter analysis (D). The results are from one representative experiment of four similar experiments (A–C) or represent the means ± S.E. of triplicate measurements in each of three experiments (D). *, p < 0.001.

Data were analyzed using analysis of variance and a paired Student’s t test to determine the level of significance between the different groups.

Immunoprecipitation—Immunoprecipitation was performed as described previously (12). Control C6 cells or cells transfected with CV or PKCδ-KD mutant were used in this study. The samples were preabsorbed with 25 μl of protein Exacta–Cruz beads for 10 min, and immunoprecipitation was performed using 4 μg/ml anti-MKP-1 for 1 h at 4 °C and then incubated with 30 μl of A/G-Sepharose for an additional hour or overnight. After 3 washes, the pellets were resuspended in 25 μl of SDS sample buffer and boiled for 5 min. The entire supernatant was subjected to Western blotting.

Statistical Analysis—The results are presented as the mean value ± S.E. Data were analyzed using analysis of variance and a paired Student’s t test to determine the level of significance between the different groups.
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of p38 and JNK that was observed after 24 h and a transient increase in the phosphorylation of Erk5 that was observed after 1 h and diminished after 4 h of treatment (Fig. 1C).

To examine the role of the different MAP kinases in the apoptotic effect of etoposide, we employed the Erk1/2 inhibitors PD98059 and U0126, the p38 inhibitor SB239063, and the JNK inhibitor SP600125. Treatment of the cells with the specific inhibitors abolished the phosphorylation of the respective MAP kinases in response to etoposide (data not shown). However, only the Erk1/2 inhibitors significantly decreased the apoptotic effect of etoposide, suggesting that only activation of this pathway played a role in etoposide effect (Fig. 1D).

**Activation of Erk1/2 by Etoposide Is Downstream of PKCδ**—PKCδ has been shown recently by us and others to mediate the apoptotic effect of etoposide (2, 3). We, therefore, examined the role of PKCδ in the activation of Erk1/2 by this drug. For these experiments we employed a PKCδ-KD mutant and siRNAs. Cells were transiently transfected with a PKCδ-KD mutant and a CV, with PKCδ siRNA and control siRNA duplexes, or with control and PKCδ RNA-mediated interference pSuper vectors. Cells were then stimulated with etoposide, and the level of p-Erk1/2 was determined. Fig. 2A demonstrates that cells overexpressing the PKCδ-KD mutant exhibited a lower phospho-Erk1/2 in response to etoposide as compared with the CV cells. Similarly, silencing of PKCδ significantly decreased the expression of PKCδ (Fig. 2B) and of phospho-Erk1/2 in etoposide-treated cells as compared with cells transfected with control siRNAs (Fig. 2C). In addition, cells transfected with PKCδ siRNAs exhibited lower level of cell apoptosis as compared with control siRNA-transfected cells (Fig. 2D). Thus, our results clearly demonstrate that etoposide induces activation of Erk1/2 downstream of PKCδ.

The Phosphorylation of PKCδ on Tyrosines 64 and 187 Is Essential for the Activation of Erk1/2 by Etoposide—In a recent study we demonstrated that the PKCδY64F and PKCδY187F mutants decreased the apoptotic effect of etoposide and proposed that tyrosine phosphorylation of PKCδ played an important role in its pro-apoptotic effect in response to etoposide (2). In this study we further examined the role of tyrosines 64 and 187 in the effect of PKCδ on the activation of Erk1/2. Mutation of tyrosine 64 and 187 to phenylalanine did not affect the basal activity of PKCδ (data not shown). Using the PKCδY64F and PKCδY187F mutants, we found that the phosphorylation of Erk1/2 was significantly decreased in cells transfected with either the PKCδY64F or the PKCδY187F mutants (Fig. 3A). Thus, the phosphorylation of PKCδ on tyrosines 64 and 187 was essential for the activation of Erk1/2 induced by etoposide. To further study the role of tyrosines 64 and 187 in the phosphorylation of Erk1/2, we employed the phosphomimetic mutants PKCδY64E and PKCδY187E. Overexpression of these mutants increased the phosphorylation of Erk1/2 after 24 h of transfection as compared with the effect of PKCδ (Fig. 3B), further supporting a role of these phosphorylation sites in Erk1/2 activation. In contrast, expression of a PKCδY155E mutant did not induce phosphorylation of Erk1/2 (Fig. 3B).

