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# PKCδ survival signaling in cells containing an activated p21$^{Ras}$ protein requires PDK1

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ABSTRACT

Protein kinase C δ (PKCδ) modulates cell survival and apoptosis in diverse cellular systems. We recently reported that PKCδ functions as a critical anti-apoptotic signal transducer in cells containing activated p21$^{Ras}$ and results in the activation of AKT, thereby promoting cell survival. How PKCδ is regulated by p21$^{Ras}$, however, remains incompletely understood. In this study, we show that PKCδ, as a transducer of anti-apoptotic signals, is activated by phosphotidylinositol 3′ kinase/phosphoinositide-dependent kinase 1 ($PI_3K$–PDK1) to deliver the survival signal to Akt in the environment of activated p21$^{Ras}$. PDK1 is upregulated in cells containing an activated p21Ras. Knock-down of PDK1, PKCδ, or AKT forces cells containing activated p21$^{Ras}$ to undergo apoptosis. PDK1 regulates PKCδ activity, and constitutive expression of PDK1 increases PKCδ activity in different cell types. Conversely, expression of a kinase-dead (dominant-negative) PDK1 significantly suppresses PKCδ activity. p21$^{Ras}$-mediated survival signaling is therefore regulated by *via* a $PI_3K$–AKT pathway, which is dependent upon both PDK1 and PKCδ, and PDK1 activates and regulates PKCδ to determine the fate of cells containing a mutated, activated p21$^{Ras}$.

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## 1. Introduction

The protein kinase C (PKC) family, comprised of 12 serine-threonine kinase isozymes, is a prominent target for cancer therapeutics [1]. PKC enzymes are functionally linked to cell growth, differentiation, survival, migration and carcinogenesis. As result, they mediate tumor cell proliferation, survival, drug resistance, invasion and metastasis [2–4]. PKCδ, as a member of the "novel" class of PKC enzymes, can be activated by diacylglycerol (DAG) and by other PKCs. Under physiological conditions, PKCδ can also be activated by various growth factors, *via* phospholipase C (PLC). PLC stimulates cells to produce DAG and inositol trisphosphate ($IP_3$) from plasma membrane phospholipids, thence activating conventional and novel PKC family members [5,6]. Conventional PKCs (PKCα and PKCβI/II) predominantly exert anti-apoptotic effects, in part through phosphorylation of the anti-apoptotic protein BCL-2 [7]. In contrast, PKCδ can be pro- or anti-apoptotic, depending upon the cell type or signal received [8]. PKCδ increases the chemotherapeutic sensitivity of a human glioma cell line [9], but promotes survival and resistance to paclitaxel and cisplatin in non-small-cell lung cancers [10]. One recent report suggested that the mechanism through which PKCδ influences cellular proliferation is through regulation of the GL1 protein in Hedgehog signaling [11]. It has alternatively been postulated, in studies using chemical kinase inhibitors, that PKCδ affects the balance of proliferation and apoptosis through regulation of the $PI_3K$–AKT pathway [12].

Phosphoinositide-dependent kinase 1 (PDK1) is a 63 kDa Ser/Thr kinase ubiquitously expressed in human tissues. It consists of an N-terminal kinase domain and a C-terminal pleckstrin-homology (PH) domain. *In vitro*, the PH domain binds PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ with higher affinity than other PIs such as PtdIns(4,5)$P_2$. The activity of PDK1 is up-regulated by binding of these $PI_3K$-generated 3′-phosphorylated phospholipids. AKT (PKB) can be partially-activated by $PI_3K$ directly, through phosphoinositide-dependent protein kinase-1 (PDK1), in certain cells. PDK-1 efficiently phosphorylates AKT at Thr-308, and over-expression of PDK1 is sufficient to partially activate AKT in transfected cells [13]. In unstimulated cells, PDK1 is located in both the cytoplasm and at the inner surface of the plasma membrane [14]. We have previously demonstrated that PKCδ is required for cell survival in both p21$^{Ras}$-transformed cells and in neoplastic cells containing mutated, activated p21$^{Ras}$, and that $PI_3K$ serves as the main downstream effector of the p21$^{Ras}$ oncoprotein to regulate cell survival through PKCδ and ultimately AKT [15]. The functional inter-relationships and relative importance of these signaling kinase components in p21$^{Ras}$-mediated apoptosis and survival, therefore, have not yet been clarified. In this present work, we report that the balance of p21$^{Ras}$-mediated survival signaling is regulated by *via* a $PI_3K$–AKT pathway, which is dependent upon both PDK1 and PKCδ, and that PDK1 activates and regulates PKCδ to determine the fate of cells containing a mutated, activated p21$^{Ras}$.

