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Molecules and Cells

Over-Expression of Phospholipase D Isozymes Down-Regulates Protein Kinase CKII Activity via Proteasome-Dependent CKIIβ Degradation in NIH3T3 Cells

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Over-expression of phospholipase D (PLD) 1 or PLD2 down-regulated CKII activity in NIH3T3 cells. The same results were found with catalytically inactive mutants of PLD isozymes, indicating that the catalytic activity of PLD is not required for PLD-mediated CKII inhibition. Consistent with this, 1-butanol did not alter CKII activity. The reduction in CKII activity in PLD-over-expressing NIH3T3 cells was due to reduced protein level, but not mRNA level, of the CKIIβ subunit. This PLD-induced CKIIβ degradation was mediated by ubiquitin-proteasome machinery, but MAP kinase and mTOR were not involved in CKIIβ degradation. PLD isozymes interacted with the CKIIβ subunit. Immunocytochemical staining revealed that PLD and CKIIβ colocalize in the cytoplasm of NIH3T3 cells, especially in the perinuclear region. PLD binding to CKIIβ inhibited CKIIβ autophosphorylation, which is known to be important for CKIIβ stability. In summary, the current data indicate that PLD isozymes can down-regulate CKII activity through the acceleration of CKIIβ degradation by ubiquitin-proteasome machinery.

INTRODUCTION

CKII is a ubiquitous serine/threonine kinase that is found in all eukaryotes so far examined and in various subcellular compartments (Issinger, 1993; Litchfield, 2003; Pinna, 1990). CKII catalyzes the phosphorylation of many cytoplasmic and nuclear proteins, including DNA-binding proteins, nuclear oncoproteins, and transcription factors (Issinger, 1993; Litchfield, 2003; Pinna, 1990). The holoenzyme of CKII is a heterotetramer, composed of two catalytic (α and/ or α′) and two regulatory (β) subunits. The α and α′ subunits are different gene products and they exhibit the catalytic activity of the enzyme. The β subunit is thought to be a regulatory subunit that stimulates the catalytic activity of the α or α′ subunits and it also mediates tetramer formation and substrate recognition (Gietz et al., 1995; Jakobi et al., 1992; Lin et al., 1991). The over-expression of the CKII catalytic subunit leads to tumorigenesis in mice that over-express myc (Seldin and Leder, 1995). Analysis using temperature-sensitive yeast mutants for the CKII gene has shown that CKII is required for cell cycle progression in both G_1 and G_2/M phases (Hanna et al., 1995). In addition, recent observations in which CKII phosphorylates procaspase-2 or caspase substrates have shown that CKII prevents apoptosis (Desagher et al., 2001; Krippner-Heidenreich et al., 2001; Shin et al., 2005). These findings suggest that CKII plays a critical role, not only in cell growth and proliferation, but also in anti-apoptosis. CKII is a second messenger independent, and the crystal structure of the α catalytic subunit has confirmed the enzyme to be constitutively active (Niefind et al., 1998). Because of its constitutive nature, it is likely that one or more potent mechanisms can inhibit the CKII when required.

Recently, it has been reported that CKII interacts with and phosphorylates the enzyme phospholipase D (PLD) (Ganley et al., 2001), which catalyzes the hydrolysis of phosphatidylcholine, the major membrane phospholipid, to form phosphatidic acid and choline. Phosphatidic acid functions as an effector in multiple physiological processes. PLD activity is thought to be involved in a broad range of physiological responses, including rapid responses such as secretion and superoxide generation, as well as long-term responses such as proliferation, differentiation, and immune response (Exton, 1997; 1999; Liscovitch et al., 2000). To date, two PLD isoforms have been characterized (Colley et al., 1997; Hammond et al., 1995; 1997). PLD1 has low basal activity and is up-regulated by small G-proteins (ARF, Rho, and Ral), classical protein kinase C (PKC) isoforms such as PKCα and β, and phosphatidylinositol 4,5-bisphosphate (PIP2) *in vitro*. In contrast, PLD2 has high basal activity, requires PIP2, and is up-regulated by ARF and PKC.

In the present study, we investigated the physiological significance of the interaction between CKII and PLD isozymes. Our results indicate that CKII activity is down-regulated in NIH3T3 cells that over-express PLD1 or PLD2. PLD is involved in the regulation of CKII activity through the proteasome-dependent

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degradation of CKIIβ. The current study demonstrates one possible inhibition mechanism for CKII.

MATERIALS AND METHODS

Antibodies

Polyclonal anti-CKIIα and monoclonal anti-CKIIβ antibodies were obtained from Calbiochem (Germany). Polyclonal anti-PLD antibody was raised against the C-terminal peptide of PLD as described elsewhere (Min et al., 2001). Polyclonal anti-ubiquitin, anti-β-actin, and anti-tubulin antibodies were obtained from Santa Cruz Biotechnology (USA), and the anti-hemagglutinin (HA) antibody was from Roche (Switzerland). Rhodamine-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG were from Invitrogen (USA).

