Phororbol Esters Induce Intracellular Accumulation of the Anti-apoptotic Protein PED/PEA-15 by Preventing Ubiquitinylation and Proteasomal Degradation*

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Phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes (PED/PEA)-15 is an anti-apoptotic protein whose expression is increased in several cancer cells and following experimental skin carcinogenesis. Exposure of untransfected C5N keratinocytes and transfected HEK293 cells to phorbol esters (12-O-tetradecanoylphorbol-13-acetate (TPA)) increased PED/PEA-15 cellular content and enhanced its phosphorylation at serine 116 in a time-dependent fashion. Ser-116 → Gly (PEDS116G) but not Ser-104 → Gly (PEDS104G) substitution almost completely abolished TPA regulation of PED/PEA-15 expression. TPA effect was also prevented by anti-sense inhibition of protein kinase C (PKC)-ζ and by the expression of a dominant-negative PKC-ζ mutant cDNA in HEK293 cells. Similar to long term TPA treatment, overexpression of wild-type PKC-ζ increased cellular content and phosphorylation of WT-PED/PEA-15 and PEDS104G, but not of PEDS116G. These events were accompanied by the activation of Ca2+, calmodulin kinase (CaMK) II and prevented by the CaMK blocker, KN-93. At variance, the proteasome inhibitor lactacystin mimicked TPA action on PED/PEA-15 intracellular accumulation and reverted the effects of PKC-ζ and CaMK inhibition. Moreover, we show that PED/PEA-15 bound ubiquitin in intact cells. PED/PEA-15 ubiquitinylation was reduced by TPA and PKC-ζ overexpression and increased by KN-93 and PKC-ζ block. Furthermore, in HEK293 cells expressing PEDS116G, TPA failed to prevent ubiquitin-dependent degradation of the protein. Accordingly, in the same cells, TPA-mediated protection from apoptosis was blunted. Taken together, our results indicate that TPA increases PED/PEA-15 expression at the post-translational level by inducing phosphorylation at serine 116 and preventing ubiquitinylation and proteasomal degradation.

Cancer cells feature both excessive proliferation and abandonment of the ability to die (1, 2). Thus, alterations of genes involved in the control of apoptosis have been implicated in a number of human malignancies. In certain lymphomas, for example, cell death is blocked by excessive production of the anti-apoptotic factor Bcl-2 (2). Similarly, some tumors prevent apoptosis by up-regulating the expression of anti-apoptotic death effector domain (DED)2-containing proteins, which, in turn, inhibit Fas from conveying signals to the death machinery (3).

Phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes (PED/PEA)-15 is a DED-containing protein originally identified in astrocytes as a protein kinase C substrate (4–6) and found overexpressed in insulin target tissues of patients with type 2 diabetes (7). Raised PED/PEA-15 levels have also been detected in several human tumor cell lines (7–9). A growing body of evidence indicates that increased PED/PEA-15 expression may provide a mechanism to escape cell death upon a number of pro-apoptotic stimuli (10–14). Moreover, in transgenic mice, overexpression of PED/PEA-15 enhances the susceptibility to develop experimentally induced skin tumors (15). The molecular mechanism of PED/PEA-15 anti-apoptotic action has been extensively investigated. In several cell types, PED/PEA-15 blocks Fas- and tumor necrosis factor-α-induced apoptosis by competing with its DED with the interaction between FADD and caspase 8 (10). In addition, in several cell lines of human glioma, PED/PEA-15 inhibits tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, thereby generating resistance to this anti-neoplastic agent (9). At variance with other anti-apoptotic proteins inhibiting caspase 8 activation via FADD trapping (3), PED/PEA-15 overexpression also prevents apoptosis induced by growth factors deprivation, UV exposure, and osmotic stimuli (11, 13).

Besides the anti-apoptotic function, a role for PED/PEA-15 in restraining cell proliferation has been proposed (16–20). It

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2 The abbreviations used are: DED, death effector domain; PED/PEA, phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKB, protein kinase B; PKC, protein kinase C; CaMK, calmodulin kinase; FADD, Fas-associated death domain; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription; PBS, phosphate-buffered saline; Ab, antibody; ASO, antisense oligonucleotides; DN, dominant-negative; WT, wild type; SO, scrambled.

