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PKCδ enhances C/EBPα degradation via inducing its phosphorylation and cytoplasmic translocation

Zhao-Xia Wu [1], Meng Zhao [1], Li Xia, Yun Yu, Shao-Ming Shen, San-Feng Han, Hui Li, Tong-Dan Wang, Guo-Qiang Chen, Li-Shun Wang *

The Department of Pathophysiology, Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao-Tong University School of Medicine, Shanghai 200025, China

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ABSTRACT

Our previous study has shown that PKCδ stimulates proteasome-dependent degradation of C/EBPα, which partially contributes to PKCδ-mediated apoptosis. However, the molecular interrelationship between these two important proteins is still unknown. In this study, we reported that C/EBPα was phosphorylated by activated PKCδ on three serines, two of which were reported for the first time. Phosphorylated C/EBPα underwent cytoplasmic translocation, which led to the inactivation of its transcriptional activity. Inactive cytoplasmic C/EBPα was finally subjected to proteasome degradation. This work reveals the exquisite molecular events linking activated PKCδ and C/EBPα degradation during cell apoptosis.

1. Introduction

Protein kinase C delta (PKCδ), a member of the novel PKC family, plays important roles in apoptotic responses, especially in DNA-damaging agents-induced apoptosis [1–3]. During apoptosis, PKCδ undergoes proteolytic activation, which generates a catalytic fragment, PKCδ-CF [4,5]. C/EBPα, the founder member of the C/EBP family of basic leucine zipper (bZIP) proteins, is well known for its crucial role in the differentiation of various cell types such as adipocytes, hepatocytes and myeloid cells [6]. However, a role for C/EBPα in cell death has also been revealed. It has been reported that C/EBPα protects Ba/F3 from apoptosis on interleukin-3 withdrawal by inducing the expression of anti-apoptotic Bcl-2 gene in hematopoietic cell lines in a manner independent of its DNA binding activity [7]. Our previous work showed that inhibition of C/EBPα expression enhanced, while its over-expression inhibited DNA-damaging agents-induced apoptosis. Moreover, C/EBPα expression was down-regulated during apoptosis, partially through induction of proteasome degradation by proteolytically activated PKCδ-enhanced ubiquitination of C/EBPα [8]. However, the underlying mechanism of PKCδ in C/EBPα regulation remains unknown. In the present work, C/EBPα is found to be subject to phosphorylation by PKCδ, which in turn changes its subcellular localization, transcriptional activity and protein stability.

2. Materials and methods

2.1. Cell culture and treatment

HEK293T cells were cultured in high-glucose (400 mg/dl) Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and maintained at 37 °C in an environment with 5% CO_2 MG132 (BIOMOL) was dissolved in DMSO and stored at −20 °C until usage.

2.2. Plasmids and constructs

Full-length human C/EBPα cDNA was amplified by PCR from pCMV-SPORT-C/EBPα plasmid (kindly provided by Dr. Gombart AFin Cedars-Sinai Medical Center, Los Angeles, CA) and subcloned into pCMV-flag expression vector to form the pCMV-flag-C/EBPα plasmid. The pEGFP-PKCδ-CF plasmid was kindly provided by Dr. Reyland ME in School of Dentistry, University of Colorado Health Sciences Center.

2.3. Luciferase assay

For luciferase assay, HEK293T cells were seeded in a 12-well plate. After 24 h, cells were transfected either with 100 ng of G-CSFR promotor-driven luciferase reporter plasmid and 4 ng of pSV-*Renilla* plasmid, or further co-transfected with pCMV-flag-C/EBPα and pEGFP-N1 or pEGFP-PKCδ-CF. The total amount of DNA was held constant by addition of empty vector. Thirty-six hours after transfection, cells were lysed and analyzed by the

* Corresponding author. Fax: +86 21 64154900.
E-mail address: jywangls@shsmu.edu.cn (L.-S. Wang).
[1] These two authors contributed equally to this work.

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Dual-Luciferase Assay system (Promega) according to the manufacturer's instructions. For each experiment, at least three independent transfections in triplicate were performed.

