Protein kinase Cζ regulates survivin expression and inhibits apoptosis in colon cancer

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Abstract. The phosphatidylinositol 3-kinase pathway transduces cell survival signals in different malignancies. Protein kinase Cζ (PKCζ) is one of the molecules involved in this pathway. In this study, we investigated the role of PKCζ in apoptosis. Short interfering RNA against PKCζ (siPKCζ) sensitized HCT116 and SW480 colon cancer cells to TRAIL-induced apoptosis. Among anti-apoptotic proteins, survivin protein and mRNA expression levels decreased after siPKCζ transfection while protein half-life did not change. The expression levels of survivin and PKCζ were correlated in 18 colon cancer specimens (r=0.72, P=0.01x10^{-4}). Chemosensitivity to 5-FU was enhanced by siPKCζ in HCT116 and SW480 cells. These results indicate that PKCζ regulates survivin expression levels and inhibits apoptosis in colon cancer cells. This study provides a rationale for targeting PKCζ in combination with chemotherapy for colon cancer treatment.

Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway transduces cell survival signals in different malignant cells (1,2). The pathway is directly activated by genetic mutations of PI3K or alterations in proteins regulating this pathway. Activating missense mutations of the PI3K catalytic subunit, PI3KCA, are reported in 4.6-26.7, 8.3-40.0, 13.6-31.6, 35.6, 1.3-4.2, 6.0-12.1 and 4.3-25.0% of brain, breast, colon, liver, lung, ovary and stomach cancer cases, respectively (3). Phosphatase and tensin homologue (PTEN) antagonizes PI3K signaling by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3). A germline mutation of PTEN leads to Cowden disease characterized by a predisposition to breast and thyroid cancer (4). KRAS is frequently mutated in various types of cancer, including colon, lung and pancreatic cancer. PI3K has been shown to be an essential target molecule in KRAS-dependent carcinogenesis in vitro and in vivo (5). Receptor tyrosine kinases reside upstream of the PI3K pathway: i) epidermal growth factor receptor (EGFR) mutations are reported in 7.6-30.6% of non-small cell lung cancer (6), ii) ERBB2 is overexpressed in 10-34% of breast cancer (7), and iii) BCR-ABL1 chimeric protein is produced and activates the PI3K pathway in chronic myeloid leukemia (8).

PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to produce PIP3, that activates downstream kinases including phosphoinositide dependent protein kinase 1 (PDK1) and AKT, which is a downstream target of PDK1 (9). Atypical protein kinase Cζ (PKCζ) is another kinase in the PI3K pathway. It is a member of protein kinase C family and is activated by PDK1 (10,11), K-RAS (12), and superoxide (13).

Reportedly, overexpression of PKCζ is associated with poor prognosis in patients with soft tissue sarcoma (14), cervical cancer (15), and prostate cancer (16). Experimentally, activation of PKCζ has been shown to confer aggressive phenotypes to cancer cells through different mechanisms, including promotion of cell proliferation (17,18), migration (13,19) and transactivation of hypoxia-inducible factors (20).

In this study, we show that PKCζ inhibits apoptotic stimuli by regulating survivin expression level in colon cancer cells.

Materials and methods

Reagents. Anti-PKCζ (sc-216), anti-actin (sc-8432), anti-Bcl-2 (sc-7382), and anti-Bcl-XL (sc-8392) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cIAP1 (AF818), anti-cIAP2 (AF817), anti-survivin (AF886), and anti-XIAP (MAB822) antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Anti-phospho-PKCζ (NB100-80026) antibody was purchased from Novus Biologicals (Littleton, CO, USA). Anti-Ran antibody was purchased from BD Pharmingen (San Jose, CA, USA). Cycloheximide and 5-fluorouracil (5-FU) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Cell culture. The human colon cancer cell lines HCT116 and SW480 that present the constitutively active PI3K pathway were used in this study (21). SW480 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich).
supplemented with 10% fetal bovine serum (FBS; HyClone Thermo Fisher Scientific, Logan, UT, USA) in humidified 5% CO2 at 37°C. HCT116 cells were cultured in the same condition except that McCoy's 5A medium (Gibco Life Technologies, Gaithersburg, MD, USA) was used instead of DMEM.