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has been described that PED/PEA-15 directly binds extracellular signal-regulated kinase 2 (ERK2) and RSK2 and prevents their nuclear translocation and transduction of biological effects (16–19). Together with the anti-apoptotic effect, this action may expand cellular senescence (20).

PED/PEA-15 is a phosphorylated protein (4, 5). It has recently been shown that PED/PEA-15 phosphorylation at specific sites controls the ability of the protein to form complexes with specific intracellular interactors (21). PED/PEA-15 serine phosphorylation has also been shown to enhance protein stability (22). Several kinases were evidenced to phosphorylate PED/PEA-15 at specific serines. Ser-104 represents the main target for PKC phosphorylation (4, 5, 23), whereas Ser-116 has been shown to be a target site for both Ca\textsuperscript{2+}-calmodulin kinase (CaMK) II (23) and protein kinase B (PKB)/Akt (22). However, the precise function of these phosphorylation sites in controlling PED/PEA-15 expression is currently unknown. Recent evidence indicates that abnormal accumulation of PED/PEA-15 may lead to derangement of cell growth and metabolism (15, 24, 25).

In this study, we have shown that phorbol esters, which are tumor promoters and inhibitors of insulin action, up-regulate PED/PEA-15 expression by inhibiting its ubiquitinylination and proteasomal targeting. This effect involves activation of CaMKII and subsequent phosphorylation of PED/PEA-15 at Ser-116. PKC-\(\zeta\) activity is required for phorbol ester-induced activation of CaMKII and for the regulation of PED/PEA-15 degradation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Media, sera, and antibiotics for cell culture and the Lipofectamine reagent were purchased from Invitrogen (Paisley, UK). Rabbit polyclonal PKC-\(\alpha\), PKC-\(\beta\), PKC-\(\delta\), PKC-\(\zeta\), and phospho-PKC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PED/PEA-15, p-Ser-104PED, and phospho-PKC antibodies were from Santa Cruz Biotechnology (Paisley, UK). Rabbit polyclonal PKC-\(\alpha\), PKC-\(\beta\), PKC-\(\delta\), PKC-\(\zeta\) antisense and scrambled sequences used were as follows: PED/PEA-15, forward, 5'-TCTGGCTCATCCGCATCC-3', reverse, 5'-TCTGGCTATCCGCATCC-3'.
umn. The column was successively washed with the following: 1 ml of 6 M guanidium-HCl, 0.1 M Na₂ HPO₄, NaH₂ PO₄ (pH 8); 2 ml of (6 M guanidium-HCl, 0.1 M Na₂ HPO₄, NaH₂ PO₄ (pH 8), protein buffer) 1:1; 2 ml of (6 M guanidium-HCl, 0.1 M Na₂ HPO₄, NaH₂ PO₄ (pH 8), protein buffer) 1:3; 2 ml of protein buffer; 1 ml of protein buffer plus 10 mM imidazole. Elution was performed with 1 ml of protein buffer plus 200 mM imidazole. Protein buffer is 50 mM Na₂ HPO₄, NaH₂ PO₄ (pH 8), 100 mM KCl, 20% glycerol, and 0.2% Nonidet P-40. The eluate was trichloroacetic acid-precipitated for further analysis by Western blot with HA antibody.

**Cell Death Analysis by Flow Cytometry**—Cells were harvested and suspended in the sample buffer (PBS + 2% fetal bovine serum; PBS + 0.1% bovine serum albumin) and washed and resuspended in 0.3 ml of PBS. After adding 0.7 ml of cold absolute ethanol, cells were fixed for at least 2 h at −20 °C, washed twice, and resuspended in 0.4 ml of PBS. Samples were then incubated with 20 μl of propidium iodide (1 mg/ml stock solution) and 2 μl of RNaseA (500 mg/ml stock solution) in dark for 30 min at room temperature. Samples were stored at 4 °C until analyzed by flow cytometry.

**RESULTS**

**Regulation of PED/PEA-15 Protein Expression by Phorbol Esters**—The expression of PED/PEA-15 is up-regulated by phorbol myristate acetate (TPA) in the mouse skin upon experimental carcinogenesis as well as in different human tumors (7–9, 15). To investigate the molecular mechanisms regulating PED/PEA-15 expression, HEK-293 cells, stably transfected with PED/PEA-15 cDNA (293PEDY1), were incubated with serum-free medium in the absence or in the presence of 1 μM TPA.

