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Phorbol 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 (PED_{S116G}) but not Ser-104 → Gly (PED_{S104G}) substitution almost completely abolished TPA regulation of PED/PEA-15 expression. TPA effect was also prevented by antisense 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 PED_{S104G} but not of PED_{S116G}. These events were accompanied by the activation of Ca^{2+}-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 PED_{S116G}, 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 proteosomal 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 (PKC) 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.

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^{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 ubiquitinylation and proteasomal targeting. This effect involves activation of CaMKII and subsequent phosphorylation of PED/PEA-15 at Ser-116. PKC-ζ 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-α, PKC-β, PKC-δ, PKC-ζ, and phospho-PKC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PED/PEA-15, p-Ser-104PED, and p-Ser-116PED and antibodies have been previously reported (7, 22). Mouse monoclonal polyubiquitinylated protein antibodies (FK1) were from Biomol International. Mouse monoclonal HA antibody were from Roche Applied Science. Phosphorothioate PKC-α, PKC-β, PKC-δ, PKC-ζ antisense and scrambled control oligonucleotides have been previously described (26–28) and were synthesized by PRIMM (Milan, Italy). PKC-ζ wild-type and dominant-negative constructs were kindly provided by Dr. M. S. Marber (St. Thomas Hospital, London, UK) and Dr. S.Gutkind (NCI, National Institutes of Health, Bethesda, MD), respectively. SDS-PAGE reagents were purchased from Bio-Rad. Western blotting and ECL reagents and radiochemicals were from Amersham Biosciences. All other reagents were from Sigma.

Plasmid Preparation, Cell Culture, and Transfection—The PED_{S104G} and PED_{S116G} mutant cDNAs were prepared by using pcDNAIIIPEDY1 cDNA (pcDNAIII containing His_6-Myc-tagged PED/PEA-15) as template with the site-directed mutagenesis kit by Promega according to the manufacturer's instructions. Stable expression of the mutants and wild-type PEDY1 cDNAs in HEK293 cells (293_{PEDY1}) was achieved as reported in Condorelli *et al.* (11). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU of penicillin/ml, 100 IU of streptomycin/ml, and 2% L-glutamine in a humidified CO_2 incubator. Transient transfection of phosphorothioate oligonucleotides and plasmid DNA in HEK293 cells was accomplished by using the Lipofectamine method according to the manufacturer's instructions. Briefly, the cells were cultured in 60-mm-diameter dishes and incubated for 24 h in serum-free DMEM supplemented with 3 μg of cDNA and 15 μl of Lipofectamine reagent. An equal volume of DMEM supplemented with 20% fetal calf serum was then added for 5 h followed by replacement with DMEM supplemented with 10% serum for 24 h before the assays.

Western Blot Analysis—For Western blotting, cells were solubilized in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 4 mM EDTA, 10 mM Na_4PO_7, 2 mM Na_3VO_4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 mg of aprotinin/ml, 1 mM leupeptin) for 60 min at 4 °C. Cell lysates were clarified at 5,000 × *g* for 15 min. Solubilized proteins were then separated by SDS-PAGE and transferred onto 0.45-μm-pore size Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with the primary (PED, etc.) antibody and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions.

Real-time RT-PCR Analysis—Total cellular RNA was isolated from C5N cells by the use of RNeasy kit (Qiagen) according to the manufacturer's instructions. For real-time RT-PCR analysis, 1 μg of cell RNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). PCR reaction mixes were analyzed using SYBR Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green quantitative PCR Super-UDG using an iCycler IQ multicolor real-time PCR detection system (Bio-Rad). All reactions were performed in triplicate, and β-actin was used as an internal standard. Primer sequences used were as follows: PED/PEA-15, forward, 5′-TTCCCGCTGTTCCCTTAGG-3′, and PED/PEA-15, reverse 5′-TCTGGCTCATCCGCATCC-3′.

Immunoprecipitation of PED/PEA-15—Cells grown in 100-mm Petri dishes were treated as indicated and washed once with ice-cold PBS and solubilized in lysis buffer for 2 h at 4 °C. Then, lysates were clarified by centrifugation at 5,000 × *g* for 20 min. 500 μg of protein lysates was immunoprecipitated with Myc or PED antibodies for 16 h. The precipitates were incubated with protein G-Sepharose beads at 4 °C for 1 h with shaking. Beads were precipitated by centrifugation at 1,000 × *g* for 5 min at 4 °C and washed five times with ice-cold washing buffer. After the final wash, the pellets were resuspended in 30 μl of 1× SDS electrophoresis buffer and heated to 95 °C for 5 min prior to protein separation by 15% SDS-PAGE. Western blot analysis was performed as described above.