## 2. Materials and methods

### 2.1. Plasmids and reagents

Cell culture reagents were purchased from GIBCO. The anti-PKCδ, and -PDK1 antibodies were ordered from BD Biosciences (San Jose,

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CA); anti-actin was purchased from Sigma (St. Louis, MO); anti-Akt was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rottlerin was purchased from EMD Chemicals (Gibbstown, NJ). Unless otherwise stated in the text, all other chemicals were purchased from Sigma. PDK1 (wild-type), PDK1-A280V(constitutively-activated), and PDK1-K114G (kinase-dead), were cloned into the pCDNA vector [16]. These vectors were kindly provided by Dr. F. Liu (University of Texas). The pEGFP-PKCδ vector was the generous gift of Dr. M.E. Reyland (University of Colorado).

### 2.2. Cell culture and transfection

Murine fibroblast cell lines NIH/3T3 and Balb were purchased from ATCC. NIH/3T3-Ras and KBalb were produced by stable expression of the v-Harvey Ras and v-Kirsten Ras genes, respectively, in these cell lines. The human pancreatic tumor cell lines MIA PaCa-2 (containing a mutated, activated Kirsten-Ras allele) and BxPc-3 (containing wild-type Ras alleles), and 293T cells were obtained from ATCC. All these cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% donor calf serum and 100 units/ml penicillin/streptomycin. Cells were cultured at 37 °C and 5% $CO_2$. Transfections were performed using Lipofectamine 2000 (InVitrogen Corporation, Carlsbad, CA) without serum, according to the manufacturer's instructions. After 5–8 h, the media were replaced with 10% serum. Transient transfectants were lysed 48 h after transfection, or selected in medium containing G418 (0.5 mg/ml) for generation of stable clones.

### 2.3. siRNA knock-down of PKCδ , PDK1 and Akt

293T cells were co-transfected with lentiviral vector pLVTHM containing one of several specific gene-targeted shRNA sequences, and other four "packaging" plasmids for co-expression of the tat, rev, gag/pol and VSV-G gene products. DNA proportions for transfection were 20:1:1:1:2. pLVTHM vectors containing scrambled shRNA sequences were used as controls. After 48 h of transfection, the supernatant fractions containing viral particles were collected every 12 h (2–3 collections) and filtered with 0.45 μm bottle-top filters. Viral supernatant fractions were concentrated by centrifugation for 3 h at 15,000 ×g at 4 °C in a Beckman SW28 rotor. Pelleted virions were placed on ice for 30 min prior to resuspension with a P200 micropipette and stored at −80 °C. The targeting sequences for PDK1, PKCδ and Akt1 were: 5′-AACTGGCAACCTCCAGAGAAT-3′, CTTTGACCAGGAGTTCCTGAA and GAATGATGGCACCTTCATTGG, respectively. Mlu I and Cla I restriction enzyme recognition sequences were added to the termini of these oligonucleotides when they were to be cloned into pLVTHM.

### 2.4. Assay of PKCδ kinase activity

PKCδ kinase activity was measured with a Kinase Assay kit (Upstate Cell Signaling). Briefly, 48 h after transfection, cells were lysed and PKCδ was immunoprecipitated by a monoclonal PKCδ antibody. The precipitates were incubated with substrate and [γ-$^{33}$P] ATP for 30 min at 30 °C. Incorporation of $^{33}$P into the substrates was subsequently quantified by scintillation counting. Kinase assays of immunoprecipitates generated by an β-actin antibody were used as negative controls in these experiments.