Treatment of 293PEDY1 cells with TPA increased PED/PEA-15 levels in a time-dependent manner (Fig. 1A). Serum deprivation alone was sufficient to reduce PED/PEA-15 protein levels by 3-fold (Fig. 1B). This decrease was totally reverted by the simultaneous exposure to TPA. Pretreatment of the cells with the protein synthesis inhibitor cycloheximide (40 μg/ml) reduced TPA effect by only 25% (Fig. 1B, left panel). Similar results were also obtained by evaluating the levels of PED/PEA-15 in C5N keratinocytes, expressing only the endogenous compendium of the protein (Fig. 1B, right panel). Moreover, as shown in Fig. 2, PED/PEA-15 mRNA was also increased in untransfected C5N cells following 6 and 20 h of TPA treatment. Thus, TPA up-regulates PED/PEA-15 mRNA and protein.
expression. However, TPA regulation is, at least in part, independent of protein synthesis.

Regulation of PED/PEA-15 Expression by Serine Phosphorylation—It has been shown that PED/PEA-15 expression is tightly regulated by its phosphorylation state (22). We therefore investigated whether TPA could induce PED/PEA-15 phosphorylation. To this end, protein extracts of TPA-stimulated 293PEDY1 and C5N cells were immunoblotted with antibodies against the phosphorylated forms of Ser-104 and Ser-116. Each filter has been reprobed with PED Ab for the normalization. The results have been analyzed by laser densitometry, and the error bars represent the mean ± S.D. of the densitometric analyses obtained in four duplicate experiments. B, HEK293 cells have been transfected with PEDWT, PEDS116G, and PEDS104G, as indicated. Next, cells were serum-starved and stimulated with 1 μM TPA for 20 h. Cell lysates were separated on SDS-PAGE and immunoblotted with PED Ab. Filters have been analyzed by laser densitometry. The autoradiograph shown is representative of five independent experiments. The error bars represent the mean ± S.D. of the densitometric analysis.

FIGURE 3. Regulation of PED/PEA-15 phosphorylation levels by TPA. A, 293PEDY1 and C5N cells were serum-starved and stimulated with 1 μM TPA for the indicated times. Cell lysates were separated on SDS-PAGE and immunoblotted either with p-PEDS104 Ab or with p-PEDS116 Ab. Each filter has been reprobed with PED Ab for the normalization. The results have been analyzed by laser densitometry, and the error bars represent the mean ± S.D. of the densitometric analyses obtained in four duplicate experiments. B, HEK293 cells have been transfected with PEDWT, PEDS116G, and PEDS104G, as indicated. Next, cells were serum-starved and stimulated with 1 μM TPA for 20 h. Cell lysates were separated on SDS-PAGE and immunoblotted with PED Ab. Filters have been analyzed by laser densitometry. The autoradiograph shown is representative of five independent experiments. The error bars represent the mean ± S.D. of the densitometric analysis.

Regulation of PED/PEA-15 Ubiquitinylation—Almost undetectable changes of Ser-104 phosphorylation (Fig. 3A).

To assess the relevance of those phosphorylation sites, PED/PEA-15 mutants bearing Ser-104 → Gly (PEDS104G) or Ser-116 → Gly (PEDS116G) substitutions were transfected in HEK293 cells. TPA treatment increased the levels of the wild-type PED/PEA-15 (PEDWT) and of PEDS104G by about 4-fold. At variance, PEDS116G expression was increased by only 2-fold upon TPA exposure (Fig. 3B), suggesting that phosphorylation of Ser-116 is required for TPA regulation of PED/PEA-15 expression.

PKC Regulation of PED/PEA-15 Expression—To identify the kinase responsible for the regulation of PED/PEA-15 expression, 293PEDY1 cells were treated with specific phosphorothioate antisense oligonucleotides (ASO) toward individual PKC isoforms (Fig. 4). Based on Western blot experiments, ASO for PKC-δ/H9254 did not significantly affect PED/PEA-15 expression levels when compared with scrambled (SO) oligonucleotide controls. The expression of the targeted PKC isoform was selectively reduced by 50%, however. At variance, ASO-mediated silencing of PKC-ζ expression (PKCζ/ASO) was accompanied by a significant 70% decrease of PED/PEA-15 levels (Fig. 4). A scrambled oligonucleotide (PKCζ/SO) did not induce any detectable change (Fig. 4). A reduction of PED/PEA-15 expression was also observed when 293PEDY1 cells were transfected with a dominant-negative (DN) PKC-ζ mutant or with PKCζ/ASO and stimulated with TPA for 20 h (Fig. 5A). CaMKII and Akt/PKB have been shown to directly phosphorylate PED/PEA-15 at Ser-116 (22, 23). Interestingly, a 75% decrease of PED/PEA-15 expression was also detected in 293PEDY1 cells treated with the CaMK inhibitor, KN-93 (Fig. 5A). Similarly, in C5N keratinocytes, TPA-induced up-regulation of PED/PEA-15 protein expression was reduced by about 70% by KN-93 treatment (Fig. 5B).