Purification of Ubiquitin-PED/PEA-15 Conjugates—Cellular ubiquitinylation assay was performed as described by Musti *et al.* (29). Briefly, 24 h after transfection, cells expressing His-tagged-PED/PEA-15 (PED/PEA-15Y1) and HA-ubiquitin were harvested in 2 ml of 6 M guanidium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8) plus 5 mM imidazole/100-mm dish and sonicated with a Branson micro-tipped sonifier for 30 s to reduce viscosity. Lysates were mixed on a rotator with 0.2 ml (settled volume) of Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) for 4 h at room temperature. The slurry was applied to a Bio-Rad Econo-Col-

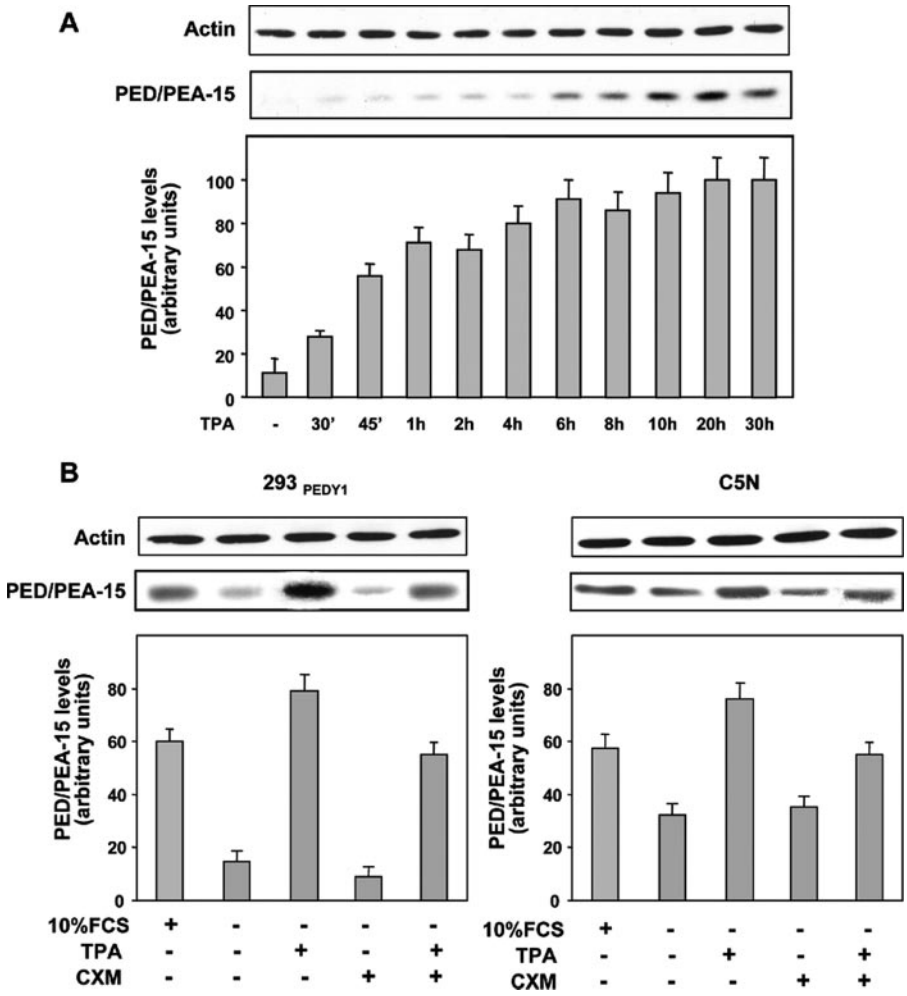

FIGURE 1. **TPA effect on PED/PEA-15 protein expression.** *A*, 293_{PEDY1} cells were serum-starved for 40 h and stimulated with 1 μM TPA for the indicated times. Cell lysates were separated on SDS-PAGE and immunoblotted with PED Ab. Filters have been analyzed by laser densitometry. The *error bars* represent the mean ± S.D. of the densitometric analyses. *B*, 293_{PEDY1} cells (*left panel*) and C5N keratinocytes (*right panel*) were serum-starved, as indicated, and treated with 1 μM TPA for 20 h in the absence or in the presence of 40 μg/ml cycloheximide (*CXM*). Cell lysates were then analyzed by PED immunoblot, and the results were quantitated by laser densitometry. The autoradiographs shown are representative of three (*A*) and four (*B*) independent experiments.

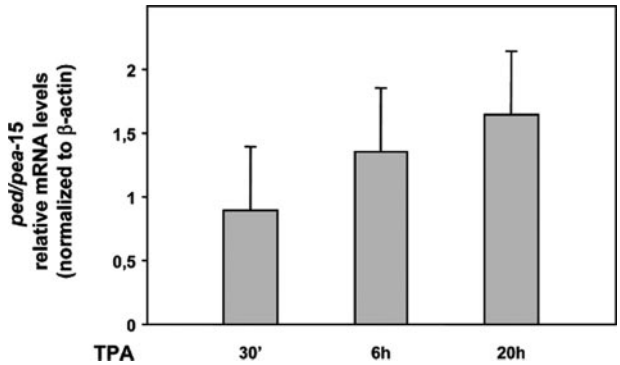

FIGURE 2. **TPA effect on PED/PEA-15 mRNA levels.** The abundance of mRNAs for PED/PEA-15 was determined by real-time RT-PCR analysis of total RNA isolated from C5N cells following treatment with TPA (1 μM) for the indicated times, using β-actin as internal standard. The mRNA levels in TPA-stimulated cells are relative to those in control cells. Each *error bar* represents the mean ± S.D. of four independent experiments in each of which reactions were performed in triplicate.