**Purification of soluble recombinant human TRAIL (rhTRAIL).** His6-tagged TRAIL expression plasmid (pQE-HTR) that expresses the extracellular portion of human TRAIL was kindly provided by Dr. W.S. El-Deiry (Penn State Hershey Cancer Institute, PA, USA). It was transformed into DH10B (Takara Bio Inc., Tokyo, Japan). Briefly, rhTRAIL was induced by 0.5 mM Isopropyl-1-thio-β-D-galactopyranoside, and purified using Ni-NTA agarose beads (Qiagen, Valencia, CA, USA) as previously described (22).

**Sub-G1 analysis.** After treatments, the cells were collected and fixed with 70% ethanol at 4°C. The cells were resuspended in phosphate-citrate buffer. After centrifugation, the pellets were treated with RNase A, stained with propidium iodide (PI), and analyzed by flow cytometry (FACSCanto, BD Bioscience, San Jose, CA, USA).

**Western blotting.** Western blotting was carried out using standard methods. Briefly, cells were sonicated in lysis buffer [20 mM Tris-HCl (pH 8.0), 135 mM NaCl, 5 mM EDTA and 1% NP-40] with a protease inhibitor cocktail (Sigma-Aldrich) and Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL, USA). The samples were separated on a Tris-glycine gel (Life Technologies) under denaturing conditions. Proteins were electro-blotted onto a nitrocellulose membrane. After incubation with antibodies, the membranes were washed, incubated with the appropriate secondary antibody, washed again, and the proteins were detected using ECL Prime Western Blotting Detection kit (GE Healthcare, Piscataway, NJ, USA). The blots were visualized using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA).

**Measurement of caspase-3 and -7 activities.** Caspase-3 and -7 activities were measured using Caspase-Glo 3/7 assay (Promega, Madison, WI, USA). Briefly, 5,000 cells/100 µl of conditioned medium were seeded into each well of 96-well plates and the cells were treated using the conditions described in each figure. Assay reagent (100 µl) was added to each well, and the cells were incubated for 1 h at room temperature (RT). The luminescence signal was measured using a Veritas™ Microplate Luminometer (Promega).

**Transduction of short interfering RNA (siRNA).** siRNAs were transfected into cells by electroporation using Amasra Cell Line Nucleofector Kit V (Lonza, Gaithersburg, MD, USA) and Nucleofector II (Lonza). The programs D-032 and L-024 were used for HCT116 and SW480, respectively, according to the manufacturer's instructions. The sequence 5'-CUACGC GCAUUGCGAAGARAA-3' was used to silence PKCζ. A non-silencing control siRNA (siNSC) was purchased from Applied Biosystems (Foster City, CA, USA).

**Quantification of BIRC5 (survivin) mRNA.** Expression of BIRC5 (survivin) mRNA was determined by quantitative reverse-transcriptase polymerase chain-reaction (RT-PCR) using the ABI PRISM 7700 sequence-detector system (Applied Biosystems). Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions. The cDNA was reverse-transcribed by TaqMan reverse transcription reagents (Applied Biosystems). The gene-specific primers and fluorescent hybridization probes for BIRC5a were as follows: 5'-AAGAACTGCGCTTCTTGGA-3', 5'-CAA CCGGACGAATGCTTTT-3' and 5'- (FAM)CAGATAGCG ACCCCCATAGAGAA (TAMRA)-3'. These were used as forward primer, reverse primer, and the TaqMan probe, respectively. For the internal control, GAPDH was quantified using primers and TaqMan probe purchased from Applied Biosystems.