Cell culture and the establishment of stable cell lines

NIH3T3 cells were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. To establish stable cell lines that over-express PDL1 or PDL2, cells were transfected with the pcDNA3.1-PLD constructs or the vector control by Lipofectamine (Bethesda Research Laboratories, USA), as described by the manufacturer. One day later, the cells were cultured in the presence of 1 mg/ml G418. After 2 weeks, the cells were grown in the same medium in the presence of 100 μg/ml G418 and were examined for protein expression by Western blotting.

To use as controls, other NIH3T3 cells were transfected with plasmids encoding catalytically inactive mutants of PLD1 (K898R) and PLD2 (K758R). When over-expressed, these mutant isoforms were shown to confer a dominant inhibitory effect on cellular PLD activity (Shen et al., 2001).

The preparation of mammalian cell extract

For Western blotting, NIH3T3 cells in 100-mm dishes were washed with an ice-cold phosphate-buffered saline (PBS), collected by scraping with a rubber policeman, and lysed in a 100 μl of ice-cold RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin]. To measure kinase activity, cells were lysed by sonication in lysis buffer [50 mM Tris-HCl (pH 8.0), 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*. The volumes of the supernatants were adjusted for equal protein concentration.

Assay for CKII activity

A standard assay for the phosphotransferase activity of CKII 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) in a total volume of 30 μl at 30°C. The reactions were started by the addition of cell lysates and incubated for 15 min. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10%. The sample was then centrifuged, and 10 μl of the supernatant was then applied to P-81 paper. The paper was washed in 100 mM phosphoric acid and radioactivity was measured by scintillation counting.

PLD activity assay

PLD activity was assessed by measuring the formation of [^{3}H]-phosphatidylbutanol (PtdBut), the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol. NIH3T3 cells grown in six-well plates were serum starved overnight in the presence of 3 μCi/ml [^{3}H]-myristic acid. The next day, the cells were washed three times with 5 ml of PBS and pre-equilibrated in a serum-free DMEM for 1 h. The cells were incubated with 0.3% butan-1-ol for 30 min. The lipids were extracted and characterized by thin-layer chromatography as previously described (Kim et al., 2004).

Western blotting

Proteins were separated on polyacrylamide gels in the presence of SDS, and transferred electrophoretically to nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBST [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20] for 2 h and then incubated with specific antibodies. The membrane was washed three times in TBST, and then treated with an enhanced chemiluminescence (ECL) system (Amersham Biosciences, USA). Some membranes were stripped in stripping buffer [50 mM Tris-HCl (pH 7.0), 2% SDS, 100 mM β-mercaptoethanol] at 50°C for 1 h with gentle shaking and then reprobed with anti-β-actin or anti-tubulin antibodies as a control for protein loading.

Preparation of mRNA

We extracted total RNA from the NIH3T3 cells and then reverse-transcribed CKII mRNA and performed PCR using specific primers for the human CKIIα and CKIIβ. The level of β-actin mRNA was measured as a control.

Purification of CKII

Human CKII tagged with maltose-binding protein (MBP) was expressed in *E. coli* and purified as described previously (Kim et al., 2005).

MBP pulldown assay and immunoprecipitation

An MBP pulldown assay was performed by incubating amylose-agarose beads with MBP-CKIIβ and cell lysates that were transfected with the pcDNA3.1-PLD1 or pcDNA3.1-PLD2 in a 200 μl of binding buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF]. The reaction was allowed to proceed for 1 h while rocking at 4°C. For immunoprecipitation, cell lysates were pre-cleared with normal mouse or rabbit IgG and protein A sepharose (Amersham Biosciences) for 1 h at 4°C. The supernatant was then incubated with anti-CKIIβ or anti-PLD antibodies and protein A sepharose with mixing for 12 h at 4°C. Then, the beads were collected by centrifugation and washed three times with PBS.

Phosphorylation assay

Immunoprecipitated proteins were washed three times with PBS and incubated with 200 ng of MBP-CKIIβ for 2 h at 4°C. Phosphorylation reactions were conducted for 5 min at 30°C in 30 μl reaction mixture containing 100 ng MBP-CKIIα and [γ-^{32}P]-GTP and then terminated by the addition of 5× SDS loading buffer. Proteins were separated on a 10% (w/v) SDS-polyacrylamide gel and visualized by Coomassie blue staining and autoradiography.