umn. The column was successively washed with the following: 1 ml of 6 M guanidium-HCl, 0.1 M Na_2 HPO_4, NaH_2 PO_4 (pH 8); 2 ml of 6 M guanidium-HCl, 0.1 M Na_2 HPO_4, NaH_2 PO_4 (pH 5.8); 1 ml of 6 M guanidium-HCl, 0.1 M Na_2 HPO_4, NaH_2 PO_4 (pH 8); 2 ml of (6 M guanidium-HCl, 0.1 M Na_2 HPO_4, NaH_2 PO_4 (pH 8), protein buffer) 1:1; 2 ml of (6 M guanidium-HCl, 0.1 M Na_2 HPO_4, NaH_2 PO_4 (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 Na_2 HPO_4, NaH_2 PO_4 (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 (293_{PEDY1}), were incubated with serum-free medium in the absence or in the presence of 1 μM TPA. Treatment of 293_{PEDY1} cells with TPA increased PED/PEA-15 levels in a time-dependent manner (Fig. 1*A*). Serum deprivation alone was sufficient to reduce PED/PEA-15 protein levels by >3-fold (Fig. 1*B*). 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. 1*B*, *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. 1*B*, *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

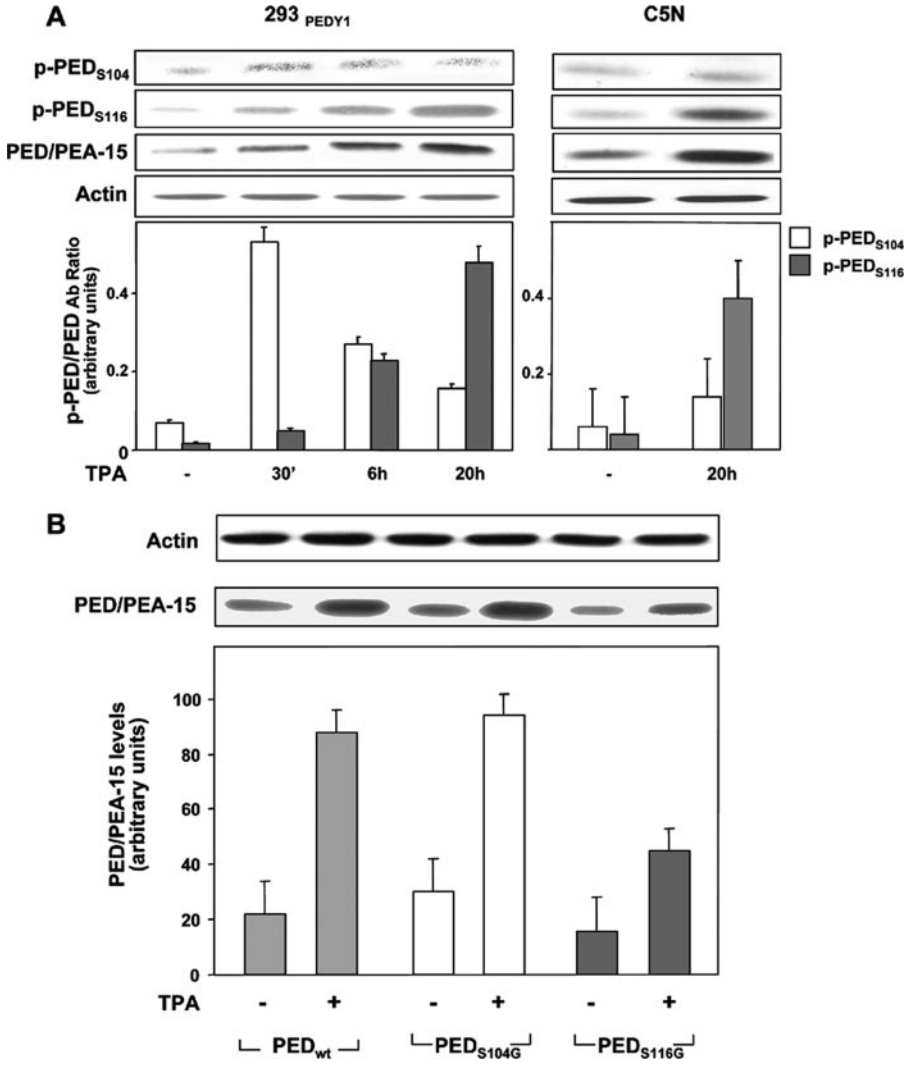

FIGURE 3. **Regulation of PED/PEA-15 phosphorylation levels by TPA.** *A*, 293_{PEDY1} and C5N cells were serum-starved and stimulated with 1 μM TPA for the indicated times. Cell lysates were separated on SDS-PAGE and immunoblotted either with p-PEDS104 Ab or with p-PEDS116 Ab. Each filter has been reprobed 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 PED_{WT}, PED_{S104G}, and $293PED_{S116G}$, 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.

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 293_{PEDY1} and C5N cells were immunoblotted with antibodies against the phosphorylated forms of Ser-104 and Ser-116 (Fig. 3*A*). In 293_{PEDY1} cells, Ser-104 phosphorylation increased within the initial 30 min of TPA exposure, declining thereafter. At variance, Ser-116 phosphorylation was barely detectable at 30 min and progressively raised for up to 20 h after TPA exposure. Similarly, in C5N keratinocytes, 20 h of TPA treatment led to a significant increase of Ser-116 phosphorylation, with almost undetectable changes of Ser-104 phosphorylation (Fig. 3*A*).

