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Identification of Evi-1 as a novel effector of PKCδ in the apoptotic response to DNA damage

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Protein kinase C delta (PKCδ), a PKC family isoform, regulates diverse signal transduction pathways during DNA damage to induce apoptosis. To explore the apoptosis mechanism that PKCδ modulates, we sought to uncover transcription factor targets of PKCδ by devising a screening strategy that utilizes ChIP-cloning and microarray analysis. Transcription factor candidates were generated with the application of public access data-mining tools and this resulted in the identification of Evi-1 as a novel PKCδ-mediated DNA damage responsive molecule. The results demonstrated that PKCδ is constitutively associated with Evi-1. PKCδ regulated Evi-1 to activate PLZF transcription upon genotoxic stress. Furthermore, both Evi-1 and PLZF were associated with DNA damage-stimulated apoptosis. Taken together, we have discovered a novel regulation of Evi-1, which transactivates PLZF, by PKCδ to induce cell death in response to genotoxic stress.

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1. Introduction

Protein kinase C (PKC) family of serine/threonine kinases plays an essential role in signal transduction pathways of diverse cellular processes including cell proliferation, differentiation, migration, and apoptosis. Protein kinase C δ (PKCδ) belongs to the novel PKCs and was the first novel PKC isozyme discovered [1]. PKCδ undergoes self-phosphorylation and is further phosphorylated on different sites depending on the stimuli [2]. While pro-survival aspect of PKCδ has been described, it is generally conceived that PKCδ is a pro-apoptotic molecule as evidenced from experiments in a variety of cell types in response to different death stimuli [3,4]. PKCδ-mediated induction of apoptosis upon genotoxic stress involves its breaking up into a 40 kDa protein catalytic domain that can be translocated into the mitochondria and nucleus by proteolysis with caspase-3 [5,6]. Association of PKCδ with the c-Abl tyrosine kinase is also important for the apoptotic response since PKCδ and c-Abl are found to be activated by inter-phosphorylation each other [7,8].

DNA damage triggers PKCδ to maneuver apoptosis events by targeting a vast spectrum of substrates such as DNA-dependent protein kinase [9], phospholipid scamblase 1 [10], lamin B [11], or Rad9 [12]. In particular, it is interesting to note that those effectors reported include TP53, a transcription factor central to DNA damage defend mechanism. TP53 Ser46 phosphorylation is regulated by PKCδ in response to DNA damage signal [13,14]. More surprisingly, PKCδ transactivates TP53 by interacting with the death promoting domain Btf, which itself is also a transcription factor to promote apoptosis [15]. Taken together, these findings indicate that transcription factors belong to one of the target categories that contribute to the PKCδ apoptotic mechanism.

In view of this, we intended to uncover unknown PKCδ regulated, directly or indirectly, transcription factors induced by genotoxic damage. For this purpose, we have developed a strategy that utilizes chromatin immunoprecipitation (ChIP)-sequencing and microarray. The strategy presented here makes the use of the public access bioinformatics tools to predict transcription factors from the data generated by two screens. By comparing the occurrence of the in silico-predicted transcription factors, the data revealed Evi-1 as a top candidate. Further experiments showed that Evi-1 transactivates promyotic leukemic zinc finger (PLZF), a previously reported downstream target of Evi-1 [16], in response to DNA damage.

2. Materials and methods

2.1. Cell culture

U2OS cells (human osteosarcoma cells expressing wild type p53 and Rb) were cultured in RPMI1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotics. Cells were treated with 5 μM rottlerin (PKCδ inhibitor; Sigma-Aldrich), 2 μg/ml adriamycin (ADR; Sigma-Aldrich), or 10 μM etoposide (ETO; Sigma-Aldrich).

Abbreviations: ChIP, chromatin immunoprecipitation; HSC, haematopoietic stem cells; PKCδ, protein kinase C δ; PLZF, promyotic leukemic zinc finger

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2.2. Construction of plasmids

PKCδ cDNA was cloned as described previously [17,18]. Evi-1 cDNA was amplified from U2OS cDNA using polymerase Pfu Ultra (Stratagene) and was cloned into pFLAG-CMV-5a (Sigma-Aldrich) and pEGFP-C1 (Clontech). Plasmids for luciferase assay were constructed as mentioned previously [16]. Briefly, the PLZF promoter region was first amplified by using the following primer set: 5′-AAAGTGGCATCTTCTCCCCAAA-3′ and 5′-TGGCTGCACAGCAGTTTGATAA-3′. P2 and P1 from the promoter region were then amplified by nested-PCR using the template obtained with the previous primer set. The nested-PCR was performed with the following forward primers: for P2 5′-GGCAGGTACCCGCGAGAGATT-GAGTATT-3′ and 5′-GTTTCTCGAGCCCAGATAAAGCAGCAGC-3′; for P1 5′-CTCTGGTACCTTTCCATCCTGCTTGTTT-3′, 5′-GTTTCTCGAGCCCAGA-TAAAGCAGCAGC-3′. These amplified DNA fragments were cloned into pGL3-Basic vector (Promega) resulting in P2-Luc and P1-Luc, respectively.

2.3. Cell transfection

Plasmid DNA was transfected by using FuGENE 6 transfection reagent (Roche). Validated PKCδ gene-specific siRNAs (short-interfering RNAs) and Evi-1 stealth siRNAs were purchased from Invitrogen (Stealth RNAi). The RNAi sequences are as follows:

PKCδ siRNA: 5′-CCAUCCACAAGAAAUGCAUCGACAA-3′,
Evi-1 siRNA: 5′-AUUGAAGCCAGAUUCUGAAGAGGGC-3′,
Evi-1 siRNA-a: 5′-AAUCAUUCACAGCUCCUGACACCGC-3′,
Evi-1 siRNA-b: 5′-UUUCGAGGCUCAGUCAGCUUUGUCC-3′.