**Determination of half-life of survivin protein.** Protein synthesis of cells was blocked using 100 µg/ml cycloheximide, incubated for different times as indicated in each figure, and subjected to western blotting using anti-survivin and anti-actin antibodies. The densitometric units of the blots were measured by the software Quantity One (Bio-Rad Laboratories) and survivin expression level was normalized to that of actin.

**Ubiquitination assay.** siPKCζ or siNSC were cotransfected with p3xFLAG-survivin expression vector to cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were treated with 10 µM MG132 (Sigma-Aldrich) for 8 h after the transfection, collected and the whole cell lysates were prepared. Ubiquitinated FLAG-tagged survivin was immunoprecipitated using anti-FLAG antibody and they were subjected to immunoblotting analysis using antibodies against ubiquitin or survivin.

**Immunohistochemical staining of tissue sections.** Colorectal cancer tissues were obtained from patients who underwent surgery at Sapporo Medical University Hospital. Written informed consent was obtained before the acquisition of these tissues. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections. Antigen retrieval was performed by boiling the sections at 120°C for 5 min in a microwave oven in preheated 0.01 M sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in ethanol for 10 min. After blocking with 1% non-fat dry milk in PBS (pH 7.4), the sections were incubated with a polyclonal anti-PKCζ or anti-survivin antibody for 1 h followed by incubation with biotinylated anti-rabbit IgG (Nichirei Bioscience, Tokyo, Japan) for 30 min. Subsequently, the sections were stained with streptavidin-biotin complex (Nichirei Bioscience), followed by incubation with 3,3'-diaminobenzidine used as the chromogen, and counterstaining with hematoxylin.

**Measurement of cell viability.** The CellTiter-Glo Luminescent Cell Viability assay (Promega) was used according to the manufacturer's instructions. Briefly, 1,000 cells were seeded in each 96-well culture plates. Cells were treated with different conditions as described in each figure, after which the assay reagent was added and incubated for 10 min at RT. The luminescent signal was measured using a Veritas Microplate Luminometer.
**Immunofluorescence staining for p65.** Cells were fixed in ice-cold 50% ethanol for 5 min. The samples were incubated with anti-p65 antibody (D14E2, Cell Signaling Technology) for 24 h at 4°C, washed, and incubated with anti-rabbit IgG Alexa Fluor 488 antibody (1/200 dilution) (Invitrogen, Carlsbad, CA, USA) for 1 h at RT. Then, the nuclei were counterstained with Hoechst 33342 in mounting medium and the fluorescence images were obtained using a Leica AF6000 fluorescence imaging system (Nussloch, Germany).

**Statistical analysis.** Statistical analysis of the data was conducted using Microsoft Excel®. Pearson’s correlation coefficient was used to determine the correlation between expression level of PKCζ and survivin in colorectal cancer clinical specimens. Statistical significance was evaluated using the Student’s t-test or χ² test.

**Results**

**PKCζ is anti-apoptotic in colon cancer cells.** To check whether PKCζ is anti-apoptotic in colon cancer cells, the effect of siRNA to PKCζ (siPKCζ) on colon cancer cell apoptosis was tested. The effectiveness of siPKCζ to protein expression was confirmed by immunoblotting (data not shown). Preliminary experiments indicated that TRAIL induced apoptosis in HCT116 and SW480 dose- and time-dependently (data not shown). Sub-G1 analysis (Fig. 1A) and caspase-3/7 assay (Fig. 1B) showed that siPKCζ
Figure 3. PKCζ regulates survivin expression at the transcriptional level. HCT116 and SW480 cells were transfected with siPKCζ or siNSC and levels of BIRC5 (survivin) mRNA was quantified by TaqMan RT-PCR. Level of BIRC5 mRNAs was normalized relative to that of GAPDH transcripts. Error bars denote standard deviation. NS, statistically not significant. *P<0.05. **P<0.01.