To assess the relevance of those phosphorylation sites, PED/PEA-15 mutants bearing Ser-104 → Gly (PED_{S104G}) or Ser-116 → Gly (PED_{S116G}) substitutions were transfected in HEK293 cells. TPA treatment increased the levels of the wild-type PED/PEA-15 (PED_{WT}) and of PED_{S104G} by about 4-fold. At variance, PED_{S116G} expression was increased by only 2-fold upon TPA exposure (Fig. 3*B*), 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-α, -β, and -δ 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 293_{PEDY1} cells were transfected with a dominant-negative (DN) PKC-ζ mutant or with PKCζ-ASO and stimulated with TPA for 20 h (Fig. 5*A*). 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 293_{PEDY1} cells treated with the CaMK inhibitor, KN-93 (Fig. 5*A*). Similarly, in C5N keratinocytes, TPA-induced up-regulation of PED/PEA-15 protein expression was reduced by about 70% by KN-93 treatment (Fig. 5*B*).

Next, we investigated whether TPA could regulate CaMKII activity in 293_{PEDY1} and in C5N cells. In this regard, CaMKII phosphorylation (Fig. 6*A*) was induced by TPA and well correlated with increased PED/PEA-15 expression levels and Ser-116 phosphorylation in 293_{PEDY1} cells (Figs. 1*A* and 3*A*). Consistently, 20 h of TPA treatment of C5N cells was accompanied by a 2.5-fold increase of CaMKII phosphorylation (Fig. 6*A*). The

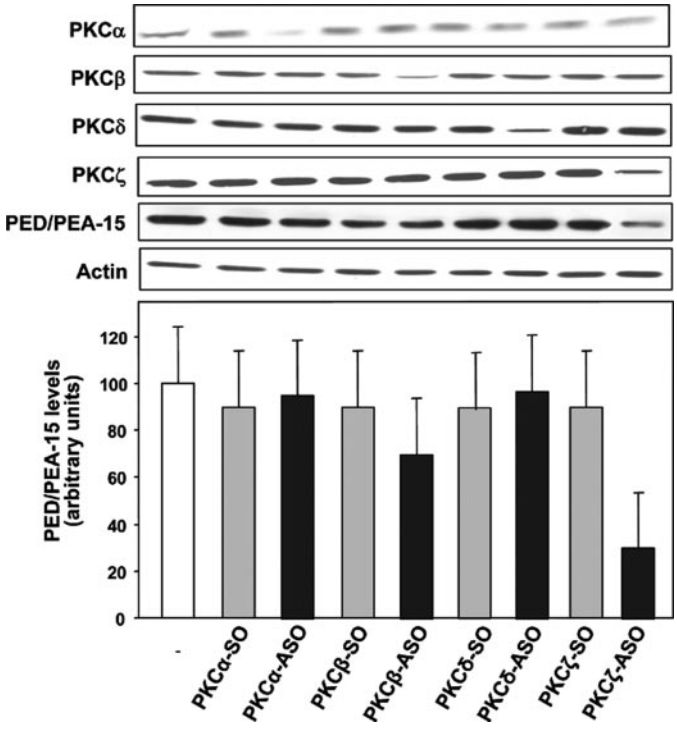

FIGURE 4. **Regulation of PED/PEA-15 expression levels by PKC isoforms.** 293_{PEDY1} cells were treated with phosphorothioate antisense (ASO) and sense (SO) oligonucleotides (3 μg/ml) directed against PKC-α, -β, -δ, and -ζ. Cell lysates were then analyzed by PED immunoblot, and the results were quantitated by laser densitometry. The autoradiograph shown is representative of five independent experiments. The *error bars* represent the mean ± S.D. of the densitometric analysis.

