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Protein kinase Cδ negatively regulates Notch1-dependent transcription via a kinase-independent mechanism in vitro

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ABSTRACT

Protein kinase Cδ (PKCδ) plays a significant role in the regulation of growth, apoptosis, and differentiation in a diversity of cell types. We investigated the effect of PKCδ on Notch1 intracellular domain (NICD)-mediated transcription with Notch transcription reporter constructs. The results indicate that co-expression of PKCδ down-regulated NICD-dependent transcription. Co-expression of a dominant negative PKCδ (K376R) variant lacking kinase activity was also able to downregulate NICD-dependent transcription, suggesting that PKCδ exerts its inhibitory effect via a kinase-independent mechanism(s). Interestingly, expression of PKCδ as well as K376R induced NICD up-regulation by inhibiting proteasome-mediated degradation of NICD, indicating that NICD protein quantity is not proportional to its transcriptional activity. When the subcellular distribution of NICD was investigated by both subcellular fractionation and immunocytochemistry, it was found that PKCδ and K376R effectively impaired proper nuclear localization of NICD, possibly via a physical association between NICD and PKCδ, which was confirmed by co-immunoprecipitation experiments. Chromatin immunoprecipitation assays revealed that both PKCδ and K376R inhibit the association of NICD with the promoter region of its target gene, *Hes1*. Furthermore, silencing of PKCδ resulted in increased NICD nuclear localization and NICD transcriptional activity in MCF-7 cells. PKCδ silencing-induced increase in anti-apoptotic survivin could not rescue apoptosis induced by doxorubicin. The data herein indicate that PKCδ can induce down-regulation of NICD transcriptional activity via a kinase-independent inhibition of NICD nuclear targeting and dissociation of NICD from target gene promoters.

1. Introduction

The Notch signaling pathway is a highly conserved mechanism that plays a significant role in the control of cell fate during various developmental processes, including cellular proliferation, differentiation and apoptotic events [1]. Notch receptors are single-pass transmembrane proteins that contain a signal peptide, a series of epidermal growth factor-like repeats in the extracellular domain involved in ligand binding, a transmembrane domain responsible for receptor activation, and an intracellular domain [2,3]. In *Drosophila*, there is one Notch receptor (dNotch) and two associated ligands (Delta and Serrate) [4]. Mammals possess four Notch receptors (Notch1, Notch2, Notch3 and Notch4) [5–7], five ligands containing the two Serrate homologs, Jagged 1 (JAG1) and Jagged 2 (JAG2) [8,9], and three Delta-like (DLL) homologs (DLL1, 3, and 4) [10–12]. Notch signaling is initiated by ligand binding with subsequent proteolytic cleavage of Notch and release of the Notch intracellular domain (NICD) [13]. The NICD then enters the nucleus and subsequently associates with a DNA binding protein to assemble a complex that activates transcription of downstream target genes including *Hes1* and *Hes5* [14–17].

The Protein Kinase C (PKC) family of serine/threonine kinases plays an important role in many cellular functions including cellular proliferation, tumor promotion, growth regulation, cell survival and programmed cell death [18]. The PKC family can be broadly subdivided into three groups: The classical/conventional PKCs (cPKCs: α, βI, βII, γ), which are calcium dependent and activated by diacylglycerol (DAG) or 12-O-tetradecanoyl-13-phorbol acetate (TPA); the novel PKCs (nPKCs: δ, ε, θ, μ) that are also activated by DAG or TPA but are calcium-independent; and the atypical PKCs (aPKCs: ζ, λ) that are calcium-independent and do not respond to DAG or TPA [19–22]. As a member of the nPKC subfamily, PKCδ contains a C-terminal catalytic region with two conserved domains (C3 and C4) essential for ATP binding, catalytic activity, and substrate binding. The N-terminus of PKCδ contains a regulatory region with an

Abbreviations: ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole; DLL, delta-like; DTT, dithiothreitol; ER, estrogen receptor; HDAC, histone deacetylase; NICD, Notch1 intracellular domain; PKCδ, protein kinase Cδ; PKCδ K376R, kinase-dead dominant negative PKCδ; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-O-tetradecanoyl-13-phorbol acetate; TSA, trichostatin

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inhibitory pseudosubstrate sequence and cysteine-rich sequences in the twin C1 domains [23–25].

Many reports suggest that Notch-induced signaling interacts with other major signaling pathways, such as NF-kB signaling, the caspase 3 pathway, the mitogen-activated protein kinase (MAPK) pathway, and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [26–29]. We previously reported that Akt downregulates transcriptional activity of NICD possibly by Akt-mediated phosphorylation of NICD and subsequent cytosolic retention of NICD [30]. While searching for a possible site of phosphorylation by Akt on NICD using the NetPhosK server (http://www.cbs.dtu.dk/services/NetPhosK) and Scansite (http://scansite.mit.edu), we found that two serine residues, Ser2152 and Ser2173, may also be phosphorylated by PKC. This led us to investigate the possible regulation of NICD transcriptional activity by PKC-dependent phosphorylation. Interestingly, we found that PKCδ, and not the classical PKCα, PKCβI and PKCβII kinases, could downregulate NICD transcriptional activity in a kinase-independent manner.

