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PKCδ stabilizes TAp63 to promote cell apoptosis

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ABSTRACT

PKCδ and p63 are respectively reported to play important roles in cell apoptosis. But there is no report on interaction between them in regulation of apoptosis. In the present study, we found that PKCδ can directly associate and up-regulate TA isoforms of p63 (TAp63) proteins via increasing their stability. PKCδ kinase activity and Thr157 site in TAp63 are crucial for this PKCδ-induced accumulation of TAp63. PKCδ can also enhance TAp63-mediated transcription and cell apoptosis. Taken together, our data indicate that PKCδ phosphorylates TAp63 proteins at Thr157 to stabilize them and promote cell apoptosis.

1. Introduction

Protein kinase C (PKC) is a family of cytoplasmic lipid-dependent serine/threonine protein kinases in mammals [1]. PKCδ is a member of novel PKC isoforms and widely present in various tissues such as brain and epithelial tissue cells. It is reported that PKCδ is Ca^{2+} independent but activated by diacylglycerol, and plays a key role in regulation of cell cycle and apoptosis in many kinds of cells [2].

PKCδ can take part in cell apoptosis through physical interaction with other apoptotic regulators. Studies demonstrate that PKCδ is phosphorylated and activated by c-Abl under oxidative stress. Activated PKCδ in turn can activate c-Abl by phosphorylation which makes mitochondria release cytochrome C and activates caspase-3, consequently promoting cell apoptosis [3]. Other studies have found that PKCδ could bind to caspase-3 directly and phosphorylate it to promote cell apoptosis [4]. PKCδ also directly phosphorylates tumor suppressor protein p53 at Ser15 and Ser46, resulting in p53 accumulation and cell death [5–7]. In the apoptotic response of cells to DNA damage, PKCδ is cleaved into a kinase-active catalytic fragment (PKCδCF), which mediates phosphorylation and accumulation of the p53 homologue p73β [8].

P63 is a new member of the p53 gene family. Due to alternative transcription starting sites, p63 gene encodes two classes of protein isotypes, TAp63 and ΔNp63. TAp63 isotypes have a full N-terminal transactivation domain (TAD) each and up-regulate lots of downstream target genes like p53, while ΔNp63 isoforms only possess incomplete TADs at their N-termini and inhibit transcriptional activity of p53, p73 and TAp63 [9–11]. Furthermore, owing to the C-terminal splicing in different ways, either type of p63 has been subdivided into at least three different subtypes: α, β, γ (TAp63α, β, γ and ΔNp63α, β, γ) [12]. In recent years, studies have shown that, TAp63 proteins play a key role in multiple apoptosis signaling pathways, and thus participate in apoptosis, despite their very low abundance in somatic cells. Bax, p21, and other downstream target genes can be activated by TAp63, causing cell cycle arrest and apoptosis [12,13].

As TAp63 proteins play important roles in apoptosis, upstream proteins of TAp63 are involved in apoptosis by interacting with them. For example, Plk1 down-regulates TAp63α by phosphorylating it at Ser52, suppressing cell apoptosis [14]; TATA-binding protein-like protein (TLP) and peptidyl-prolyl isomerase Pin1, can promote cell apoptosis by up-regulating TAp63α [15,16]. It has been reported that p53 and p73β are both up-regulated by PKCδ, inducing cell apoptosis [6–8]. Whether PKCδ has effect on p63 is previously unknown. Here we demonstrate that PKCδ stabilizes TAp63 proteins likely via phosphorylating them at Thr157, resulting in enhanced TAp63-mediated transactivation and cell apoptosis.

2. Materials and methods

2.1. Cell culture and drug treatment

HeLa, H1299 and HCT116($p53^{-/-}$) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) with 10%

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heat-inactivated fetal bovine serum (FBS, Hyclone) and 1% penicillin G/streptomycin (Hyclone) at 37 °C in a humidified 5% CO_2 incubator. For activation or inhibition of PKCδ, 200 nM Phorbol 12-myristate 13-acetate (PMA, Sigma) or 5 μM rottlerin (Sigma) was supplemented 24 h post-transfection and was incubated for 24 more hours.

2.2. Transfection

Cells were transfected with pcDNA-c-Myc-TAp63α, pcDNA-c-Myc-TAp63γ, pcDNA-c-Myc-ΔNp63α, pcDNA-c-Myc-ΔNp63γ, pKV-PKCδ or pGFP-PKCδ plasmid using LipofectAMINE 2000 (Invitrogen). Supplement of 50 ng GFP plasmid pEGFP-N1 was used for each transfection, as a transfection efficiency control. To knockdown PKCδ, HeLA cells were transfected with siRNA directed against PKCδ or scramble control (Gene Pharma) using LipofectAMINE 2000. After 48 h, cells were harvested for analysis.

2.3. Immunoprecipitation (IP)

Cells were lysed in IP lysis buffer [20 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.5% Nonidet P-40, 10 mM KCl, 0.5 m MEDTA, 10% glycerol, 1.5 mM $MgCl_2$]. Cell lysates containing 500 g total protein were pre-cleared with normal mouse IgG (Santa Cruz) and protein G-sepharose (GE health) for 1 h. Then the supernatant was incubated with anti-c-Myc or anti-GFP or normal mouse IgG (Santa Cruz) for 2 h and precipitated with protein G-Sepharose for 2 more hours. The precipitates were washed with lysis buffer three times at 4 °C, resuspended in 30 μL of 2× SDS sample buffer and heated at 100 °C for 10 min, followed by immunoblot (IB) analysis.