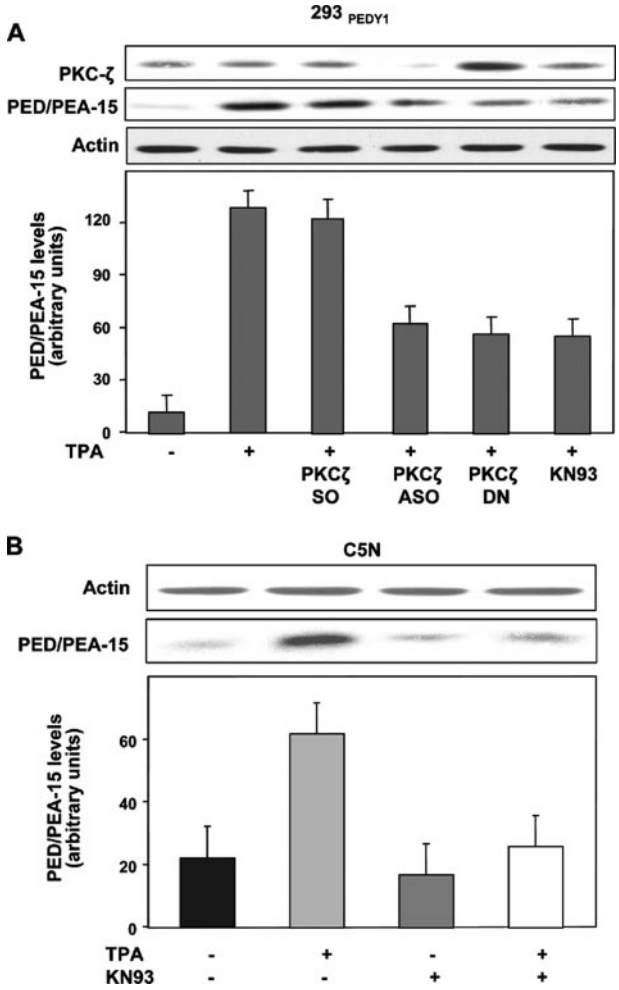

FIGURE 5. **Regulation of PED/PEA-15 expression levels by PKCζ and CaMK.** 293_{PEDY1} (*A*) and C5N (*B*) cells were serum-starved and treated with 1 μM TPA or with 10 μM KN-93 for 20 h in the absence or in the presence of PKCζ-ASO and PKCζ-SO or DN-PKCζ, as indicated. Cell lysates were then analyzed by PKC-ζ (*upper part*) or PED Ab (*lower part*) immunoblot, and the results were quantitated by laser densitometry. The autoradiographs shown are representative of four (*A*) and three (*B*) independent experiments. The *error bars* represent the mean ± S.D. of the densitometric analyses.

expression of DN-PKC-ζ and the treatment of 293PEDY1 cells with PKCζ-ASO reduced by about 65% TPA-induced CaMKII activation (Fig. 6*B*). 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 293_{PEDY1} and C5N cells (Fig. 6*C*).

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. 7*A*). 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 PED_{WT} and PED_{S104G} but not of PED_{S116G} (Fig. 7*B*). Indeed, PKC-ζ-mediated changes of PED_{WT} and PED_{S104G} were prevented by KN-93, which, instead, had no effect on the regulation of PED_{S116G} 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, 293_{PEDY1} 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. 8*A*). Lactacystin treatment also prevented PED/PEA-15 degradation induced by KN-93 in the 293_{PEDY1} cells (data not shown). In addition, lactacystin, at variance with TPA, increased the expression of PED_{S116G} at a similar extent as PED_{WT} (Fig. 8*B*), 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. 8*B*). 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 ubiquitinylated (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. 9*A*). A typical smear was observed in cells co-transfected with both constructs, indicating that PED/PEA-15 is a ubiquitinylated protein (Fig. 9*A*).

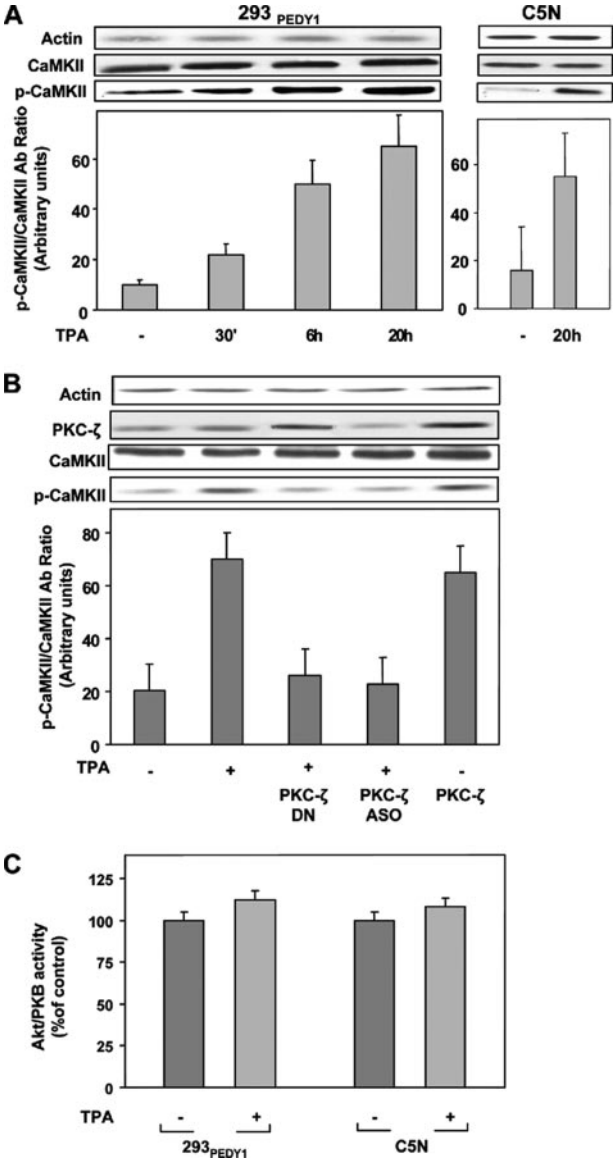

FIGURE 6. **TPA and PKC-ζ effect on CaMKII phosphorylation.** *A*, 293_{PEDY1} cells were serum-starved and treated with 1 μM TPA for the indicated times. Cell lysates were analyzed by p-CaMKII immunoblot. Filters were then reprobed with CaMKII Ab for normalization, and the results were quantitated by laser densitometry. The autoradiograph shown is representative of five (for 293_{PEDY1}) and three (for C5N) independent experiments. The *error bars* represent the mean ± S.D. of the densitometric analysis. *B*, 293_{PEDY1} cells were serum-starved and treated with 1 μM TPA for 20 h in the absence or in the presence of PKCζ-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 analysis. *C*, 293_{PEDY1} and C5N 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 three independent experiments in triplicate.