To further examine the specificity of the tyrosine phosphorylation of PKCδ on Erk1/2 activation, we employed the apoptotic stimulus TRAIL, which activates PKCδ and induces its phosphorylation on tyrosine 155 (9). We found that similar to etoposide, TRAIL also induced phosphorylation of Erk1/2; however, this effect was transient and decreased after 60 min (Fig. 3C). The activation of Erk1/2 by TRAIL was not mediated by PKCδ or its phosphorylation on tyrosine 155 as a PKCδ-KD mutant or a PKCδY155F mutant did not alter the activation of Erk1/2 by TRAIL (Fig. 3C). Similarly, the PKCδY155F mutant did not inhibit the phosphorylation of Erk1/2 induced by etoposide (Fig. 3D).

**The Nuclear Localization of PKCδ Is Independent of Its Tyrosine Phosphorylation and Does Not Play a Role in the Activation of Erk1/2 by Etoposide**—We previously demonstrated that etoposide induced nuclear translocation of PKCδ (2) and that nuclear localization of PKCδ induced cell apoptosis (33). Because PKCδ and phospho-Erk1/2 exhibited similar nuclear translocation, we examined the role of the nuclear localization of PKCδ in the phosphorylation of Erk1/2. PKCδ-KD, PKCδY64F, and the PKCδY187F mutants inhibited the activation of Erk1/2, whereas the PKCδY155F mutant did not have any inhibitory effect. We, therefore, examined whether these mutants exhibited different translocation in response to etoposide. As demonstrated in Fig. 4A, the PKCδY187F and the...
PKC\textsubscript{\textgamma}H9254 Y64F mutants translocated to the nucleus in response to etoposide similar to PKC\textsubscript{\textgamma}H9254, further supporting our previous report that phosphorylation on these tyrosine residues does not play a role in the nuclear translocation of PKC\textsubscript{\textgamma}H9254 (2). In contrast, the PKC\textsubscript{\textgamma}KD mutant did not undergo nuclear translocation in response to etoposide at either 3 h (data not shown) or 24 h post-treatment (Fig. 4B), suggesting that the activity of PKC\textsubscript{\textgamma} was essential for its nuclear translocation. The lack of a role of the phosphorylation of tyrosine 187 and 64 or that of tyrosine 155 in the nuclear localization of PKC\textsubscript{\textgamma} was further demonstrated by the use of the phosphomimetic mutants, PKC\textsubscript{\textgamma}Y64E and PKC\textsubscript{\textgamma}Y187E. These mutants increased the phosphorylation of Erk1/2 but exhibited similar localization to that of PKC\textsubscript{\textgamma}-GFP with no enhanced accumulation in the nucleus (Fig. 4C). Likewise, the PKC\textsubscript{\textgamma}Y155E mutant did not show enhanced nuclear localization (Fig. 4C). Because PKC\textsubscript{\textgamma} has been reported to shuttle in and out of the nucleus (34), we also examined the mobility of PKC\textsubscript{\textgamma} and the PKC\textsubscript{\textgamma} tyrosine mutants in the nucleus using FRAP analysis. As presented in Fig. 4D, mutation of tyrosines 64 and 187 to phenylalanine did not affect the mobility of PKC\textsubscript{\textgamma} in the nucleus, and the FRAP of the nuclear PKC\textsubscript{\textgamma} was identical for the three proteins. Collectively, these results suggest that although PKC\textsubscript{\textgamma}-KD and the PKC\textsubscript{\textgamma} tyrosine mutants displayed differential subcellular localization after stimulation with etoposide, they similarly inhibited the activation of Erk1/2, suggesting that the nuclear localization of PKC\textsubscript{\textgamma} was not essential for this process. Importantly, the PKC\textsubscript{\textgamma}Y155F mutant, which did not inhibit the phosphorylation of Erk1/2 by etoposide, translocated to the nucleus in response to etoposide (9), further suggesting that the phosphorylation of PKC\textsubscript{\textgamma} on a specific tyrosine residue and not its nuclear translocation are associated with the effect of PKC\textsubscript{\textgamma} on Erk1/2 activation.