Immunocytochemical staining

NIH3T3 cells were seeded on four-well microchamber slides (Nunc, USA) and transfected the next day with pcDNA3.0-HA-CKIIβ and control vector pcDNA3.1, pcDNA3.0-HA-CKIIβ and pcDNA3.1-PLD1, or pcDNA3.0-HA-CKIIβ and pcDNA3.1-PLD2. Forty-eight hours after transfection, the cells were fixed with 4%

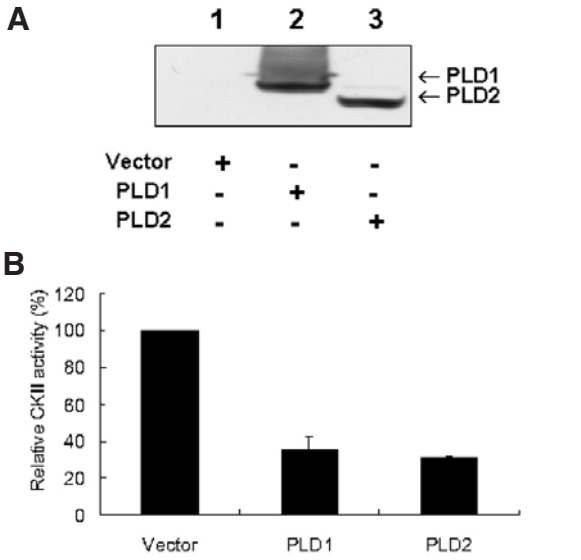

Fig. 1. The overexpression of PLD isozymes inhibits CKII activity in NIH3T3 cells. (A) Protein extracts from NIH3T3 cells stably expressing the control vector (lane 1), pcDNA3.1-PLD1 (lane 2), or pcDNA3.1-PLD2 (lane 3) were electrophoresed on a 12% (w/v) SDS-polyacrylamide gel, and visualized by Western blotting with anti-PLD antibody. (B) CKII kinase activity was measured in lysates from NIH3T3 cells stably expressing PLD1 or PLD2 using the specific CKII substrate peptide RRREEE-TEEE. The ^{32}P incorporation in the substrate peptide was measured by scintillation counting. Bars and error bars represent the relative activity of CKII and the range of duplicate experiments, respectively.

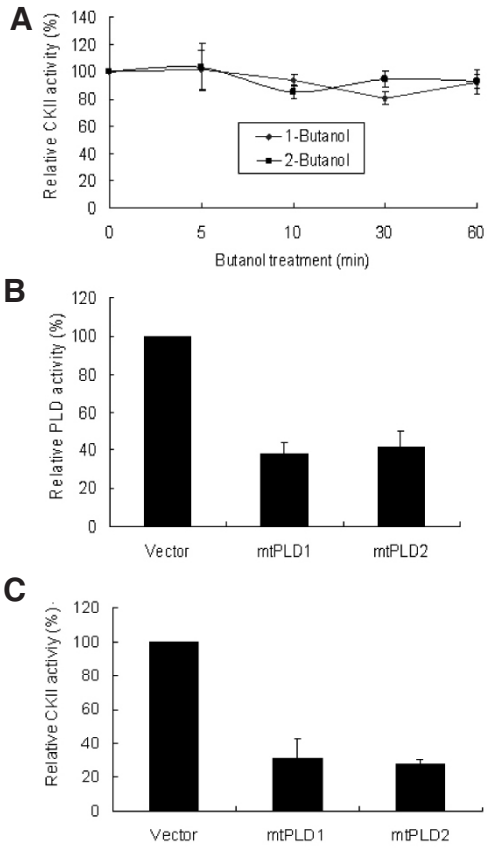

Fig. 2. PLD activity is not required for the inhibition of CKII by PLD. (A) NIH3T3 cells were treated with 0.5% 1-butanol or 2-butanol for 10 to 60 min prior to harvest. CKII activity was measured in the lysates from these cells using the specific CKII substrate peptide. The ^{32}P incorporation in the substrate peptide was measured by scintillation counting. (B) NIH3T3 cells that over-expressed PLD dominant negative mutants (mtPLD1 and mtPLD2) or vector were cultured in six-well plates and labeled with 3 μCi/ml [^{3}H]-myristic acid. After being washed, the cells were preincubated with DMEM, 0.1% BSA, and 0.3% 1-butanol for 30 min. The radioactivity incorporated into phosphatidylbutanol was measured as described in "Materials and Methods". (C) CKII kinase activity was measured in lysates from NIH3T3 cells that over-expressed PLD dominant negative mutants using the specific CKII substrate peptide. The ^{32}P incorporation in the substrate peptide was measured by scintillation counting. Data are expressed as the mean of the means ± S.D. of three independent experiments.

paraformaldehyde in PBS for 10 min at room temperature and permeabilized in 0.25% Triton X-100 before being blocked with 2% bovine serum albumin in PBS. Primary antibodies (anti-HA antibodies at a dilution of 1:100, anti-PLD antibodies at 1:200) were added at room temperature for 1 h. The secondary antibodies were rhodamine-conjugated goat anti-rabbit IgG (1:200) and FITC-conjugated donkey anti-mouse IgG (1:200). The 4′,6-Diamidino-2-phenylindole (DAPI; Molecular Probes, USA) was used to counterstain nuclei, and fluorescence signals were detected using a Carl Zeiss Axioplan 2 microscope (Moon et al., 2007).

