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Research paper

Regulation of protein kinase CK2 catalytic activity by protein kinase C and phospholipase D2

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ABSTRACT

We have previously demonstrated that phospholipase D2 (PLD2) overexpression antagonizes protein kinase CK2 (CK2) inhibition-mediated cellular senescence. In the current paper, we show the molecular mechanism of CK2 activation by PLD2 and protein kinase C (PKC). Elevated expression and chemical activation of PLD2 increased the catalytic activity of CK2 and PKC in human colon cancer HCT116 and embryonic kidney HEK293 cells, whereas utilization of PLD2 chemical inhibitors and siRNA suppressed this activity. Inhibition of PKC in these cells suppressed PLD2-induced CK2 activation, suggesting that PLD2 enhances CK2 activity through PKC. Overexpression of the PKC isoforms: PKCα, PKCβ, and PKCζ, but not PKCγ, stimulated CK2 activity in the cells. Importantly, purified conventional PKC (cPKC) and atypical PKC (aPKC) enzymes enhanced CK2 activity and phosphorylated serines 194 and 277 within CK2α as well as serine 148 within CK2β. Furthermore, PKC-mediated phosphorylation of these serines on CK2α was crucial for the stimulation of CK2 activity. Taken together, the present results suggest that PLD2 can stimulate CK2 activity through PKC-mediated CK2α phosphorylation.

1. Introduction

Protein kinase CK2 (CK2) is a ubiquitous serine/threonine kinase that catalyzes the phosphorylation of a large number of cytoplasmic and nuclear proteins. The holoenzyme of CK2 is a heterotetramer, composed of two catalytic (α and/or α′) and two non-catalytic (β) subunits. The β subunit enhances the catalytic activity of the α or α′ subunit and mediates tetramer formation and substrate recognition [1,2]. CK2 expression levels have been shown to be strongly elevated in various types of cancer cells [3–6]. These findings suggest that CK2 plays a critical role in cell growth and proliferation. However, the regulatory mechanism of CK2 activity remains largely unknown.

Phospholipase D (PLD) catalyzes hydrolysis of the membrane phospholipid phosphatidylcholine to produce the lipid second messenger, phosphatidic acid (PA), and choline [7]. PLD has two isoforms, PLD1 and PLD2. It has been demonstrated that PLD activity prevents the apoptosis induced by overexpression of c-Src or treatment with H_2O_2 [8,9]. Joseph et al. have reported that PLD2 overexpression can overcome the cell cycle arrest induced by high-intensity Raf signaling [10]. In addition, enhanced PLD expression has been shown in various human tumor types such as colon, breast, kidney, and gastric cancers [11,12]. Point mutations in *PLD2* have been identified in breast cancer, and PLD2 expression levels were shown to strongly correlate with tumor size and survival in colorectal carcinoma patients [13,14]. Therefore, elevated PLD2 activity contributes to the generation of prosurvival signals that suppress apoptosis and to cell proliferation.

Protein kinase C (PKC) isozymes are serine/threonine kinases that are involved in a variety of cellular processes such as cell proliferation, survival, differentiation, and motility. There are at least 11 PKC isoforms that are classified into three subgroups according to their structure and biochemical properties [15,16]. The classical, or conventional, PKCs (cPKCs; α, βI, βII and γ) are diacylglycerol (DAG) sensitive and Ca^{2+} responsive. The novel PKCs (nPKCs; δ, ε, η and θ) are DAG sensitive but Ca^{2+} insensitive. The

Abbreviations: CK2, protein kinase CK2 (also, CKII); DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; FBS, fetal bovine serum; PA, phosphatidic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PIP2, phosphatidylinositol 4, 5-bisphosphate; PKC, protein kinase C; aPKC, atypical PKC; cPKC, conventional PKC; nPKC, novel PKC; PLD, phospholipase D; PMA, phorbol myristate acetate; PS, phosphatidyl serine; SDA, S194/277A; SDE, S194/277E; SDS, sodium dodecyl sulfate; siRNA, small-interfering RNA; WT, wild-type.

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atypical PKCs (aPKC; ζ and λ/ι) are not regulated by either DAG or Ca^{2+} but are dependent on PA, phosphatidyl serine (PS), and inositol lipids. All PKC isoforms are composed of a C-terminal protein kinase domain and an N-terminal regulatory domain. The distinctiveness of the regulatory domains contributes to the unique cellular function of individual PKC isoforms [17]. Increased activation of specific PKC isozymes has been observed in different cancer types. Thus, PKC isozymes are thought to be good targets for cancer therapy [18]. In this study, we demonstrate novel roles of PLD2 and PKC in CK2 activation, in which PLD2 acts upstream of PKC, which responds by phosphorylating serines 194 and 277 on the CK2α subunit, leading to the stimulation of CK2 activity.