Next, we investigated whether TPA could regulate CaMKII activity in 293PEDY1 and in C5N cells. In this regard, CaMKII phosphorylation (Fig. 6A) was induced by TPA and well correlated with increased PED/PEA-15 expression levels and Ser-116 phosphorylation in 293PEDY1 cells (Figs. 1A and 3A). Consistently, 20 h of TPA treatment of C5N cells was accompanied by a 2.5-fold increase of CaMKII phosphorylation (Fig. 6A). The
expression of DN-PKC-ζ and the treatment of 293PEDY1 cells with PKCζ-ASO reduced by about 65% TPA-induced CaMKII activation (Fig. 6B). Conversely, overexpression of the wild-type PKC-ζ led to >3-fold increase of CaMKII activity. At variance, Akt/PKB activity was not induced following 20 h of TPA treatment of both 293PEDY1 and C5N cells (Fig. 6C).

Moreover, TPA treatment and PKC-ζ overexpression increased by >5-fold the phosphorylation of PED/PEA-15 at Ser-116. Pretreatment of 293PEDY1 cells with KN-93 almost completely reverted both TPA- and PKC-ζ-induced phosphorylation of PED/PEA-15 (Fig. 7A). TPA-induced Ser-116 phosphorylation was also reduced by KN-93 in C5N keratinocytes. Consistent results were obtained in transiently transfected HEK293 cells by analyzing the expression of PEDWT and PEDS104G but not of PEDS116G (Fig. 7B). Indeed, PKC-ζ-mediated changes of PEDWT and PEDS104G were prevented by KN-93, which, instead, had no effect on the regulation of PEDS116G expression.

Regulation of PED/PEA-15 Ubiquitinylation—We hypothesized that PED/PEA-15 protein accumulation within the cell was due to decreased degradation. To investigate the mechanisms regulating PED/PEA-15 degradation, 293PEDY1 cells were treated with the proteasomal inhibitor lactacystin. Lactacystin (30 μM) inhibited the degradation of PED/PEA-15 induced by serum deprivation by 70% and almost completely reverted the effect of the PKCζ-ASO (Fig. 8A). Lactacystin treatment also prevented PED/PEA-15 degradation induced by KN-93 in the 293PEDY1 cells (data not shown). In addition, lactacystin, at variance with TPA, increased the expression of PEDS116G at a similar extent as PEDWT (Fig. 8B), suggesting that PED/PEA-15 phosphorylation at the Ser-116 was required to escape degradation. Following lactacystin treatment of the untransfected C5N cells, PED/PEA-15 protein levels were also increased by 2.5- and 3-fold, respectively, in the absence or in the presence of TPA (Fig. 8B). In both cases, the incubation with KN-93 did not significantly reduce lactacystin effect on PED/PEA-15 protein levels. Thus, CaMK block was overcome by proteasome inhibitors.

These data were consistent with the hypothesis that PED/PEA-15 is largely degraded within the proteasomal compartment. Proteasome-targeted proteins are usually ubiquitylated (30). His-tagged PED/PEA-15 and HA-tagged ubiquitin have been transfected, alone or in combination, in HEK293 cells, and PED/PEA-15-bound ubiquitin was detected by Western blot with HA antibodies (Fig. 9A). A typical smear was observed in cells co-transfected with both constructs, indicating that PED/PEA-15 is a ubiquitylated protein (Fig. 9A).
Next, 293PEDY1 cells were incubated in serum-free medium and treated with TPA for 20 h or transfected with wild-type PKC-ζ. PED/PEA-15 immunoprecipitates were then blotted with FK1 antibodies, which recognize polyubiquitinylated proteins. Interestingly, PED/PEA-15 ubiquitinylation was 2.5-fold increased by serum starvation. At the opposite, it was reduced by ~2-fold by TPA treatment and by overexpression of PKC-ζ (Fig. 9B). Both TPA and PKC-ζ failed to decrease PED/PEA-15 ubiquitinylation in the presence of KN-93. Also, ubiquitinylation of the PEDS104G mutant was reduced in a manner comparable with that of PEDWT, whereas that of the PEDS116G mutant did not significantly change (Fig. 9C).