2. Materials and methods

2.1. Plasmids, Vectors and siRNA nucleotides

Mouse Notch1 NICD was as described [30]. HA-tagged PKC isozymes (PKCα, PKCβI, PKCβII, PKCδ), and dominant-negative forms of PKC isozymes (PKCα, PKCβI, PKCβII, PKCδ) were kindly donated by Dr. Su-Jae Lee (Hanyang University, Seoul, Korea). pEGFPN1-PKCδ and dominant negative forms of pEGFPN1-PKCδ were kindly provided by Dr. Stuart H. Yupsa (Bethesda, MD, USA). The plasmid, pBJ5-HDAC1-Flag, was obtained from Dr. Jongbok Yoon (Yonsei University, Seoul, Korea). The plasmid, pcDNA3-Flag-HDAC1 (H141A), was a gift from Dr. Sung Hee Baek (Seoul National University, Seoul, Korea). siRNA nucleotides against PKCδ and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and transfection

Two types of cells, 293 T and Cos7 (ATCC), were maintained in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin (Welgene, Daegu, Korea). Cells were incubated at 37 °C under 5% CO_2 in a humidified incubator. 293 T and Cos7 cells were transfected using calcium phosphate in HEPES buffered saline as described previously [31]. MCF-7 cells were transfected using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocols.

2.3. Antibodies and reagents

PKCδ, mouse Myc-Tag (9B11), rabbit c-Jun (60A8), mouse HA-tag (6E2), and Akt and p-Akt (Ser473) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Actin (C-2), rabbit HA-probe (Y-11), and rabbit β-tubulin (H-235), mouse Hes1 (E-5) and survivin (D-8) antibodies were purchased from Santa Cruz Biotechnology. The 26S proteasome inhibitor, MG132, was obtained from Calbiochem. PKCδ siRNA and control siRNA were purchased from Santa Cruz Biotechnology .

2.4. Reporter assays

Cells grown on 12-well plates were transfected with 1.5 μg total DNA containing 0.5 μg reporter construct [6×Notch response elements (6×NRE-Luc); 4×CSL-Luc; HES1-Luc, gifts from Dr. Hee-Sae Park (Chonnam National University, Korea)] and analyzed 48 h after transfection. Dual luciferase assays were performed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.5. Immunoblot analysis

All cells were lysed and processed 48 h post-transfection. Transfected cells were washed once with phosphate-buffered saline (PBS) and lysed with a lysis buffer [20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% Triton X-100, 0.1% SDS, 20 mM NaF, 1 mM Na_3VO_4, 1×protease inhibitor (Roche, Indianapolis, IN)]. All protein samples were resolved by 10% SDS–PAGE after boiling 10 min in SDS sample buffer. For immunoblot analysis, proteins were electro-transferred onto nitrocellulose membranes in Tris-glycine-methanol buffer for 2 h. The membranes were blocked with 5% skim milk in standard TBST buffer for 1 h, incubated overnight with appropriate dilutions of primary antibody in TBST containing 5% bovine serum albumin. The membranes were then washed three times with TBST and incubated with HRP-conjugated secondary antibody solution for 2 h. The blots were then visualized using the WEST-ZOL® plus Western Blot Detection System (iNtRON Biotechnology, Seoul, Korea).

2.6. Cycloheximide decay assays

Cells were seeded at a density of 5×10^5 cells/well in 6-well dishes. After 24 h, cells were treated with 20 μg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) to block further protein synthesis. The cells were harvested at each indicated time point and subjected to immunoblot analysis.

2.7. Immunostaining

For immunostaining, 293 T cells were seeded at a density of 3×10^5 cells/well on glass coverslips in 6-well plates, transfected with 4 μg total DNA (2 μg Myc-NICD and 2 μg pEGFP-N1-PKCδ or 2 μg pEGFP-N1-PKCδ K376R), and processed 48 h post-transfection. Cells were washed with PBS, fixed with 3.8% paraformaldehyde in PBS for 10 min and permeabilized with 0.05% Triton X-100 in PBS for 10 min at ambient temperature. The fixed cells were washed three times with PBS for 5 min each and blocked with 3% skim milk in PBS for 30 min. Primary antibody (80 μl per coverslip) was diluted in 1% skim milk and added to each coverslip and incubated for 1 h. The samples were then washed 3 times with PBS and incubated with 80 μl of secondary antibody solution [anti-rabbit IgG-Cy3 (In vitrogen) in 1% skim milk in PBS]. The samples were washed three times for 10 min with PBS, and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) in the first wash. Coverslips were mounted on glass slides and observed by fluorescence microscopy (Olympus BX50).

2.8. Cell fractionation assays

In a 100-mm dish, 293 T cells were seeded at a density of 1×10^6 cells and subsequently transfected with 5 μg of total DNA. Forty-eight hours post-transfection, the cells were washed with ice cold PBS, incubated in cytoplasmic extraction buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]and agitated for 10 min at 4 °C. After addition of NP-40 (final 0.5%), the samples were further agitated for 10 min at 4 °C. The samples were then subjected to centrifugation at 13,000 rpm for 5 min. The resulting supernatant was collected as the cytosolic fraction. Nuclear pellets were washed three times with cold PBS and resuspended in a nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). The nuclear extracts were agitated for 10 min at 4 °C then centrifuged at 13,000 rpm at 4 °C. The resulting supernatants were collected as the nuclear fraction.