Figure 4. Downregulation of PKCζ does not affect the protein half-life of survivin. (A) HCT116 and SW480 cells were transfected with siPKCζ or siNSC, incubated for 24 h, after then half-life of survivin protein was monitored as described in Materials and methods. Left, western blotting; right, densitometric units of western blots represented as line graphs. (B) HCT116 and SW480 cells were transfected with p3xFLAG-survivin and subjected to immunoblotting analysis using anti-survivin antibody. (C) Ubiquitination status of survivin protein was analyzed as described in Materials and methods.
survivin is regulated by PKCζ. To determine the molecular mechanism by which PKCζ inhibits apoptosis in colon cancer cells, expression levels of anti-apoptotic proteins were analyzed in cells transfected with siPKCζ and siNSC. As shown in Fig. 2, among the anti-apoptotic proteins analyzed in this study, survivin was the only protein downregulated by siPKCζ in both HCT116 and SW480 cells. siPKCζ reduced the expression levels of survivin in HCT116 and SW480 cells in a time-dependent manner while overexpression of PKCζ upregulated survivin expression level in HCT116 cells (data not shown). These results show that expression level of survivin is regulated by PKCζ in colon cancer cells.

PKCζ regulates survivin expression at transcriptional level. Generally, survivin is regulated by two different mechanisms: i) regulation at the transcriptional level or ii) by post-translational modifications. First, mRNA level of BIRC5 (survivin) was quantified by TaqMan RT-PCR. As shown in Fig. 3, siPKCζ reduced BIRC5 transcript expression level in HCT116 and SW480 cells. Half-life of survivin protein in cells silenced with siPKCζ (Fig. 4A) or in HCT116 cells overexpressing

Figure 5. Survivin and PKCζ expression levels correlate in colon cancer specimens. (A) Immunostaining for survivin (left) and PKCζ (right) in serial sections obtained from colon cancer tissues. Representative specimens after immunostaining for survivin and PKCζ are shown. Bright field images of immunostained tissues were captured using a digital camera (original magnification, x100). (B) Relationship between immunostaining intensities of survivin and PKCζ in colon cancer specimens. Brightness of immunostaining was analyzed using Photoshop Elements software. Immunostaining intensities of survivin and PKCζ in cancer cells were calculated as a brightness ratio relative to non-neoplastic stromal cells.

Enhanced TRAIL sensitivity both in HCT116 and SW480 cells. Conversely, overexpression of PKCζ conferred TRAIL resistance to HCT116 cells (data not shown). These results show that PKCζ is anti-apoptotic in colon cancer cells.
PKCζ (data not shown) did not differ from that of the control cells. We were unable to find a condition that shows difference in ubiquitination status of survivin in HCT116 cells transduced with siNSC and siPKCζ (Fig. 4B and C). These results indicate that PKCζ regulates survivin expression at the transcriptional level.

Correlation of survivin and PKCζ expression levels in colon cancer. Expression levels of survivin and PKCζ were investigated in colon cancer specimens. Immunohistochemical analysis confirmed that survivin and PKCζ were expressed in 18 samples examined in this study. Generally, expression levels of these proteins were higher in cancer cells than neighboring normal cells (Fig. 5A). Moreover, expression levels of survivin and PKCζ were positively correlated (Fig. 5B, r=0.72, P=3.01x10⁻⁴).

siPKCζ sensitizes colon cancer cells to 5-FU. We next checked whether siPKCζ sensitizes colon cancer cells to 5-FU. SW480 cells were relatively more chemo-resistant than HCT116 cells (Fig. 6A). Transfection of siPKCζ enhanced caspase-3/7 activity in HCT116 and SW480 cells and sensitized HCT116 and SW480 cells to 5-FU (Fig. 6B).

Discussion

In this study, we showed that PKCζ inhibits apoptosis by regulating survivin expression at the transcriptional level in colon cancer cells. Moreover, expression level of PKCζ and survivin correlated in colorectal cancer specimens, and silencing of PKCζ sensitized colon cancer cell lines to 5-FU, one of the key drugs in colon cancer chemotherapy (23).