2.4. Extraction of nuclear and cytoplasmic proteins

After transfection with the indicated plasmids and treatment, the HEK293T cells were harvested and washed three times with cold phosphate-buffered saline (PBS). Nuclear and cytoplasmic protein fractions were extracted using NE-PER extraction reagent (Pierce Protein Research Products, ThermoFisher Scientific, Rockford, IL) according to the manufacturer's protocol and used for Western blot.

2.5. Immunofluorescent analysis

HEK293T cells were collected onto slides and then co-transfected with pCMV-flag-C/EBPα and pEGFP-N1 or pEGFP-PKCδ-CF for 24 h. Cells were fixed with 4% paraformaldehyde for 15 min and then permeated with 0.25% Triton X-100 for 10 min at room temperature, followed by blocking in 10% bovine serum albumin for 30 min. Slides were sequentially incubated with anti-flag antibody (1:100) and Alexa 594-conjugated secondary antibody (1:100), followed by nuclear counterstaining with 4′-6-diamidino-2-phenylindole (DAPI) for 3–5 min to localize the nucleus and observed by confocal microscopy.

2.6. Transfection and immunoprecipitation

After HEK293T cells were co-transfected with pCMV-flag-C/EBPα and pEGFP-N1 or pEGFP-PKCδ-CF using the Lipofectamine 2000 transfection reagent (invitrogen) for 24 h. Cells were lysed and sonicated in the buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Np-40, 1 mM EDTA, plus protease inhibitors) and cell extracts were incubated with mouse anti-flag M2-agarose affinity gel (Sigma, St. Louis, MI) overnight at 4 °C. The immunoprecipitates were washed three times with the lysis buffer and then were eluted by rehydration buffer (containing 8 M urea, 2% (m/v) CHAPS, 25 mM DTT and 0.002% bromophenol blue) for two-dimensional electrophoresis or the 2× SDS sample buffer and assessed by Western blot with the indicated antibodies.

2.7. Two-dimensional electrophoresis (2-DE)

After total protein was diluted in rehydration buffer, and then applied onto 7 cm IPG strips (NL, pH 3–10, Bio-Rad). The first dimension was carried out on a Protean IEF Cell system (Bio-Rad) as described previously [9]. After IEF, the IPG strips were equilibrated in the buffer (6 M urea, 20% glycerol, and 2% SDS in 0.05 M Tris–HCl buffer, pH 8.8) containing 2% w/v DTT and 2.5% w/v iodoacetamide sequentially. The second dimensional separation was carried out on 10% SDS–PAGE gel, followed by Western blot for C/EBPα or Coomassie Brilliant Blue staining. Protein spots of interest were excised, transferred into the ZipPlate micro-SPE Plate wells (Millipore, Billerica, MA). The proteins were digested according to the manufacturer's protocol (Millipore).

2.8. SYPRO Ruby staining

Proteins on 2 DE gel were detected using the SYPRO Ruby Protein Gel Stain (Molecular probes). The gel was fixed in a solution containing 10% acetic acid/50% methanol for 30 min, then 7% acetic acid/10% methanol for 30 min. Next, the gel was incubated in the undiluted stock solution of SYPRO Ruby for 90 min, and destained with 7% acetic acid/10% methanol for 30 min. After rinsing with H_2O for 10 min, digital images were acquired using a Fluorophorestar 3000 image capturesystem (Anatech, Japan) with 470 nm excitation and 618 nm emission for SYPRO Ruby detection.

2.9. Pro-Q Diamond staining

Phosphoproteins on 2 DE gel were detected using Pro-Q Diamond Phosphoprotein Stain (Molecular probes). The gel was fixed in a solution containing 10% aceticacid/50% methanol for 30 min two times, and then the gel was washed with MilliQ H_2O for 10 min twice. Next, the gel was incubated in an undiluted stock solution of Pro-Q Diamond for 90 min, and destained with three successive washes (30 min per wash) in 50 mM sodium acetate (pH 4.0), 20% (v/v) acetonitrile. Digital images were acquired using the Fluorophorestar 3000 image capture system with 520 nm excitation and 575 nm emission for Pro-Q Diamond detection.