### 2.5. DNA profile analyses

Cells were harvested and resuspended in a 35% ethanol/DMEM solution for 5 min at room temperature, then stained with propidium iodide at 50 μg/ml containing RNase (25 units/ml) and incubated in the dark for 30 min, then subjected to flow cytometric analysis on a FACScan flow cytometer (BD Biosciences, San Jose, CA).

### 2.6. Co-immunoprecipitation assays

Cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA) plus protease inhibitor cocktail (Sigma), and proteins of interest were immunoprecipitated by a 1 h incubation with the respective antibody (2–5 μg/ml), followed by a 30-min incubation with protein A (Sigma). Immune complexes were washed 3 times with Tris-buffered saline and resolved by SDS-PAGE.

### 2.7. Protein stability assays

Cells were incubated with 100 μg cycloheximide/ml for the time periods indicated. Cell extracts were prepared as above. Proteins (100 μg per lane) were analyzed by 10% SDS-PAGE electrophoresis and immunoblotting.

### 2.8. Cell proliferation assays

$5\times10^4$ cells were plated on 6-well plates and infected with lentivirus expressing PDK1-shRNA or with control shRNA. Cells were enumerated by trypan blue staining from the second day to the sixth day post-infection.

### 2.9. Statistical analyses

Results are expressed as mean±S.D. Statistical analysis was performed by using Student's *t*-test, and $p$ values $<0.05$ were considered significant.

## 3. Results

### 3.1. $p21^{Ras}$-activity up-regulates PDK1 expression, and acts through PDK1 to regulate PKCδ expression

We previously demonstrated that $PI_3K$ is the main effector of $p21^{Ras}$ which plays the critical pro-survival role in $p21^{Ras}$-mediated apoptosis, acting *via* PKCδ to activate AKT and thereby protecting the cells from apoptotic stimuli [15]. Because PDK1 is a known downstream effector of $PI_3K$, we sought to determine if PDK1 was involved in this tumor survival signaling process. To investigate a role of PDK1, PDK1 expression was assayed in two human pancreatic tumor cell lines: MIA PaCa-2 (containing a mutated, activated $p21^{Ras}$) and BxPc-3 (containing wild-type $p21^{Ras}$), and two "matched" pairs of murine cell lines: KBalb (Balb cells containing a mutated, activated Kirsten $p21^{Ras}$) and Balb (containing wild-type $p21^{Ras}$), and NIH-Ras (NIH cells containing a mutated, activated Harvey $p21^{Ras}$) and NIH-3T3 cells (containing wild-type $p21^{Ras}$). PDK1 protein expression in MIA PaCa-2 cells (activated $p21^{Ras}$) was 60–80% higher than in BxPc-3 cells (Fig. 1A). Levels of PDK1 were also higher in Balb cell lines and NIH/3T3 cell lines expressing an (activated) v-Ki- or v-Ha-$p21^{Ras}$ (respectively) than in their respective parental counterpart cells containing wt-$p21^{Ras}$ ("paired" NIH cell lines not shown).

Activated $p21^{Ras}$ or constitutive activation of its effector $PI_3K$, elevates the levels of PKCδ in cells as well as PKCδ kinase activity [15]. When PDK1 levels were knocked down with a specific PDK1-siRNA in a lentiviral vector, PKCδ expression was coordinately decreased at least 50% in the MIA PaCa-2 cells (activated $p21^{Ras}$) and the KBalb cells (activated $p21^{Ras}$). In the BxPc-3 and Balb cells, where basal levels of PDK1 were already quite low, no significant effects of PDK1 knockdown on PKCδ levels were detected.

The regulation of PKCδ by PKD1 was not at the level of transcription, as PKCδ transcript levels, assessed by quantitative RT-PCR, did not vary as a function of $p21^{Ras}$ activity (data not shown). To determine if PDK1 affected PKCδ protein stability, the half-life of PKCδ protein was examined in cell lines transfected with either a constitutively active PDK1 (PDK1-A280V) or the empty vector. The

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