**Etoposide Induces Activation of MEK1 and Down-regulation of MKP-1**—The sustained phosphorylation of Erk1/2 by etoposide can be mediated by activation of MEK1 or by decreased dephosphorylation by MAP kinase phosphatases. Etoposide induced initial phosphorylation of MEK1 with similar kinetics to that of Erk1/2. The increase in pMEK1 was observed after 1 h of etoposide treatment but gradually decreased after 8 h of treatment (Fig. 5A). Thus, the sustained activation of Erk1/2 cannot be explained solely on the basis of MEK1 activation. We, therefore, examined the effect of etoposide on the expression of the MAP kinase phosphatases, MKP-1, MKP-2, and MKP-3. We found that etoposide induced a significant down-regulation of MKP-1 which was evident after 12 h of etoposide treatment and persisted up to 24 h thereafter (Fig. 5B). In contrast, we did
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FIGURE 4. Subcellular localization of PKCδ and the PKCδ tyrosine mutants in etoposide-treated cells. C6 cells were transfected with PKCδ or with the PKCδ tyrosine mutants PKCδY64F and PKCδY187F tagged with GFP. After 24 h the cells were stimulated with etoposide (50 μM) for 24 h, and the localization of PKCδ was determined using confocal microscopy. The percentage of cells with nuclear PKCδ is marked (A). The role of PKCδ activity in its nuclear translocation in etoposide-treated cells was determined by comparing the translocation of PKCδ-GFP and a PKCδ-KD GFP using confocal microscopy (B). C6 cells were transfected with the phosphomimetic PKCδ mutants PKCδY64E, PKCδY155E, and PKCδY187E tagged with GFP, and their subcellular localization was compared with that of PKCδ-GFP (C). FRAP analysis of PKCδ-GFP and the tyrosine mutants PKCδY64F-GFP and PKCδY187F-GFP was determined in etoposide-treated cells as described under “Experimental Procedures” (D). The results are from one representative experiment of three similar experiments. WT, wild type.

not find significant changes in the expression of MKP-2 and MKP-3 by etoposide (Fig. 5C). To examine the role of MKP-1 in the activation of Erk1/2 and in the apoptotic effect of etoposide, we silenced the expression of this protein by specific siRNA duplexes. We found that silencing of MKP-1 significantly decreased its expression after 48 h, increased the phosphorylation of Erk1/2 (Fig. 5D), and enhanced the apoptotic effect of etoposide as compared with that of control siRNA transfected cells (Fig. 5E).

PKCδ Mediates the Decrease in MKP-1 Expression Induced by Etoposide—We then examined the role of PKCδ and its tyrosine phosphorylation in the down-regulation of MKP-1 expression using the PKCδ-KD and PKCδ tyrosine mutants. Overexpression of PKCδ-KD abolished the decrease in MKP-1 expression induced by etoposide after 4 and 24 h (Fig. 6A), and similar results were obtained with the PKCδY187F and PKCδY64F mutants (Fig. 6B), suggesting that the down-regulation of MKP-1 was dependent on PKCδ and its phosphorylation on tyrosines 64 and 187.

MKP-1 has been shown to undergo down-regulation via ubiquitylation and proteasomal degradation (35). To examine the role of this pathway in the down-regulation of MKP-1 by etoposide, we first employed the proteasome inhibitor MG-132 and found that treatment of the cells with MG-132 abolished the decrease in MKP-1 expression induced by etoposide (Fig. 6C). We then examined the ubiquitylation of MKP-1 in etoposide-treated cells and found that etoposide induced polyubiquitylation of MKP-1 (Fig. 6D), further suggesting that the degradation of MKP-1 is via the ubiquitin-proteasome system. Because PKCδ induced down-regulation of MKP-1, we examined its role in the ubiquitylation of this protein using a PKCδ-KD mutant. As presented in Fig. 6E, overexpression of the PKCδ-KD mutant abolished the ubiquitylation of MKP-1 by etoposide, as compared with cells overexpressing CV.