Functional Relevance of Ser-116 Phosphorylation—To further investigate the relevance of PED/PEA-15 phosphorylation on its anti-apoptotic action, 293PEDY1 cells have been deprived of serum for 20 h in the absence or in the presence of TPA (Fig. 10). As expected, TPA exposure largely rescued the cell death induced by serum starvation. TPA effect was also mimicked by PKC-ζ overexpression in 293PEDY1 cells (Fig. 10A). However, the incubation with KN-93 prevented both TPA and PKC-ζ

![Figure 6](image1.png)

**FIGURE 6. TPA and PKC-ζ effect on CaMKII phosphorylation.** A, 293PEDY1 cells were serum-starved and treated with 1 μM TPA for the indicated times. Cell lysates were analyzed by p-CaMKII immunoblot. Filters were then reprobed with CaMKII Ab for normalization, and the results were quantitated by laser densitometry. The autoradiograph shown is representative of five (for 293PEDY1) and three (for CSN) independent experiments. The error bars represent the mean ± S.D. of the densitometric analysis. B, 293PEDY1 cells were serum-starved and treated with 1 μM TPA for 20 h in the absence or in the presence of PKC-ζ ASO or of wild-type or a dominant-negative PKC-ζ mutant. Cell lysates were then analyzed by immunoblot with p-CaMKII and CaMKII Abs, and the results were quantitated by laser densitometry. The autoradiograph shown is representative of four independent experiments. The error bars represent the mean ± S.D. of the densitometric analyses. C, 293PEDY1 and CSN cells were serum-starved and treated with 1 μM TPA for 20 h. Akt/PKB activity has been measured as described previously (35). The error bars represent the mean ± S.D. of the densitometric analyses obtained in four duplicate experiments.

![Figure 7](image2.png)

**FIGURE 7. Regulation of PED/PEA-15 phosphorylation and expression by CaMKII.** A, 293PEDY1 and CSN cells were serum-starved and treated with 1 μM TPA for 20 h or transfected with a pcDNAIII plasmid containing a PKC-ζ cDNA, in the absence or in the presence of 10 μM KN-93. Cell lysates were then analyzed by immunoblot with p-PEDS116 and PED Abs. The results have been analyzed by laser densitometry, and the error bars represent the mean ± S.D. of the densitometric analyses obtained in four duplicate experiments. B, HEK293 cells transfected with PEDWT, PEDS104G, and PEDS116G alone or in combination with PKC-ζ cDNA and further incubated in the absence or in the presence of 10 μM KN-93. Cell lysates were then analyzed by PKC-ζ and PED immunoblot, and the results were quantitated by laser densitometry. The autoradiographs shown are representative of four independent experiments. The error bars represent the mean ± S.D. of the densitometric analyses.
rescue of cell death, suggesting that CaMKII-induced phospho-
rylation of PED/PEA-15 at Ser-116 was required for this effect
(Fig. 10A). To further sustain this hypothesis, we have tested
TPA protection from cell death in HEK293 cells transfected
with either PEDWT or PEDS116G. Although normally inducing
survival of serum-starved cells transfected with PEDWT, TPA
effect was 2-fold reduced in cells overexpressing PEDS116G
(Fig. 10B).

**DISCUSSION**

Elevated expression of the anti-apoptotic protein PED/
PEA-15 has been found in transformed cell lines and confers
resistance to apoptotic stimuli (7–11, 15, 22). An increase of
PED/PEA-15 levels is also detected in the papillomatous skin of
dimethylbenzanthracene/TPA-treated mice upon experimen-
tal carcinogenesis protocols (15), further indicating that raised
PED/PEA-15 expression may play a role in cellular transforma-
tion. In this work, we have investigated the molecular mechanisms
through which the tumor-promoting agent TPA affects PED/PEA-15
expression. Two lines of evidence indicate that, at least in part, PED/
PEA-15 expression is regulated by TPA at the post-translational level.
Firstly, similar to previous observations in mouse skin and in keratino-
cyte cell lines (15), phorbol esters up-regulate PED/PEA-15 protein
expression in HEK293 cells ectopically expressing the PED/PEA-15
cDNA under the transcriptional control of the cytomegalovirus pro-
moter. In addition, in these cells, as well as in untransfected keratino-
cytes, PED/PEA-15 regulation by TPA also occurs in the presence of
the protein synthesis inhibitor cycloheximide. The evidence that
TPA effect was partially reduced by cycloheximide, however, sug-
gests that additional regulation may occur at the transcriptional level.
Indeed, PED/PEA-15 mRNA levels are also significantly
increased in untransfected C5N

