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Intracellular zinc increase affects phosphorylation state and subcellular localization of protein kinase C delta (δ)

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ABSTRACT

Protein kinase C delta (PKCδ) is a Ser/Thr-specific kinase involved in many fundamental cellular processes including growth, differentiation and apoptosis. PKCδ is expressed ubiquitously in all known cell types, and can be activated by diacylglycerol, phorbol esters and other kinases. Multiple lines of evidence have indicated that the mode of activation greatly influences the role PKCδ plays in cellular function. Divalent metal ions, such as zinc are released as a response to cellular stress and injury, often resulting in oxidative damage and cell death. In this study, we evaluate the effect increased concentrations of intracellular zinc has on the phosphorylation state and subcellular localization of PKCδ. More specifically, we demonstrate that intracellular zinc inhibits the phosphorylation of PKCδ at Thr^{505} in a concentration-dependent manner and facilitates the translocation of PKCδ from the cytosol to the Golgi complex. Analysis of a PKCδ structural model revealed a potential His-Cys3 zinc-binding domain adjacent to residue Thr^{505} and suggests that interaction with a Zn^{2+} ion may preclude phosphorylation at this site. This study establishes zinc as a potent modulator of PKCδ function and suggests a novel mechanism by which PKCδ is able to "sense" changes in the concentration of intracellular zinc. These findings illuminate a new paradigm of metal ion-protein interaction that may have significant implications on a broad spectrum of cellular processes.

1. Introduction

Protein kinase C delta (PKCδ) is a member of the PKC family of Ser/Thr kinases that are expressed in brain, heart, spleen, lung, liver, ovary, pancreas and adrenal gland [1, 2]. PKCδ activity has been implicated in a wide range of cellular processes including proliferation [3, 4], differentiation [5] and apoptosis [6–8]. Structurally, PKCδ consists of a C-terminal catalytic domain and N-terminal regulatory domain [9, 10]. Together with PKC ε, η and θ, PKCδ is classified as a 'novel kinase', which can be activated by diacylglycerol (DAG) or phorbol esters and is calcium-independent [11]. PKCδ is the most well-studied novel protein kinase, and it has recently been discovered that PKCδ can have both pro- and anti-apoptotic function [6, 12]. Despite these advancements however, a consensus on the functional nature of PKCδ remains to be established. This disparity likely exists because the activity of PKCδ is highly dependent on cell type, phosphorylation state, external stimuli and intracellular localization [10, 13, 14].

The biological functions of proteins within the PKC family are primarily controlled by their subcellular localization [13, 15, 16] and phosphorylation state [17]. Aside from being activated by DAG and phorbol esters, PKCδ can also be activated by other kinases. Regulation of PKCδ function by phosphorylation is quite complex [17]; PKCδ has been shown to be phosphorylated at up to 17 sites, including eight Ser/Thr residues and nine Tyr residues [18]. Several of these sites, such as Ser^{643} and Ser^{662}, remain constitutively phosphorylated and have little known effect of PKCδ function [18]. Conversely, phosphorylation of PKCδ within its C-terminal domain activation loop at Thr^{505} has been associated with increased kinase activity [18]. PKCδ is unique from all the other kinases in the novel kinase subfamily, because it does not require phosphorylation at Thr^{505} for catalytic function [19]. These observations have led to speculation that phosphorylation at Thr^{505} ($pThr^{505}$) is used to fine-tune PKCδ substrate specificity [19, 20]. The complex nature of PKCδ phosphorylation may explain the multifaceted and stimulus-depended functions of PKCδ, making it an interesting target for investigation.

Zinc is the most abundant intracellular trace mineral, being found in the cytosol, organelles and nucleus of all cell types [21]. Zinc is required for the function of hundreds of enzymes [22] and is crucial for

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stabilizing the folded structure of many non-enzymatic proteins [23]. Zinc is unique among transition metals in that it is redox inert in biological systems and has only one valence state: Zn(II) [24]. The electron donors such as oxygen, nitrogen and sulfur limit zinc interactions with proteins [24]. As a cofactor, zinc may remain bound during the lifetime of proteins that rely on it for catalytic function. Other proteins, however, may bind free zinc reversibly with dissociation rates corresponding with functional requirements. These dissimilar binding attributes allow zinc to play highly important roles in cell physiology [25, 26]. Despite its inert redox potential, concentrations of intracellular free zinc are tightly regulated through organelle compartmentalization [27] and binding to metallothioneines [25, 28]. This high level of control is achieved by transmembrane proteins that transport zinc in and out of intracellular compartments. Proteins such as Zrt and Irt-like protein function to transport zinc ions (Zn^{2+}) into the cytosol from outside the cell or from vesicles, whereas proteins such as ZnT control the efflux of Zn^{2+} from the cytoplasm to outside the cell or into vesicles [29]. While zinc is considered an essential element required for normal physiological function, dysregulated zinc homeostasis has been implicated in pathogenesis of human disease. For example, zinc deficiencies can result in oxidative damage to DNA and lead to increased cancer risk [30]. Furthermore, inefficient zinc transport has been linked to the onset of neurodegenerative diseases [31, 32], depression [33, 34] and type II diabetes [35, 36]. Finally, numerous reports have shown that concentrations of intracellular zinc increase during hypoxia [37–40] and that such increases in zinc can precede cell death in neurons [39]. More recently, zinc has emerged as a potent modulator of protein phosphorylation in cells. Indeed, it has been shown that increases in intracellular zinc concentrations can facilitate the phosphorylation of proteins such as tau [41], insulin-like growth factor receptor [42], and insulin-like growth factor-1 [43]. The inhibitory effects of zinc on protein phosphorylation have also been reported [44] however, less is known about how zinc influences this process.