2.10. In-gel digestion and LC-MS/MS Analysis

The protein bands of C/EBPα were cut out of SDS–PAGE gels stained with Coomassie Brilliant Blue and transferred into the ZipPlate micro-SPE Platewells (Millipore, Billerica, MA). The proteins were digested according to the manufacturer's protocol (Millipore), as described previously [9]. For LC-MS analysis, the peptides were lyophilized using a SpeedVac (ThermoSavant, USA), and resuspended in 20 μl 0.1% formic acid/2% acetonitrile. All mass spectrometric experiments were performed on a LTQ orbitrap "XL" mass spectrometer (Thermo Fisher Scientific, San Jose, CA) connected with a Paradigm MDLC nanoflow LC system (Michrom BioResources, USA) via an ADVANCE Spray Source LC-MS interface (Michrom BioResources, USA). The peptide mixture was loaded onto a 15 cm with 0.1 mm inner diameter column packed with Magic C18AQ 3-μm Reversed Phase resins (Michrom BioResources, USA), and separated within a 120 min linear gradient from 100% solvent A (0.1% formic acid/2% acetonitrile/98% water) to 35% solvent B (0.1% formic acid/100% acetonitrile) at a flow rate of 500 nl/min. The spray voltage was set to 1.5 kV and the temperature of ion transfer capillary was 160 °C. The mass spectrometer was operated in positive ion mode and employed in the data-dependent mode to automatically switch between MS and MS/MS using the Tune and Xcalibur 2.5.5 software package. One full MS scan from 350 to 1800 m/z was acquired at high resolution $R = 100{,}000$ (defined at $m/z = 400$), followed by fragmentation of the ten most abundant multiply charged ions (singly charged ions and ions with unassigned charge states were excluded). In CID mode, two activated ion modes were achieved, except that MS2 data-dependent mode was operated on both total peptides extracted from in-gel digestion and enriched phosphopeptides, multistage activation (MSA) with excitation at pseudo mass losses of 97.97, 48.99 and 32.66 Da from the precursor was performed on enriched phosphopeptides to analyze the phosphopeptide sample in two parallel technical replicates. Ions selected for MS/MS were limited on a dynamic exclusion list for 30 s.

2.11. Western blots

Western blots were performed as described [10]. The protein lysates were separated by SDS–PAGE and then transferred to nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% non-fat dry milk solution for 1 h at room temperature and then incubated in primary antibody dissolved in block solution at 4 °C overnight. The proteins were probed by antibodies against Lamin B (Santa Cruz Biotech, Santa Cruz, CA), β-actin (Merck, Darmstadt), C/EBPα (Cell Signaling, Beverly, MA) and GFP (Sigma–Aldrich). After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Dako Cytomation, Glostrup, Denmark) corresponding to the primary

antibody for 1 h at room temperature, and detections were performed by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to the manufacture's instructions.

2.12. Statistical analysis

All experiments were repeated at least for three times with the same results. The Student's *t*-test was used to compare the difference between two different groups. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Activated PKCδ alters the subcellular localization of C/EBPα protein

Previously, we found that activated PKCδ results in the increased proteasome-dependent degradation of C/EBPα [8]. Here, by co-transfection of C/EBPα with the catalytic fragment of PKCδ (PKCδ-CF), we found that activated PKCδ also inhibited the transcriptional activity of C/EBPα (Fig. 1A). However, MG132 treatment which accumulated C/EBPα protein in the presence of PKCδ-CF did not rescue the transcriptional activity of C/EBPα (Fig. 1A), indicating that C/EBPα was inactivated by activated PKCδ. Interestingly, subcellular fractionation showed that PKCδ-CF dramatically increased the level of cytoplasmic C/EBPα and reduced that of nuclear C/EBPα (Fig. 1B). Immunofluorescence staining also confirmed that C/EBPα underwent cytoplasmic translocation when co-expressed with PKCδ-CF (Fig. 1C). Considering the fact that C/EBPα is a transcription factor which requires nuclear localization to exert its function, we speculated that cytoplasmic translocation of C/EBPα might be the major mechanism of PKCδ-CF mediated inactivation of C/EBPα.