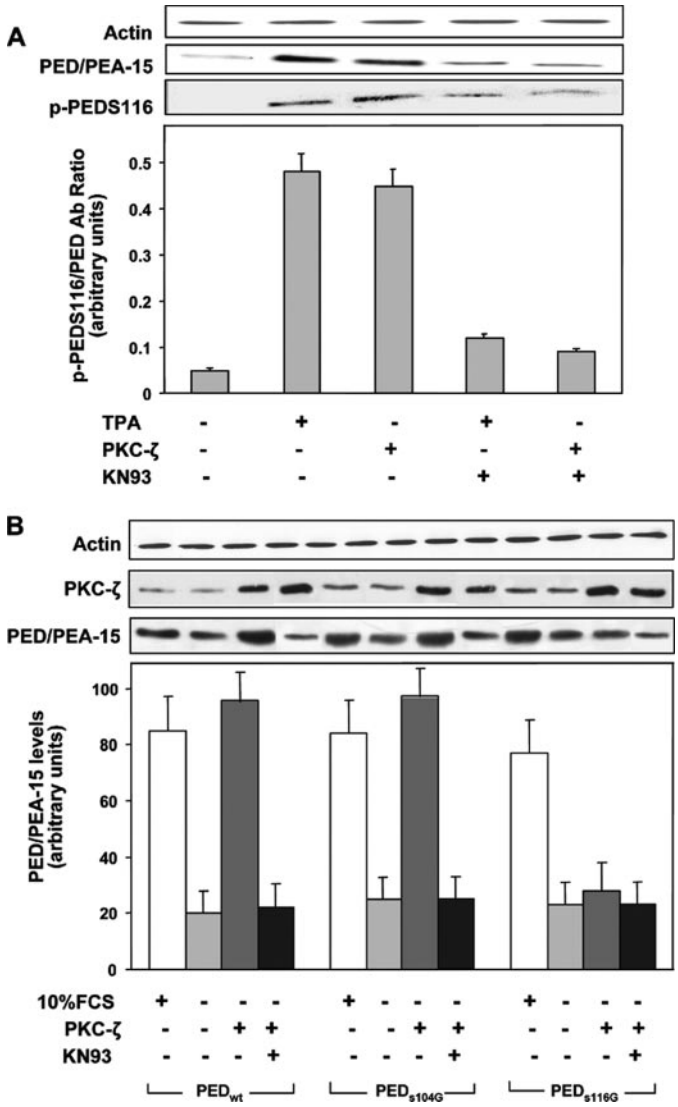

FIGURE 7. **Regulation of PED/PEA-15 phosphorylation and expression by CaMKII.** *A*, 293_{PEDY1} and C5N cells were serum-starved and treated with 1 μM TPA for 20 h or transfected with a pcDNAIII plasmid containing a PKC-ζ cDNA, in the absence or in the presence of 10 μM KN-93. Cell lysates were then 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 PED_{WT}, PED_{S104G}, and PED_{S116G} 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.

Next, 293_{PEDY1} 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. 9*B*). Both TPA and PKC-ζ failed to decrease PED/PEA-15 ubiquitinylation in the presence of KN-93. Also, ubiquitinylation of the PED_{S104G} mutant was reduced in a manner comparable with that of PED_{WT}, whereas that of the PED_{S116G} mutant did not significantly change (Fig. 9*C*).

Functional Relevance of Ser-116 Phosphorylation—To further investigate the relevance of PED/PEA-15 phosphorylation on its anti-apoptotic action, 293_{PEDY1} 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. 10*A*). However, the incubation with KN-93 prevented both TPA and PKC-ζ