DISCUSSION

PKCδ plays a major role in the regulation of cell apoptosis (1, 36); however, the downstream signaling pathways activated by PKCδ are not fully characterized. In this study we explored the role of PKCδ in the phosphorylation of Erk1/2 and its role in the apoptotic effect of etoposide. We found that etoposide induced a sustained activation of Erk1/2 and the translocation of phospho-Erk1/2 to the nucleus. Etoposide induced activation of other members of the MAP kinase family albeit to a different degree; however, the activation of these pathways did not contribute significantly to the apoptotic effect of etoposide. In contrast, the phosphorylation of Erk1/2 by etoposide contributed to its apoptotic effect, and inhibition of the Erk pathway decreased cell apoptosis induced by this drug. Erk1/2 has been extensively studied for its role in cell apoptosis and survival (31, 32, 37, 38) which is dependent on the duration of
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Etoposide induces down-regulation of MKP-1. C6 cells were treated with etoposide for different periods of time, and the phosphorylation (p) of MEK1 (A) and expression of MKP-1 (B) were determined using Western blot analysis. The effect of etoposide was also examined on the expression of MKP-2 and MKP-3 (C). MKP-1 expression was silenced using specific siRNA duplexes, and the expression of MKP-1 and the phosphorylation of Erk1/2 were determined after 48 h (D). Cells were then treated with etoposide for an additional 36 h, and cell apoptosis was determined using propidium iodide staining and fluorescence-activated cell sorter analysis (E). The results are from one representative experiment of three similar experiments (E). *, p < 0.05.

FIGURE 5. Etoposide induces down-regulation of MKP-1. C6 cells were treated with etoposide for different periods of time, and the phosphorylation (p) of MEK1 (A) and expression of MKP-1 (B) were determined using Western blot analysis. The effect of etoposide was also examined on the expression of MKP-2 and MKP-3 (C). MKP-1 expression was silenced using specific siRNA duplexes, and the expression of MKP-1 and the phosphorylation of Erk1/2 were determined after 48 h (D). Cells were then treated with etoposide for an additional 36 h, and cell apoptosis was determined using propidium iodide staining and fluorescence-activated cell sorter analysis (E). The results are from one representative experiment of three similar experiments (E). *, p < 0.05.

One of the factors that could account for the different effects of PKCδ and the PKCδ mutants is their different pattern of translocation. Indeed, PKCδ translocates to the nucleus in response to etoposide, and its nuclear localization has been implicated in its apoptotic function (2, 33, 34). In a previous study we reported that the phosphorylation of PKCδ on tyrosines was not important for its nuclear translocation using a PKCδ mutant in which 5 tyrosines (Tyr-52, -64, -155, -187, and -177) were mutated to phenylalanine (2); however, the role of the individual tyrosine residues was not examined. Because phospho-Erk1/2 and PKCδ exhibited similar nuclear translocation in response to etoposide, we examined whether the inhibitory effect of the PKCδ-KD, PKCδY64F, and PKCδY187F mutants on the phosphorylation of Erk1/2 in response to etoposide was due to differences in their nuclear localization. We found that, although the PKCδ-KD and the PKCδ tyrosine mutants similarly inhibited the activation of Erk1/2, they exhibited different patterns of translocation in response to etoposide. Thus, the PKCδ-KD mutant did not translocate to the nucleus, whereas the PKCδY64F and PKCδY187F mutants translocated to the nucleus similar to PKCδ. The lack of effect of the phosphorylated tyrosines 64 and 187 on the nuclear translocation of activation of Erk1/2 by etoposide, as both PKCδY64F and PKCδY187F mutants inhibited this activation.