**FIGURE 8. Effect of lactacystin on PED/PEA-15 expression.** A. 293PEDY1 cells were treated with either PKCζ-
ASO or PKCζ-SO and further incubated in the absence or in the presence of 30 μM lactacystin (Lact), as indicated. Cell lysates were then analyzed by PED immunoblot, and the results were quantitated by laser densi-
tometry. The autoradiograph shown is representative of four independent experiments. The error bars represent the mean ± S.D. of the densitometric analysis. FCS, fetal calf serum. B. HEK293 cells transfected with
PEDWT or PEDS116G were treated with 1 μM TPA or 30 μM lactacystin for 20 h as indicated. C5N cells were
serum-starved and incubated with TPA (1 mM), KN-93 (10 mM), and lactacystin (30 μM) as indicated. Cell lysates
were then analyzed by PED immunoblot, and the results were quantitated by laser densitometry. The autora-
diographs shown are representative of four (for HEK293) and three (for C5N) independent experiments. The error bars represent the mean ± S.D. of the densitometric analyses.

is involved in TPA control of PED/PEA-15 expression since
there is no sustained Akt/PKB activation upon TPA exposure of
HEK293 cells and C5N keratinocytes. CaMKII is a more likely
candidate. Indeed, Kubes et al. (23) have reported that CaMKII
may also phosphorylate PED/PEA-15 at Ser-116 and, consist-
ent with findings in other cell types (31), we found that TPA
increases CaMKII activity in HEK293 and in C5N cells (Fig. 6).
In addition, the timing of CaMKII activation closely parallels
PED/PEA-15 phosphorylation at Ser-116 following TPA stimu-
lation. Finally, pharmacological inhibition of CaMKII with
KN-93 almost totally blocked TPA-induced Ser-116 phosphor-
ylation. At variance, Ser-104 phosphorylation was rapidly
induced by TPA and then decreased upon prolonged incuba-
tion. Ser-104 is known to be directly phosphorylated by PKC
following endothelin-1 treatment of astrocytic cells (23). The
same occurs with TPA since the down-regulation of conven-
tional PKC isoforms after long term exposure was accompanied
Regulation of PED/PEA-15 Ubiquitylation

A, HEK293 cells were transfected with His-Myc-PED/PEA-15 and HA-ubiquitin (HA-Ub) alone or in combination. Upon purification of His-Myc-PED/PEA-15 and separation on SDS-PAGE, filters were probed with HA Ab. The autoradiograph shown is representative of five independent experiments. B, 293PEDY1 cells were serum-starved and treated with 1 μM TPA for 20 h or transfected with PKC-ζ C3A in the absence or in the presence of 10 μM KN-93. Cell lysates were separated on SDS-PAGE and immunoblotted with FK1 and PED Abs. The results have been analyzed by laser densitometry, and the error bars represent the mean ± S.D. of the densitometric analyses obtained in four duplicate experiments. C, fetal calf serum. C, HEK293 cells transfected with PEDWT, PEDS104G, and PEDS116G were serum-starved and treated with 1 μM TPA for 20 h. Cell lysates were separated on SDS-PAGE and immunoblotted with FK1 and PED Abs. The results have been analyzed by laser densitometry, and the error bars represent the mean ± S.D. of the densitometric analyses obtained in four duplicate experiments.

by a decline of Ser-104 phosphorylation. However, genetic silencing of conventional and novel PKC isoforms, which are canonical intracellular targets of TPA, further argued against the involvement of Ser-104 phosphorylation by PKC in direct regulation of PED/PEA-15 expression. Consistent with this, the Ser-104 → Gly mutant, but not the Ser-116 → Gly mutant, was equally sensitive to TPA action as the wild-type PED/PEA-15. Altogether, these observations indicate that insulin and phorbol esters use different pathways to regulate PED/PEA-15 protein expression, both converging at the level of Ser-116 phosphorylation. For instance, whereas Akt/PKB is the major candidate kinase for the insulin action (22), CaMKII may mediate PED/PEA-15 phosphorylation at the Ser-116 in response to TPA. The finding that LY294002 inhibition of phosphatidylinositol 3-kinase activity also reduces TPA effect on PED/PEA-15 expression (data not shown) may be due to decreased activity of other downstream molecules different from Akt/PKB.