Previously, the group of Quilet-Mary has shown that PKCζ is a component of DISC and inhibits Fas ligand-induced apoptosis in leukemic cells (24,25). PKCζ is also known to phosphorylate mitochondrial protein Bax to abrogate its pro-apoptotic function (26). PKCζ plays an essential role in the activation of the nuclear factor (NF)-κB cell survival pathway (27). The result obtained in this study, upregulation of survivin, is a novel mechanism by which PKCζ in inhibiting apoptosis.

Survivin is a multifunctional protein that belongs to the inhibitor of apoptosis protein (IAP) family and confers chemo- and radio-resistance to cancer cells (28,29). Survivin inhibits apoptosis by stabilizing and enhancing anti-apoptotic activity of X-linked IAP, and by sequestering pro-apoptotic mitochondrial protein Smac/DIABLO (30-33). Transcription of BIRC5 is regulated by several distinct mechanisms. It
is transcriptionally upregulated by TCF4, STAT3, PML4 and c-REL, and downregulated by p53 (34). ERK and AKT pathways have been shown to cooperate in the translational regulation of survivin (35). Among these transcription factors and pathways, the NF-κB pathway is activated by PKCζ (27,36). Thus, involvement of NF-κB in regulation of BIRC5a by PKCζ was studied. As shown in Fig. 7, siPKCζ reduced the activation of NF-κB. This result suggests that the NF-κB pathway may be involved in survivin regulation by PKCζ.

Proteins are synthesized and degraded equally at steady state. The effect of siRNAs on the level of expression of a protein largely depends on its turnover. Protein with a short half-life elicits dramatic decrease after gene-silencing whereas transient knock-down of a gene presents little impact on the expression level of proteins presenting longer half-life (37). From the results of Fig. 4A and previous studies (38), survivin has a short half-life. siPKCζ reduced the expression level of BIRC5 mRNA to ~70% compared to siNSC in HCT116 and SW480 cells (Fig. 3). However, it decreased survivin protein levels to less than half of that of cells transfected with siNSC (Fig. 2). Results from this gene-silencing experiment indicate that approximately one third of BIRC5 mRNA is dependent on PKCζ and that targeting PKCζ is sufficient to reduce survivin expression levels for sensitization of colon cancer cells to apoptotic stimuli (Figs. 1 and 6).

TRAIL belongs to the TNF superfamily and gathers expectations as an anticancer drug because it kills a wide variety of transformed cells while sparing normal cells in vitro and in vivo (39). Recombinant human TRAIL and agonistic antibodies against its cognate receptors are undergoing clinical trials; they show low toxicity, although, small therapeutic effects have been observed when they are used as a monotherapy (40). Studies in the field are currently focusing on TRAIL-resistance mechanisms and finding TRAIL sensitizers. Results of this study revealed that PKCζ may be a potential therapeutic target to overcome TRAIL-resistance in colon cancer.

The PKC family consists of 10 serine/threonine protein kinases, which are divided into three subfamilies based on their dependency on phospholipids and Ca²⁺. PKC isoforms are regulators of cell life and death. Generally, PKCα, PKCζ, PKCζ and PKCζ/λ are anti-apoptotic; PKCδ is pro-apoptotic (41). Atypical PKCs, PKCζ and PKCζ/λ, share homology with each other and PKCζ/λ is considered as an oncogene (42). This study leaves two unexplained aspects. The first is the determination of the common and distinct functions of PKCζ and PKCζ/λ in the apoptotic pathway. AKT is another anti-apoptotic kinase in the PI3K pathway that is dependent of PI3P. Thus, the second unexplained aspect is the possibility of crosstalk between AKT and PKCζ. Accumulating evidence led us to understand that there are tuned wirings in the apoptotic pathway (43-45). Revealing the relative contributions as well as crosstalk of these anti-apoptotic proteins to the cell death pathway require further studies.

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References