3.2. PKCδ enhances phosphorylation of C/EBPα

Next, we sought to investigate the mechanism of C/EBPα translocation induced by activated PKCδ. Considering the fact that PKCδ plays its roles in cell growth, survival, differentiation and apoptosis by phosphorylating a series of substrates [11,12] and protein phosphorylation was always associated with subcellular localization [13,14], we evaluated whether activated PKCδ could induce phosphorylation of C/EBPα. To do this, flag-C/EBPα was co-transfected into 293T cells with GFP or GFP-PKCδ-CF. Twenty-four hours later, C/EBPα protein was immunoprecipitated with anti-flag M2 beads and isolated by two-dimensional electrophoresis. The gel was sequentially stained by Pro-Q Diamond for phosphoprotein and SYPRO Ruby for total protein. As shown in Fig. 2A, phosphorylated C/EBPα detected by Pro-Q was significantly increased in GFP-PKCδ-CF-expressing cells, while the same levels of total C/EBPα between GFP and GFP-PKCδ-CF-expressing cells were detected by SYPRO-Ruby. Furthermore, we identified three phosphorylation sites of C/EBPα protein in GFP-PKCδ-CF-expressing cells, including S21 (top panel, Fig. 2B), S266 (middle panel, Fig. 2B) and S277 (bottom panel, Fig. 2B), whereas no phosphosite of C/EBPα was identified in GFP-expressing cells. Notably, S266 and S277 were identified as C/EBPα phosphosites for the first time. These experiments indicated that ectopically expressed PKCδ-CF enhanced the phosphorylation of C/EBPα protein.

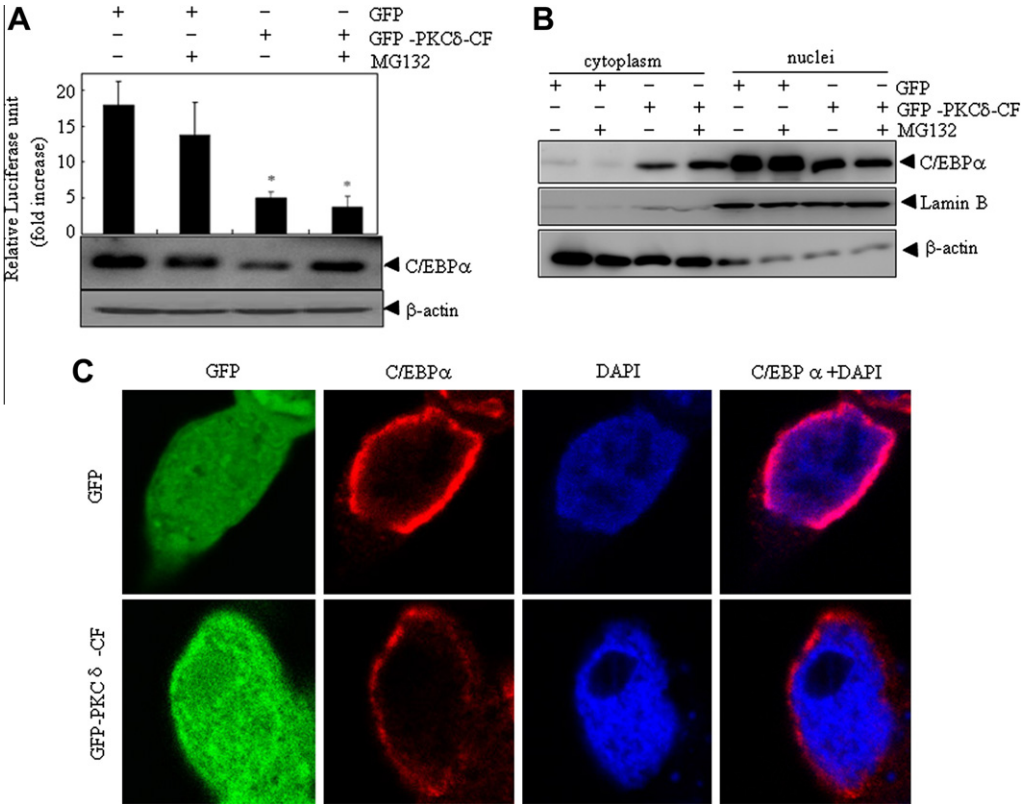

Fig. 1. The translocation of C/EBPα induced by PKCδ. HEK293T cells were transfected with C/EBPα and GFP or GFP-PKCδ-CF for 24 h. Then, cells were treated with or without 20 μM MG132 for 5 h before harvest. (A) Luciferase activities were determined. Mean luciferase activity is shown relative to the cells without transfection of C/EBPα. Error bars represent SD ($n = 3$) (*$P < 0.05$). (B) The lysates from nucleus or cytoplasm fractions were analyzed by Western blots for C/EBPα, Lamin B and β-actin. (C) Cells were stained with anti-flag antibody plus Alexa 594-conjugated secondary antibody for C/EBPα protein, followed by nuclear counterstaining with DAPI to localize the nucleus, and observed by confocal microscopy.