Indeed, a pivotal role has emerged for PKC-ζ in TPA regulation of PED/PEA-15 expression. Both the antisense reduction of PKC-ζ and the expression of a dominant-negative PKC-ζ mutant led to a decrease of TPA-regulated PED/PEA-15 phosphorylation at Ser-116, accompanied by a reduction of PED/PEA-15 protein levels. This led us to hypothesize that PKC-ζ could either directly phosphorylate Ser-116 or directly affect CaMKII activity. No PED/PEA-15 phosphorylation at Ser-116 was induced in vitro by active recombinant PKC-ζ (data not shown). At variance, inhibition of PKC-ζ expression and/or function in HEK293 cells almost completely abolished CaMKII induction by TPA, supporting the hypothesis that PKC-ζ could affect PED/PEA-15 expression by acting upstream of CaMKII. Accordingly, PKC-ζ-increased CaMKII activity was paralleled by raised Ser-116 phosphorylation and PED/PEA-15 expression levels. Whether PKC-ζ is directly activated by phorbol esters is still debated (32–34). Alternatively, however, prolonged exposure of the cell to TPA, which is known to down-regulate conventional PKC isoforms, may up-regulate PKC-ζ activity by removing the tonic inhibitory constraint exerted by the firsts on the latter. This is consistent with our previous observation, indicating that PKC-α hyperactivation causes a downstream inhibition on PKC-ζ (24, 35).

Regulation of PED/PEA-15 phosphorylation may be a common event, which contributes to protection from apoptosis, driven by either PKC-ζ (36–38) or CaMKII (39–41). Intriguingly, we have previously described that PED/PEA-15 overexpression inhibits insulin induction of PKC-ζ, thereby impairing glucose uptake (24, 35). It is now emerging that PKC-ζ activation instead up-regulates PED/PEA-15 protein levels, which in turn, may negatively affect PKC-ζ function. This is also in agreement with recent evidence showing that forced expression of PKC-ζ may inhibit insulin and growth factor signaling (42–44).

Recently, Renganathan et al. (21) have proposed that PED/PEA-15 phosphorylation at specific residues is important in enabling its interaction with selected intracellular proteins. In particular, phosphorylation at Ser-116 promotes its binding to FADD and plays an important role in protecting cells from apoptosis (9, 10, 21). Here, we show that Ser-116 phosphorylation is also involved in preventing PED/PEA-15 degradation in the 26 S proteasome. Indeed, lactacystin treatment mimicked the effect of TPA and prevented PED/PEA-15 protein loss follow-
Regulation of PED/PEA-15 Ubiquitinylation

CaMKII activation and PED/PEA-15 phosphorylation at Ser-116 are relevant for this effect. Thus, we have shown that phorbol esters up-regulate PED/PEA-15 expression by controlling its proteasomal degradation. PKC-ζ and CaMKII activities are necessary to enable TPA-dependent phosphorylation of PED/PEA-15 at Ser-116. This phosphorylation prevents ubiquitylation and proteasomal targeting and induce PED/PEA-15 intracellular accumulation, thereby enhancing its anti-apoptotic action.

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FIGURE 10. TPA-mediated regulation of cell death by PED/PEA-15 phosphorylation. A, 293PEDWT cells were serum-starved and treated with 1 μM TPA for 20 h or transfected with PKC-ζ in the absence or in the presence of 10 μM KN-93, as indicated. Cell suspensions were stained with propidium iodide and analyzed by flow cytometry. Data are presented as the percentage of the results obtained in three triplicate experiments. B, HEK293 cells transfected with PEDWT or PEDS116G were serum-starved and treated with 1 μM TPA for 20 h, as indicated. Cell suspensions were stained with propidium iodide and analyzed by flow cytometry. Data are presented as the percentage of the results obtained in three triplicate experiments.
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