2.4. Immunoblot (IB)

Cells were collected, washed with phosphate-buffered saline, and resuspended in lysis buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 1 mM phenylmethylsul-fonyl fluoride (PMSF), 1 μg/mL leupeptin, 1 μg/mL pepstatin, 2 μg/mL aprotinin, 10 mM NaF, 1 mM EDTA, and 1 mM Na_3VO_4]. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad). An equal amount of protein was loaded into every well and separated on a 10% SDS–PAGE, then transferred to polyvinylidene difluoride membrane (Bio-Rad), and hybridized to an appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody for subsequent detection with ECL (Millipore). Immunoblot analysis was performed with anti-p63 (Santa Cruz Biotechnology), anti-pho-PKCδ (Cell Signaling Technology), anti-PKCδ (Santa Cruz Biotechnology), anti-GFP (Santa Cruz Biotechnology), anti-β-actin (Santa Cruz Biotechnology) or anti-PARP (Zenable bioscience). Densitometric analysis was performed with Image Lab software (Bio-Rad).

2.5. Site-directed mutagenesis

S147A, T157A, S319A and TS349, 350AA mutant TAp63α plasmids were generated with the site-directed mutagenesis kit (Stratagene), using pcDNA-c-Myc- TAp63α as a template.

2.6. Protein stability assay

To test protein stability of TAp63α, HeLa cells were co-transfected with TAp63α and GFP plasmids plus PKCδ or empty vector. 24 h post-transfection, cells were supplemented with 50 μg/mL cycloheximide (CHX, Sigma) to block the biosynthesis of nascent proteins. Then cells were cultured for additional indicated durations to harvest. Cell lysates were used for immunoblot analysis. Cells were collected at the indicated time points for IB analysis. β-actin was used as a loading control and GFP as a transfection efficiency control.

2.7. Luciferase assays

H1299 cells were transfected with a mixture of Bax-Luc and pRL-TK-Renilla. Cells were harvested at 36 h after transfection and lysed in Passive Lysis Buffer (Promega). Lysates were analyzed for firefly and Renilla luciferase activities using the Dual Luciferase Reagent Assay Kit (Promega).

2.8. Flow cytometry analysis (FACS)

Cells were fixed in 70% ethanol at 4 °C overnight and stained with 50 μg/mL propidium iodide (Sigma), supplemented with 80 μg/mL RNase A (Sigma) for 40 min at 37 °C in the dark. Then, cells were subjected to FACS analysis with FACScan flow cytometer (Becton Dickson). Data were analyzed using the Cell Quest program.

3. Results

3.1. PKCδ can associate and up-regulate TAp63

It has been reported that PKCδ can mediate phosphorylation of the tumor suppressors p53 and p73β, increasing their protein levels in cells [5–8]. Since p63 proteins are homologues of p53 and p73β, we wondered if PKCδ has similar effects on p63 isoforms. To this end, we co-transfected HeLa cells, which are deficient in p63 proteins, with PKCδ and different TA or ΔN isoforms of p63 plasmids, respectively, using GFP plasmid as a transfection efficiency control. Cell lysates were subjected to immunoblot analysis. The results showed that, TAp63α and TAp63γ were up-regulated in the case of overexpression of PKCδ, but the two isoforms of ΔNp63 were not affected (Fig. 1A). This suggests that PKCδ can induce an accumulation of TAp63 proteins in cells.

To extend these findings, rottlerin, which is a PKCδ chemical inhibitor [17], was used to treat the HeLa cells which were transfected with TAp63α plus PKCδ or vector control. Our data demonstrate that PKCδ-mediated TAp63α up-regulation was abrogated by rottlerin (Fig. 1B). On the other hand, activation of endogenous PKCδ with its potent activator, phorbol 12-myristate 13-acetate (PMA) [18], can significantly increase protein level of transfected TAp63α in HeLa cells, while simultaneous supplement of rottlerin can obviously impair this effect (Fig. 1C). Further study demonstrates that siRNA-mediated specific knock-down of endogenous PKCδ can also significantly abrogate PMA-induced TAp63α accumulation (Fig. 1D). These data suggest that PKCδ up-regulates TAp63 due to its kinase activity.

To examine whether PKCδ directly interacts with TAp63, HeLa cells were transfected with c-Myc-tagged TAp63α and GFP-tagged PKCδ plasmids. The cell lysates were subjected to immunoprecipitation (IP) with anti-C-myc. Immunoblot (IB) analysis demonstrated that the precipitate contains both TAp63α and PKCδ (Fig. 1E). To ensure this finding, anti-GFP was used to precipitate PKCδ. The results confirmed that TAp63α can be coimmunoprecipitated with PKCδ (Fig. 1F). On the other hand, we failed to detect physical interaction between PKCδ and ΔNp63α with co-immunoprecipitation assay (data not shown).

In a summary, these results suggest that PKCδ physically interacts with TAp63 and induces accumulation of TAp63 proteins in cells due to the kinase activity of PKCδ.