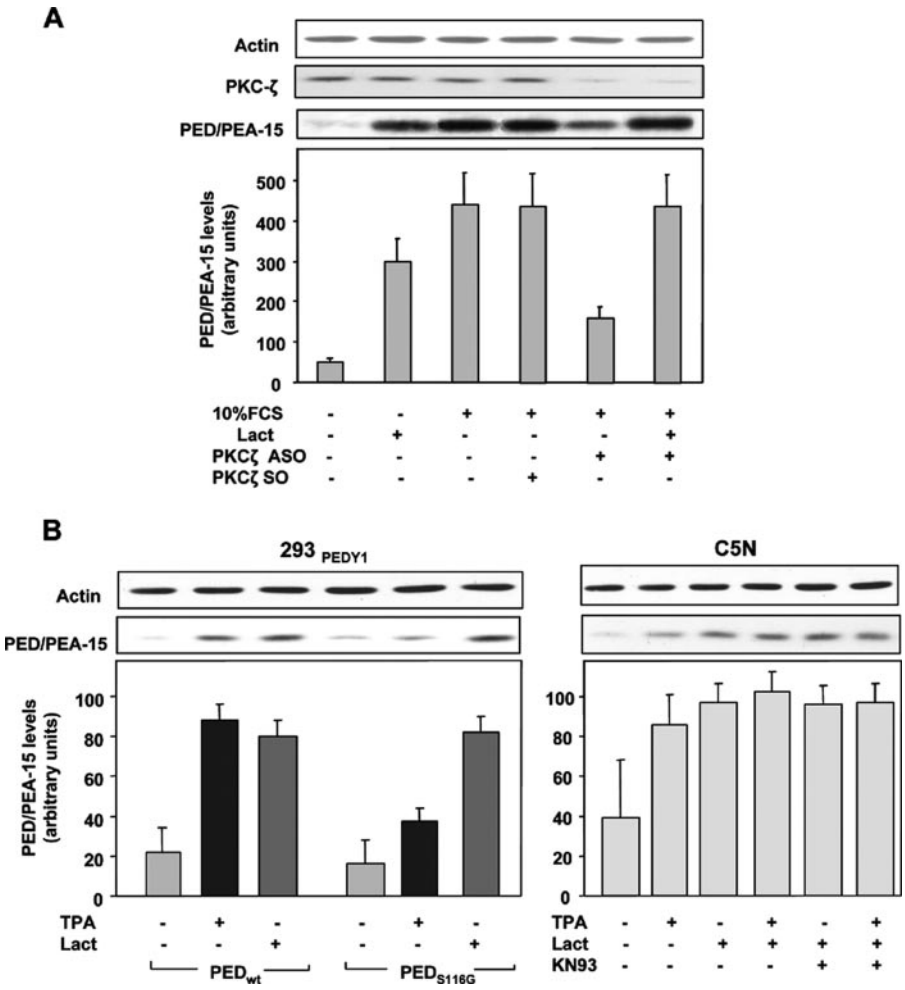

FIGURE 8. **Effect of lactacystin on PED/PEA-15 expression.** *A*, 293_{PEDY1} cells were treated with either PKCζ-ASO or PKCζ-SO and further incubated in the absence or in the presence of 30 μM lactacystin (*Lact*), as indicated. Cell lysates were then analyzed by PED immunoblot, and the results were quantitated by laser densitometry. 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 PED_{WT} or PED_{S116G} 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 mM) as indicated. Cell lysates were then analyzed by PED immunoblot, and the results were quantitated by laser densitometry. The autoradiographs 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.

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 keratinocyte 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 promoter. In addition, in these cells, as well as in untransfected keratinocytes, 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, suggests that additional regulation may occur at the transcriptional level. Indeed, PED/PEA-15 mRNA levels are also significantly increased in untransfected C5N cells following TPA stimulation.

Nonetheless, PED/PEA-15 phosphorylation is a major event for the regulation of its stability (22). Here, we show that Ser-116 is the key phosphorylation site enabling TPA regulation of PED/PEA-15 expression. We have previously described that Ser-116 phosphorylation by Akt/PKB increases PED/PEA-15 half-life following insulin stimulation (22). It is unlikely that Akt/PKB 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, consistent 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 stimulation. Finally, pharmacological inhibition of CaMKII with KN-93 almost totally blocked TPA-induced Ser-116 phosphorylation. At variance, Ser-104 phosphorylation was rapidly induced by TPA and then decreased upon prolonged incubation. 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 conventional PKC isoforms after long term exposure was accompanied

rescue of cell death, suggesting that CaMKII-induced phosphorylation of PED/PEA-15 at Ser-116 was required for this effect (Fig. 10*A*). To further sustain this hypothesis, we have tested TPA protection from cell death in HEK293 cells transfected with either PED_{WT} or PED_{S116G}. Although normally inducing survival of serum-starved cells transfected with PED_{WT}, TPA effect was >2-fold reduced in cells overexpressing PED_{S116G} (Fig. 10*B*).

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 experimental carcinogenesis protocols (15), further indicating that raised PED/PEA-15 expression may play a role in cellular transforma-