The study we reported that the phosphorylation of PKCδ on tyrosines was not important for its nuclear translocation using a PKCδ mutant in which 5 tyrosines (Tyr-52, -64, -155, -187, and -177) were mutated to phenylalanine (2); however, the role of the individual tyrosine residues was not examined. Because phospho-Erk1/2 and PKCδ exhibited similar nuclear translocation in response to etoposide, we examined whether the inhibitory effect of the PKCδ-KD, PKCδY64F, and PKCδY187F mutants on the phosphorylation of Erk1/2 in response to etoposide was due to differences in their nuclear localization. We found that, although the PKCδ-KD and the PKCδ tyrosine mutants similarly inhibited the activation of Erk1/2, they exhibited different patterns of translocation in response to etoposide. Thus, the PKCδ-KD mutant did not translocate to the nucleus, whereas the PKCδY64F and PKCδY187F mutants translocated to the nucleus similar to PKCδ. The lack of effect of the phosphorylated tyrosines 64 and 187 on the nuclear translocation of its activation and subcellular localization (37). Thus, Erk1/2 has been shown to protect cortical neurons from the apoptotic effect of flavanones (39) and to play a role in cell survival after oxidant injury (31). In contrast, Erk1/2 activation played a role in cisplatin-induced cell apoptosis (40) and in glutamate-induced neuronal cell death (35).

We found that the activation of Erk1/2 by etoposide was downstream of PKCδ and was dependent on the phosphorylation of PKCδ on tyrosines 64 or 187. Tyrosine phosphorylation has emerged as an important factor in the regulation of the activity and role of PKCδ in multiple cellular processes, including cell apoptosis (2, 12, 13, 15–17). PKCδ is phosphorylated on tyrosines 64 and 187 in etoposide-treated cells (2), on tyrosine 155 in TRAIL-treated cells (9), and on tyrosines 311 and 332 in H2O2-treated cells (13). We have previously reported that the phosphorylation of PKCδ on tyrosines 64 and 187 in response to etoposide was essential for etoposide apoptotic effect (2). Our current results demonstrate that the phosphorylation of PKCδ on these two tyrosine residues was also essential for the
PKCδ was further demonstrated by the finding that the phosphomimetic mutants PKCδY64E and PKCδY187E did not accumulate in the nucleus and showed similar subcellular localization to that of PKCδ. Furthermore, we did not find a detectable difference in the mobility of PKCδ and the PKCδ tyrosine mutants in the nucleus of etoposide-treated cells using FRAP analysis. Thus, our results demonstrate that the phosphorylation of PKCδ on tyrosines 64 and 187 did not affect the nuclear translocation of PKCδ and that the translocation of PKCδ to the nucleus was not necessary for the activation of Erk1/2 by etoposide.

We demonstrated that the sustained activation of Erk1/2 by PKCδ was dependent on its phosphorylation on specific tyrosine residues. Thus, TRAIL, which induces phosphorylation of PKCδ on tyrosine 155 (9), induced a transient activation of Erk1/2 that was independent of PKCδ, suggesting that when PKCδ is phosphorylated on tyrosine 155 it does not activate the Erk1/2 pathway. Thus, the transient activation of Erk1/2 by TRAIL, as compared with the persistent activation by etoposide, may be due to a PKCδ-independent activation of this pathway by TRAIL. Interestingly, the activation of Erk1/2 by TRAIL acts as a pro-survival pathway in glioma cells.2 Mutation of tyrosine 155 to phenylalanine exhibited similar nuclear translocation to that of the PKCδY64F and PKCδY187F mutants in response to etoposide but did not inhibit the activation of Erk1/2 or the apoptotic effect of etoposide as did the other tyrosine mutants. These results further indicate that the nuclear translocation of PKCδ is not associated with the activation of Erk1/2 by etoposide and suggest that the phosphorylation of PKCδ on specific tyrosine residues (Tyr-64 and 187) is necessary for the activation of Erk1/2. The mechanism by which phosphorylation of PKCδ on distinct residues affects the activation of specific signaling pathways is not yet understood but can be due to the altered affinity of the phosphorylated PKCδ toward different substrates or to its ability to specifically bind different signaling molecules via SH2 or PTB domains.