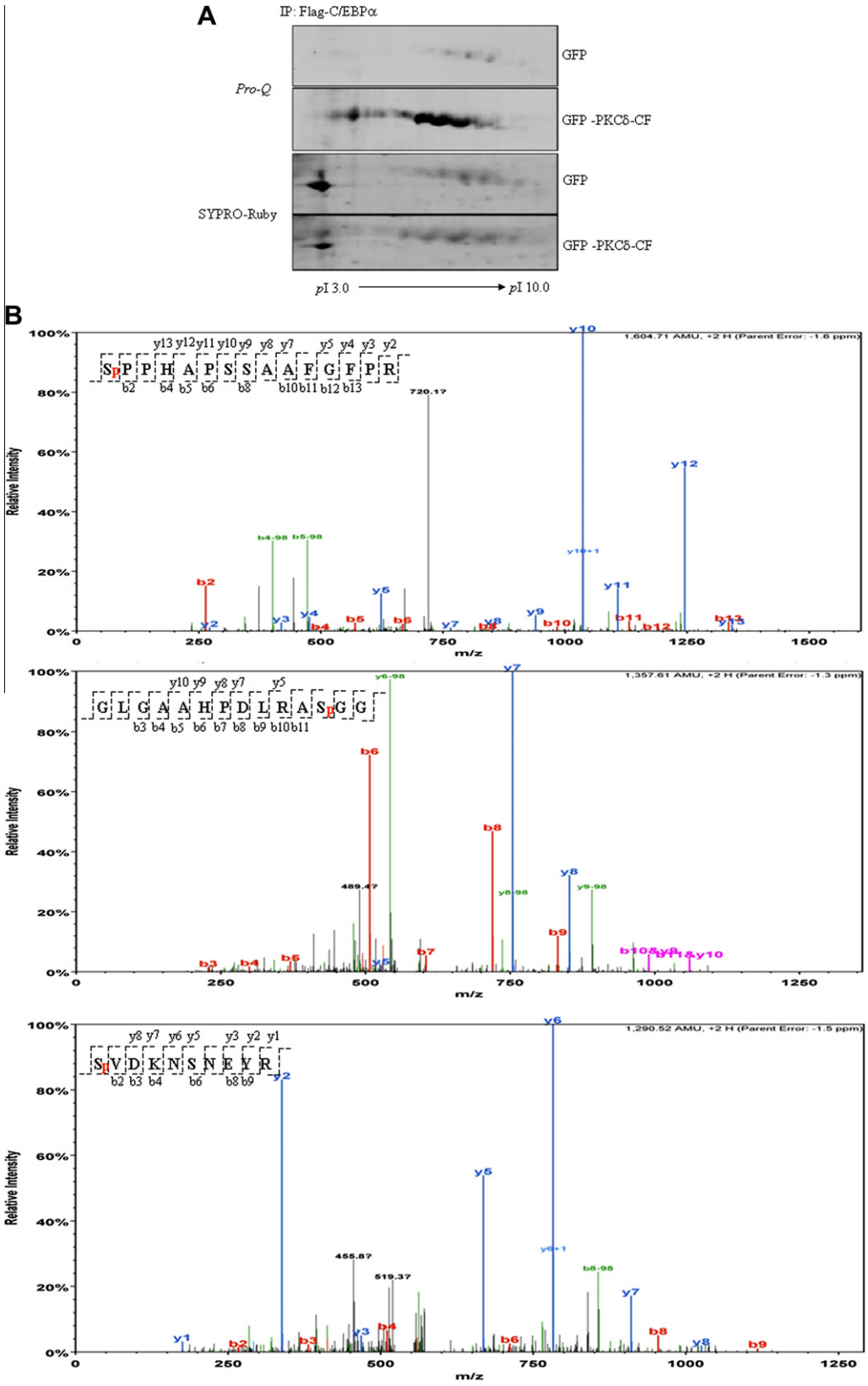

Fig. 2. The phosphorylation of C/EBPα activated by PKCδ. (A) HEK293T cells were transfected with C/EBPα and GFP or GFP-PKCδ-CF for 24 h. Cell lysates were immunoprecipitated with anti-flag M2 beads, and precipitates were isolated by 2DE. The 2DE gels were sequentially stained by Pro-Q Diamond to detect the phosphorylated residues of proteins and by SYPRO Ruby to detect the protein. The grayscale of proteins displayed by both staining was in proportion to the intensity of phosphoprotein or protein respectively. (B) The unique phosphopeptides of C/EBPα were only discovered in precipitates by anti-flag M2 beads in cells with PKCδ-CF, but not founded in the lysates in cells with GFP. The labeled MS spectra of three phosphopeptides of C/EBPα, including Phospho-Ser21 at top panel, Phospho-Ser266 at middle panel, and Phospho-Ser277 at bottom panel. Blue letter, y ion; red letter, b ion; red "p", phosphosite. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Dephosphorylation of C/EBPα reverses the inhibitory effect of PKCδ-CF on C/EBPα

To further validate the phosphorylation of C/EBPα induced by activated PKCδ, C/EBPα protein purified with anti-flag M2 beads was treated by Lambda protein phosphase (λ-PPase). The reaction products were separated by two-dimensional electrophoresis and Western blot was used to detect C/EBPα protein. The results showed that five C/EBPα spots (spots a–e) were detected in GFP-expressing cells and four spots (spots b–e) disappeared after λPPase treatment, indicating spots b–e were phosphorylated ones. As expected, these spots (spots b–e) increased significantly in GFP-PKCδ-CF-expressing cells and λPPase treatment similarly eliminated them (Fig. 3A).