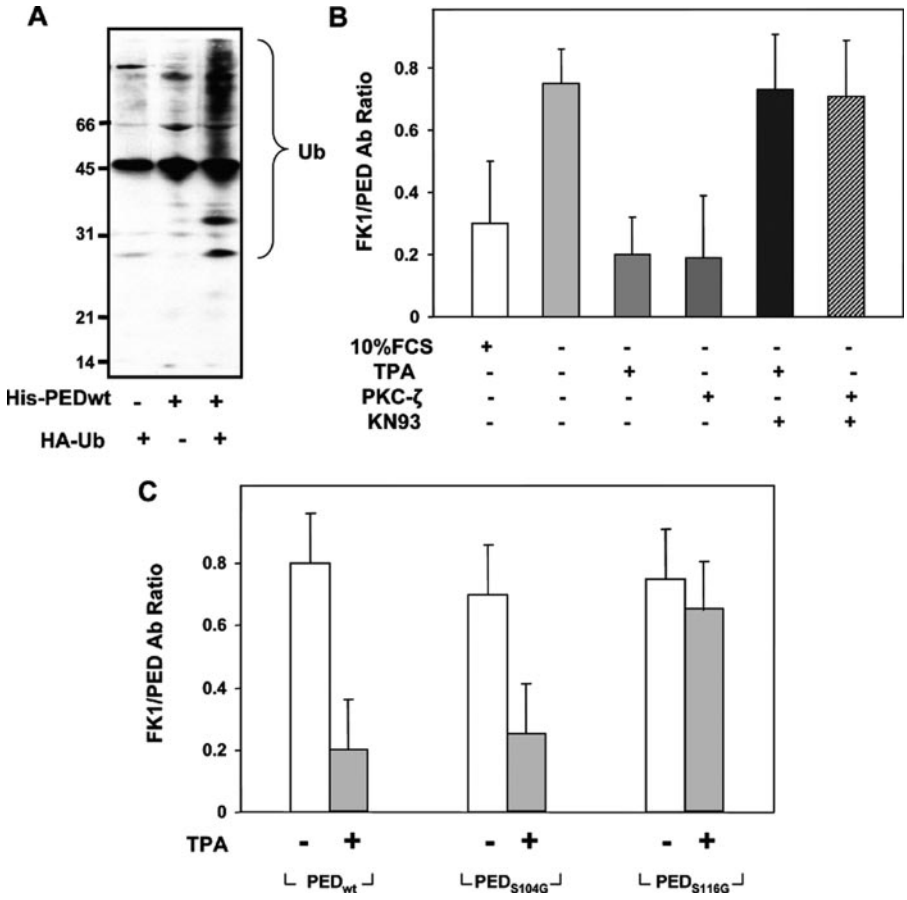

FIGURE 9. **Regulation of PED/PEA-15 ubiquitinylation.** *A*, HEK293 cells were transfected with His-Myc-PED/PEA-15 and HA-ubiquitin (*HA-Ub*) alone or in combination. Upon purification of His-Myc-PED/PEA-15 and separation on SDS-PAGE, filters were probed with HA Ab. The autoradiograph shown is representative of five independent experiments. *B*, 293$_{PEDY1}$ cells were serum-starved and treated with 1 μM TPA for 20 h or transfected with PKC-ζ cDNA 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. *FCS*, fetal calf serum. *C*, HEK293 cells transfected with PED$_{WT}$, PED$_{S104G}$, and PED$_{S116G}$ 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-

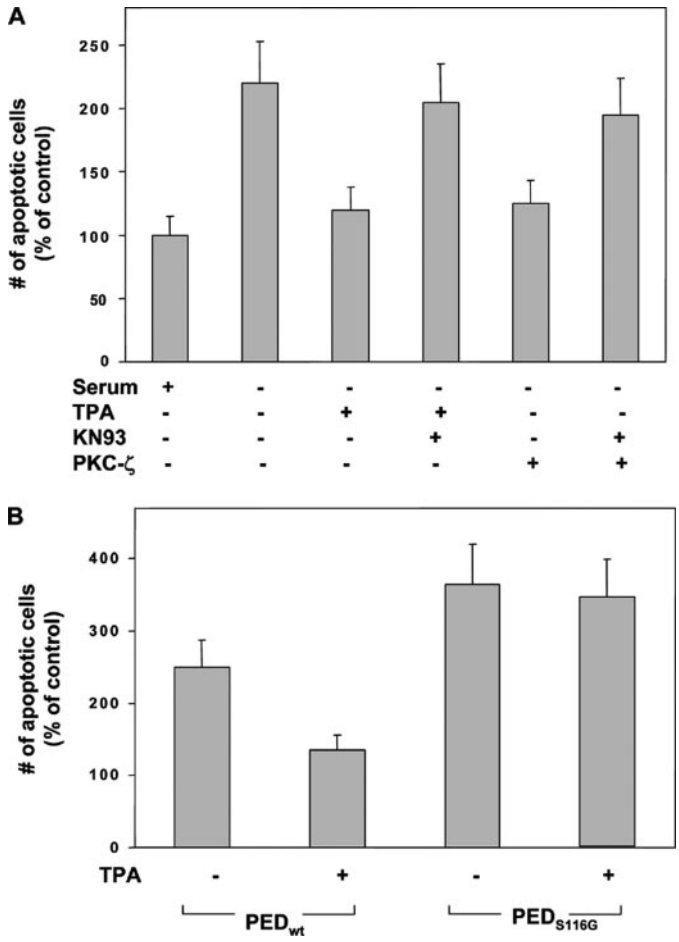

FIGURE 10. **TPA-mediated regulation of cell death by PED/PEA-15 phosphorylation.** *A*, 293$_{PEDY1}$ cells were serum-starved and treated with 1 μM TPA for 20 h or transfected with PKC-ζ, in the absence or in the presence of 10 μM KN-93, as indicated. Cell suspensions were stained with propidium iodide and analyzed by flow cytometry. Data are presented as the percentage of value obtained with cells kept in serum only. Values represent the mean ± S.D. of the results obtained in four triplicate experiments. *B*, HEK293 cells transfected with PED$_{WT}$ or PED$_{S116G}$ 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 value obtained with cells kept in serum only. Values represent the mean ± S.D. of the results obtained in three triplicate experiments.

ing growth factor deprivation or PKC-ζ silencing. Different from TPA, however, lactacystin also rescued the expression levels of the non-phosphorylatable Ser-116 → Gly mutant, indicating that phosphorylation at this site may confer the ability to escape proteasomal degradation. Proteasomal targeting and degradation are typical features of ubiquitinylated proteins (30). Sur and Ramos (45) have recently shown that vanishin, a death effector domain protein with a high degree of homology with PED/PEA-15, is ubiquitinylated. We now present evidence that PED/PEA-15 directly binds ubiquitin. Treatment with TPA as well as PKC-ζ overexpression reduced the ubiquitinylation of wild-type PED/PEA-15 but had no effect on the Ser-116 phosphorylation-deficient mutant. Preserved ubiquitinylation was also observed in the presence of KN-93, indicating that CaMKII phosphorylation plays an important role in the regulation of PED/PEA-15 expression by controlling its ubiquitinylation state. Finally, both KN-93 and Ser-116 → Gly substitution reduced TPA anti-apoptotic action, suggesting that 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 ubiquinylation and proteasomal targeting and induce PED/PEA-15 intracellular accumulation, thereby enhancing its anti-apoptotic action.

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

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