Transfection of 50 nM siRNAs was performed using Lipofectamine RNAi MAX (Invitrogen) as according to the manufacturer's protocol.

2.4. Semi-quantitative RT-PCR analysis

Isolation of total RNA from cells was performed using TRIsure (Nippon Gene) according to the manufacturer's protocol. Total RNA (400 ng) was amplified using SuperScript III One Step RT-PCR System with Platinum Taq Kit (Invitrogen) according to the manufacturer's instruction.

2.5. Immunoblotting and immunoprecipitation

Cell lysates were prepared as described elsewhere [19,20]. Briefly, cultured cells were washed once with chilled PBS and resuspended in lysis buffer [50 mM Tris/HCl, pH 7.6, containing 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT (dithiothreitol), 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1% (v/v) Tergitol NP-40]. Cell lysates were centrifuged at 12,500*g* for 10 min at 4 °C. Immunoprecipitation was performed by mixing cell lysates with Flag-tagged agarose (Sigma-Aldrich) or GFP-tagged agarose (Nacalai Tesque) for overnight with rotation at 4 °C. Western blotting was carried out by SDS/PAGE separation of the denatured cell lysates and transferred onto nitrocellulose membranes. The membranes were incubated with anti-Evi-1 (Santa Cruz Biotechnology), anti-PLZF (Santa Cruz Biotechnology), anti-tubulin (Sigma-Aldrich), anti-GFP (Nacalai Tesque), or anti-FLAG (Sigma-Aldrich) for 1–4 h at room temperature. After washing, the membranes were incubated with anti-rabbit or anti-mouse IgG–peroxidase conjugate (Santa Cruz Biotechnology). The antigen–antibody complexes were visualized by chemiluminescence (PerkinElmer).

2.6. In vitro luciferase assay

Luciferase activities were measured at 48 h post-transfection using the Bright-Glo Luciferase assay system (Promega) according to the manufacturer's protocol. The relative increase in activity compared with cells transfected with pGL-3 basic vector was determined as described [14,21].

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as previously described [15]. Briefly, $1–5 \times 10^7$ cells were fixed and sonicated. Sonicated cell mixture (50 μl) was used as input. Immunoprecipitation was performed with anti-PKCδ (Santa Cruz Biotechnology) or anti-Evi-1 (Cell Signaling) for 2 h or overnight at 4 °C before incubation with 30 μl of Protein A–Sepharose beads (Amersham) for 1 h at 4 °C. The beads were washed and chromatin complexes were eluted. DNA recovered from ChIP was blunt-ended with T4 DNA polymerase (Takara) before cloning into pBluescript SK vector. Sequencing was performed with T7 promoter and T3 promoter primers. For ChIP analysis, quantitative real-time PCR was performed by using SYBR Green PCR Master Mix (Applied Biosystems) according to the instruction manual. The data was normalized for the level of input control. Primer sequences are listed in Table 1.

2.8. Microarray analysis

Total RNA was isolated from cells using an RNeasy kit (Qiagen). Total RNA (5 μg) was used to start the protocol of One-Cycle cDNA Synthesis and to label cDNA, following the manufacturer's protocol (Affymetrix). Before making a cocktail solution, 20 μg of biotin-labeled cDNA was fragmented to 35–200 bases, and 15 μg of cDNA fragment was used to prepare the cocktail solution. The solution was applied into a GeneChip Human Genome U133 plus 2.0 array (Affymetrix) and hybridized for 16 h at 45 °C. After hybridization, the arrays were washed and stained using Fluidic station 450 according to the manufacturer's protocol, EukGE-WS2v5_450, and were then scanned using the Affymetrix GeneChip Scanner 3000. Analysis of the data was performed as previously described [22]. The complete expression datasets have been submitted to the online data repository ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/), under accession E-MEXP-2643.

2.9. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays

Cells cultured in poly-D-lysine-coated eight-well chamber slides, at 5,000 cells per well, were transfected with siRNAs and then treated with ETO. Apoptotic cells were detected with DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instruction.

3. Results

3.1. Identification of Evi-1 as a PKCδ downstream target

In an attempt to identify transcription factors that are regulated by PKCδ, we embarked upon the ChIP-based cloning and sequencing

Table 1
Primers used for RT-PCR and ChIP assays.

	Primer sequences
GAPDH	Forward: 5′-AAGGCTGTGGGCAAGGTCATCCCT-3′
	Reverse: 5′-TTACTCCTTGGAGGCCATGTGGGC-3′
Evi-1	Forward: 5′-TTCCTTCCGGCTTGTACCACAGGC-3′
	Reverse: 5′-ACTTTCCAGATCAGAGCCGAGG-3′
PKCδ	Forward: 5′-ACGGCGCTGGAGAAATACG-3′
	Reverse: 5′-CCTCTTTGGCTTCCTCACT-3′
PLZF	Forward: 5′-AGGCTGACGCTGTATTGAGC-3′
	Reverse: 5′-TCTCAGCCGCAAACTATCCAG-3′
PLZFp (ChIP assay)	Forward: 5′-ACTGATACCCAGGTTTCCCTTAAA-3′
	Reverse: 5′-AGAGCTATGTCCTTGGGTCCTG-3′
PLZFp-CR (ChIP assay)	Forward: 5′-AGCCACCACCTCCCAGGTT-3′
	Reverse: 5′-AACTCCAGATTGCCACTGG-3′

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