Next, in order to find out whether phosphorylation of C/EBPα by PKCδ-CF is associated with the translocation and consequent inactivation and degradation of C/EBPα, we sought to dephosphorylate C/EBPα in GFP-PKCδ-CF-expressing cells. To this end, we screen a series of phosphatases and found that protein phosphatase 1 catalytic subunit gamma (PP1CCC) could most effectively block C/EBPα translocation induced by activated PKCδ (Fig. 3B). Concomitantly, PP1CCC also completely restored the expression level and transcriptional activity of C/EBPα (Fig. 3C). Taken together, these results showed that phosphorylation of C/EBPα by PKCδ-CF is responsible for the cytoplasmic translocation and consequent inactivation and degradation of C/EBPα.

4. Discussion

In this study, we have delineated a molecular pathway by which C/EBPα is degraded by PKCδ (Fig. 3D). This pathway is triggered by activation of PKCδ, and activated PKCδ enhanced the phosphorylation of C/EBPα, which consequently led to its cytoplasmic translocation, transcriptional inactivation, ubiquitination and degradation. Notably, by mass spectrometry, three phosphosites of C/EBPα were identified. Moreover, dephophorylation of C/EBPα inhibited its translocation and degradation and rescued its transcriptional activity. As reported, phosphorylation can occur on several Ser or Thr residues in human C/EBPα protein including Ser-21, Ser-226, Ser-230 and Ser-234, which are homologous in mouse and rat [15–19]. Phosphorylation of these Ser or Thr residues markedly affects its transcriptional activity [20]. Phosphosites of Ser-266 and Ser-277 in human C/EBPα revealed in this study have not been reported, and will be valuable for further investigation. However, we did not provide evidence that C/EBPα was directly phosphorylated by activated PKCδ. Possible kinases downstream of PKCδ remains to be investigated.

In summary, our results demonstrated that activated PKCδ could induce phosphorylation of C/EBPα, which led to cytoplasmic translocation, inactivation and degradation of C/EBPα. Furthermore, two new phosphosites of C/EBPα were identified. These results would shed new sights for understanding mechanisms of PKCδ-C/EBPα interaction during apoptosis and new functions of C/EBPα.