PKCδ induced sustained activation of Erk1/2, which can be regulated by its phosphorylation by MEK1 and by its dephosphorylation by MKPs (25, 26, 41). We found that etoposide induced phosphorylation of MEK1 after 2 h of etoposide treatment. However, this phosphorylation declined after 8 h, and therefore, cannot solely mediate the sustained phosphorylation of Erk1/2. In contrast, we found a large decrease in MKP-1 expression in the etoposide-treated cells that persisted up to 24 h post-treatment. MKP-1 belongs to a family of MKPs that can dephosphorylate the MAP kinase family on tyrosine and threonine residues (26, 27). Silencing of MKP-1 increased the phosphorylation of Erk1/2 and enhanced the apoptotic effect of etoposide, suggesting a role of MKP-1 in the sustained phosphorylation of Erk1/2 by etoposide. MKP-1 acts also as a p38 and JNK phosphatase (42, 43) and may contribute to the phosphorylation of these two kinases by etoposide. However, because inhibition of p38 and JNK did not abrogate the apoptotic effect of etoposide, the major effect of MKP-1 is in enhancing the apoptotic effect of etoposide by the sustained activation of Erk1/2. In contrast to its effect on Erk1/2 activation, etoposide induced only a transient phosphorylation of Erk5. The difference in the activation of Erk1/2 and Erk5 by etoposide can be due to differences in the effect on this drug on the activation of MEK1 and MEK5, which phosphorylate Erk1/2 and Erk5, respectively, and to the effect of etoposide on the activation of different MKPs, which dephosphorylate these two proteins (26).

MKP-1 has been shown to undergo degradation by the ubiquitin-proteasome pathway in various cellular systems (35, 44). Recently, Erk1/2 has been implicated in the degradation of MKP-1 via this pathway, suggesting a positive feedback mechanism (30). Interestingly, the expression of MKP-1 is regulated by different members of the PKC family. Both PKCε and PKCζ induce up-regulation of MKP-1 (46, 47), whereas PKCδ has been shown to induce its degradation (35, 48). Thus, PKCδ induced the degradation of MKP-1 in glutamate-treated neuronal cells (35) and in ethanol-treated hepatocyte-like cells (48). Interestingly, in both cases PKCδ was tyrosine-phosphorylated, although the specific tyrosine residues in those studies were not identified. We also found that the tyrosine phosphorylation of PKCδ was associated with the degradation of MKP-1 and identified tyrosines 64 and 187 as the essential phosphorylation sites for this effect. The mechanisms by which PKCδ induces the degradation of MKP-1 are not yet understood, but both our study and others implicate the role of the ubiquitin-proteasome system in this response. Indeed, etoposide induced the ubiquitination of MKP-1 in a PKCδ-dependent manner. PKCδ may affect the degradation of MKP-1 by its phosphorylation or the phosphorylation of an upstream kinase or may affect an E3 ubiquitin ligase by direct or indirect phosphorylation. Studies exploring the mechanisms of the PKCδ effect in MKP-1 degradation are currently under way.

In summary, we found that phosphorylation of PKCδ on tyrosines 64 and 187 induces sustained activation of Erk1/2 by the degradation of MKP-1, which mediates the apoptotic effect of etoposide. The PKCδY64F and PKCδY187F mutants exerted a dominant negative effect on the activation of the Erk1/2 pathway and the apoptotic function of etoposide. Tyrosine mutants have been shown to act as dominant negative mutants for various functions of PKCδ (2, 8, 9, 12, 13, 14, 45), and this effect may be mediated by their altered affinity toward different substrates or their modified ability to associate with various signaling molecules and docking proteins. Thus, in addition to identifying a novel mechanism for the apoptotic effect of etoposide, the results of this study further emphasize the importance of the tyrosine phosphorylation of PKCδ in its apoptotic effect and as a molecular switch for the activation of specific downstream signaling pathways.

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