The current study was designed to investigate how increases in intracellular zinc concentrations affect the phosphorylation state and subcellular trafficking of PKCδ. We report here that increasing concentrations of intracellular free zinc significantly inhibits phosphorylation of PKCδ at a key phosphorylation site: Thr^{505}, in a concentration-dependent manner; and influences PKCδ translocation from cytosol to the Golgi complex. We also employed molecular modeling software to gain additional insight into how zinc might affect the structure of PKCδ. These studies show that zinc may bind to a novel His-Cys3 binding pocket that is adjacent to Thr^{505}. We therefore propose a mechanism by which PKCδ is able to 'sense' concentrations of intracellular zinc by coordinating it to a His-Cys3 binding site. Importantly, this binding event repositions His^{197} so that it blocks access to Thr^{505}, effectively inhibiting phosphorylation at this site. This new paradigm enhances our understanding of zinc·PKCδ interactions and implicates zinc as a potent modulator of PKCδ activity.

2. Materials and methods

2.1. Reagents and chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent zinc dye, FluoZin-3, AM was purchased from Life Technologies (Waltham, MA). Microscopy supplies were purchased from VWR (Radnor, PA). HeLa cells and cell culture reagents were purchased from ATCC (Manassas, VA). Immunoblot materials were purchased from Bio-Rad (Hercules, CA) and Invitrogen (Waltham, MA). Antibodies were from Cell Signaling Technology (Danvers, MA) and Abcam (Cambridge, MA).

2.2. Cell culture

HeLa cells (laboratory passage 4–14) were maintained in Eagle's minimum essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS) under a humidified atmosphere (95%) containing 5% CO_2 at 37 °C. Cells were subcultured every other day using standard trypsinization methods, according to ATCC recommendations.

2.3. Detection of intracellular free zinc levels

HeLa cells were seeded at medium density onto glass-bottom Petri dishes (P35G-4.5-14-C; MatTek Corp, Ashland, MA). Following seeding, the cells were allowed to incubate under a humidified atmosphere (95%) containing 5% CO_2 at 37 °C for at least 24 h before experimentation. Prior to treatments, the cells were washed three times with warm physiological buffer (25 mM HEPES, 125 mM NaCl, 3 mM KCl, 1.28 mM $CaCl_2$, 1.1 mM $MgCl_2$ and 5 mM glucose, pH 7.4). For zinc detection, FluoZin-3, AM was added to the cell media at final concentration of 1 μM and cells were incubated for 60 min at room temperature. After incubation with the dye, cells were washed three times with physiological buffer and allowed to "rest" at room temperature for 30 min. To ensure removal of incident extracellular dye, the cells were washed one final time with physiological buffer before observation by fluorescence microscopy. For treatments, either pyrithione alone or a combination of pyrithione and zinc chloride was added to the media and the cells were allowed to incubate for 10 min at 37 °C. Final concentrations of pyrithione and zinc chloride were 10 μM and 50 μM respectively. Images were collected using a Motic AE31 microscope outfitted with a QImaging Retiga 1300i camera with a 40 × /0.75 Olympus objective. Image-Pro Plus 6.2 (Media Cybernetics) was used to collect and analyze the data. Images were collected every 30 s and zinc was added after 60 s of baseline fluorescence was recorded. To detect changes in intracellular fluorescence, regions of interest (ROI) were selected manually by highlighting areas of the cytosol, excluding the nucleus. Background fluorescence was measured from multiple regions of the same images that contained no cells. Changes in fluorescence (ΔF) were determined using Eq. (1):

$$\Delta F = (F_{measure} - F_0)/F_0 \quad (1)$$

where $F_{measure}$ is measured fluorescence and F_0 is average fluorescence at baseline before the application of zinc. Each fluorescent measurement was background subtracted.

2.4. PKCδ subcellular localization (western blot)

HeLa cells were seeded at medium density onto 35 mm plastic Petri dishes in 2 mL EMEM supplemented with 5% FBS. Cells were allowed to attach to the plates under a humidified atmosphere (95%) containing 5% CO_2 at 37 °C for 24 h before treatments. Immediately prior to treatment, the cells were washed three times with 1 mL of physiological buffer. Depending on experimental conditions, washed cells were then switched to physiological buffer supplemented with either 10 μM pyrithione or 10 μM pyrithione and 50 μM zinc chloride and allowed to incubate for 30 min at 37 °C. All pyrithione and zinc chloride solutions were made fresh before the experiments. Following incubation, the cells were washed with cold PBS and harvested by scraping. The cells were then counted and viable cells were identified using Trypan Blue. Cell number and volumes were adjusted to ensure that control and zinc-treated samples had similar numbers of viable cells. Cells were lysed in cold PBS supplemented with protease inhibitor cocktail (Sigma-Aldrich, P2714) by rapidly passing the cells 20 times through a 27-gauge needle. Lysis efficiency was monitored by observing samples under brightfield microscopy; lysis was deemed complete when no whole cells were observed. Cell homogenates were then centrifuged at 1500 rpm (900 × *g*) at 4 °C for 10 min to pellet unbroken cells and nuclei. The resulting supernatant was then spun at 55,000 rpm (100,000 × *g*) for at 4 °C for 60 min using an Optima TLX Ultracentrifuge (Beckman Coulter, Brea, CA). The supernatant, which contained soluble cytosolic proteins, was heated to 95 °C for 5 min in 1 × Laemmli buffer to prepare the samples