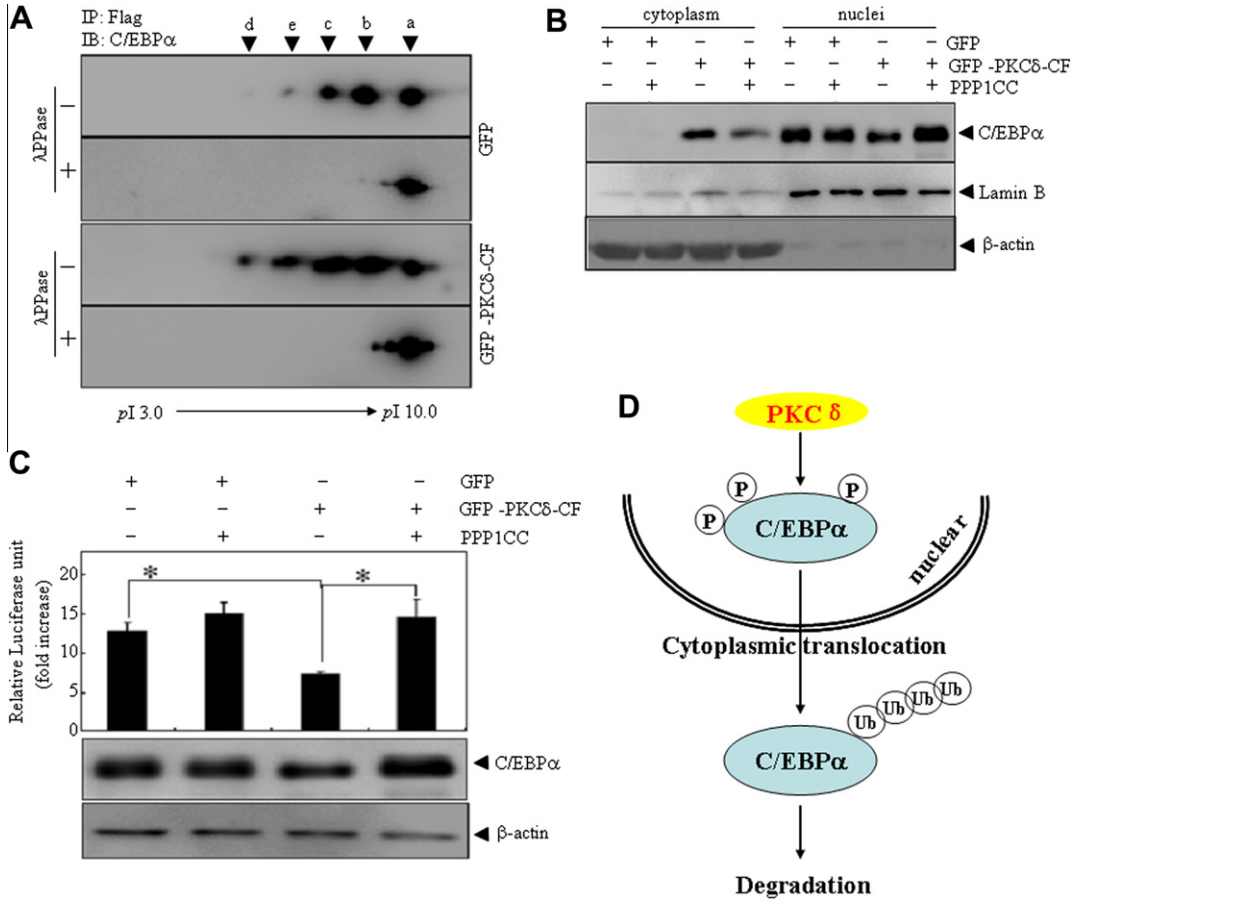

Fig. 3. The suppression of C/EBPα translocation and degradation by dephosphorylation. HEK293T cells were transfected with C/EBPα and other plasmids as indicated for 24 h. (A) Cell lysates were immunoprecipitated with anti-flag M2 beads, and the precipitates were treated with or without λPPase for 3 h, and then isolated by 2DE, followed by Western blot analysis. (B) Lysates of nuclei and cytoplasm fractions were detected by Western blot for C/EBPα. Lamin B and β-actin were as internal and quality control. (C) Cell extracts were prepared and luciferase activities were measured. Mean luciferase activity is shown relative to the cells without transfection of C/EBPα. Error bars represent SD ($n = 3$) ($^*P < 0.05$). (D) Molecular pathway regulating C/EBPα degradation by PKCδ.

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