2. Materials and methods

2.1. Materials

Antibodies against CK2α, CK2β, PLD, PKCγ, PKCζ, and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-PKCα and anti-PKCβ1 antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and BD Biosciences (Bedford, MA, USA), respectively. Purified cPKC enzyme, which consists primarily of α, β, and γ isoforms, and PKC peptide substrate (AAKIQASFRGHMARKK) were obtained from Promega (Madison, WI, USA). Purified PKCζ and PKCγ enzymes were from Invitrogen (Carlsbad, CA, USA). Gö6983 was purchased from Calbiochem (Darmstadt, Germany). The CK2 peptide substrate (RRREEETEEE) was synthesized using an automatic synthesizer (Model 431A, Applied Biosystems, Inc., Foster City, CA, USA) and purified by reverse phase chromatography. [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ, USA). The adenovirus dominant-negative (Ad-DN)-PKCζ, which was generated by mutating the ATP binding site (K281M) from human PKC, was a gift from Dr. C. H. Woo (Youngnam University, Korea). Human CK2α and CK2β subunits were expressed in *Escherichia coli* and purified as previously described [19].

2.2. Cell culture and preparation of cell extract

HCT116 human colon cancer cells and HEK293 human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) under a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. For preparation of cell extract, cells were lysed by sonication in lysis buffer [50 mM Tris–HCl (pH 7.5), 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1% Nonidet P-40, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and 4 mM p-nitrophenyl phosphate]. The particulate debris was removed by centrifugation at 12,000 × *g* for 15 min. The volumes of the supernatants were adjusted to achieve equal protein concentration.

2.3. Transfection and infection studies

The PLD2 siRNA used in our study was 5′-AAGAGGUGGCUGGUGGUGAAGdTdT-3′. Negative control siRNA was 5′-GCUCAGAUCAAUACGGAGAdTdT-3′. Cells were transfected with siRNA, pcDNA-PLD2, -PKC, or -CK2α by Lipofectamine (Invitrogen), according to the manufacturer's instructions. Five hours after transfection, the medium was changed and the cells were grown for another two days before being harvested. Adenovirus containing dominant negative PKCζ (Ad-DN-PKCζ) and control Ad-LacZ cDNA was kindly provided by Dr. Woo (Youngnam University, Korea) and was used for infection of cells as described previously [20].

2.4. Western blotting

Western blotting was performed as described previously [21].

2.5. Assay for PLD activity

PLD activity was evaluated using a PLD activity colorimetric assay kit (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's instructions. In this assay, PLD cleaves choline from phosphatidylcholine. The free choline is then oxidized to yield an intermediate product that reacts with PLD probes to produce color [optical density (OD): 570 nm].

2.6. CK2 and PKC activity assays

The standard assay for phosphotransferase activity of CK2 was conducted in a reaction mixture containing 20 mM Tris–HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP in the presence of 1 mM synthetic peptide substrate (RRREEETEEE) to a total volume of 30 μl at 30 °C. Cell lysates (30 μg of protein) were added to initiate the reactions and were incubated for 15 min. For the PKC activity assay, cell lysates (30 μg of protein) were incubated with a kinase buffer [20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.25 mM EGTA, 0.4 mM $CaCl_2$, 100 μg/ml phosphatidyl serine] containing 50 μM PKC peptide substrate (AAKIQASFRGHMARKK) and 100 μM [γ-^{32}P]ATP in a total volume of 30 μl at 30 °C for 5 min. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10%. The mixture was then centrifuged, and 10 μl of supernatant was applied to P-81 phosphocellulose filter paper. The paper was washed in 100 mM phosphoric acid, and then radioactivity was measured by scintillation counting. To analyze the CK2 activity upon dephosphorylated β-casein, the reaction mixtures were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The gels were dried and autoradiographed.

2.7. Phosphoamino acid analysis

CK2 phosphorylated by PKC was separated by SDS-polyacrylamide gel electrophoresis. After staining of the gel with Coomassie blue, CK2α and CK2β protein bands were excised and the proteins were extracted in 50 mM NH_4HCO_3 (pH 7.4), including 0.5% β-mercaptoethanol and 0.1% SDS. The proteins were precipitated in 15% trichloroacetic acid with 20 μg of bovine serum albumin as carrier, rinsed in 100% ethanol, and hydrolyzed in 6 N HCl for 1 h at 110 °C. The hydrolysate was lyophilized and resuspended in 7 μl of pH 1.9 buffer (7.8% acetic acid and 2.2% formic acid) including phosphoamino acid standards. The samples were spotted on cellulose thin-layer plates, and electrophoresis was achieved using pH 1.9 buffer. Standards were visualized with ninhydrin and ^{32}P-labeled phosphoamino acids were uncovered by autoradiography.

2.8. Site-directed mutagenesis

Point mutations in CK2 α and β subunits were induced by using the QuickChange site-directed mutagenesis kit (Stratagene, LaJolla, CA, USA), according to the manufacturer instructions. The primers to identify putative PKC phosphorylation sites were as follows: CK2α^{S194A} (5′-GTCCGAGTTGCTGCCCGATACTTCAAAGG-3′); CK2α^{S194E} (5′-GTCCGAGTTGCTGAGCGATACTTCAAAGG-3′); CK2α^{S277A} (5′-CTTGGGCAGACACGCTCGAAAGCGATGG-3′); CK2α^{S277E} (5′- CTTGGGCAGACACGCTCGAAAGCGATGG-3′); CK2β^{S148A} (5′- CACACCCAAGGCATCAAGACACC-3′); CK2β^{S148E} (5′-CACACCCAAGGAATCAAGACACC-3′); and CK2β^{S205A} (5′-CCAAGCCGCCGCCAACTTCAAGA-3′). All of the mutants were confirmed by nucleotide sequencing.