RESULTS

Over-expression of PLD isozymes inhibits CKII activity in NIH3T3 cells

To determine whether PLD isozymes regulate CKII activity, normal mouse fibroblast NIH3T3 cells were transfected with plasmids encoding PLD1 or PLD2. The empty vector (pcDNA3.1) was used as a control. After selection for G418 resistance, the over-expression of PLD1 and PLD2 was examined by immunoblotting with an anti-PLD antibody. As shown in Fig. 1A, the overexpression of PLD1 and PLD2 was detectable in NIH3T3 cells that were transfected with pcDNA3.1-PLD1 and pcDNA3.1-PLD2, respectively. We examined the CKII activity in these cell extracts by using the synthetic peptide substrate RRREEETEEE. The extract from NIH3T3 cells that were transfected with pcDNA3.1-PLD contained less than 40% of the CKII activity detected in cells transfected with the empty vector (Fig. 1B). These results indicate that the overexpression of either PLD1 or PLD2 results in a reduction of CKII activity in NIH3T3 cells, suggesting that both PLD1 and PLD2 are involved in downregulating CKII activity.

PLD activity is not required for the PLD-induced inhibition of CKII activity

Next we explored whether the catalytic activity of PLD is required for CKII inhibition. First, NIH3T3 cells were treated with 0.5% 1-butanol, an inhibitor of phosphatidic acid production by PLD, for 5 to 60 min prior to harvest. A secondary alcohol, 2-butanol, was used as a control. CKII activity was not altered in cells treated with 1-butanol or 2-butanol, indicating that PLD activity was not required for the PLD-mediated inhibition of CKII activity (Fig. 2A). To confirm that PLD activity was not linked to CKII inhibition, the catalytically inactive mutants of PLD1

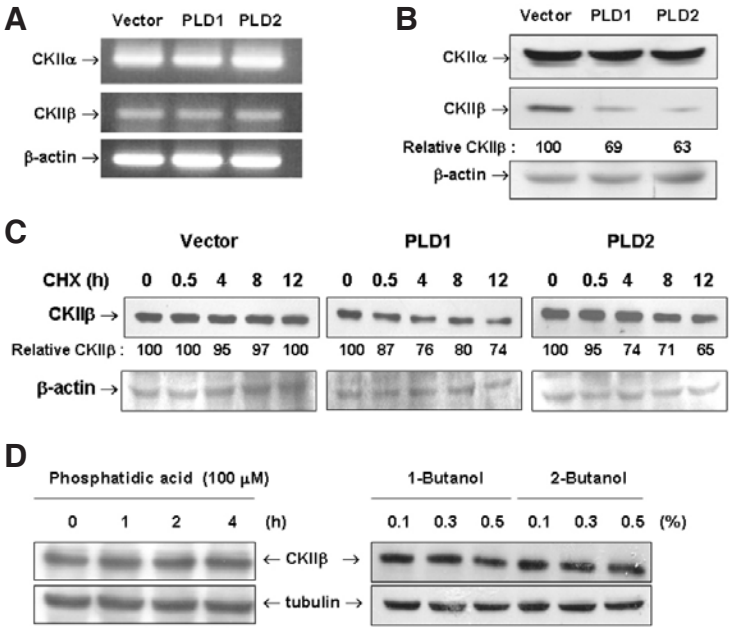

Fig. 3. The overexpression of PLD induces CKIIβ protein degradation. (A) Total RNA was extracted from NIH3T3 cells stably expressing the control vector (lane 1), pcDNA3.1-PLD1 (lane 2), or pcDNA3.1-PLD2 (lane 3) and reverse transcribed using CKIIα- or CKIIβ-specific primers and reverse transcriptase. Primers to β-actin RNA were used as a control. The levels of CKIIα and CKIIβ were unchanged relative to expression in vector-transfected cells. (B) Protein extracts from NIH3T3 cells stably expressing PLD1 or PLD2 were electrophoresed on a 12% (w/v) SDS-polyacrylamide gel and visualized by Western blotting with anti-CKIIα, -CKIIβ, or -β-actin antibodies. Only CKIIβ protein was diminished in the PLD-over-expressing cells. (C) NIH3T3 cells stably expressing the control vector (left panel), pcDNA3.1-PLD1 (middle panel), or pcDNA3.1-PLD2 (right panel) were treated with cycloheximide (CHX) for the indicated times. Protein extracts from the cells were then electrophoresed on a 12% (w/v) SDS-polyacrylamide gel, and visualized by Western blotting with anti-CKIIβ or anti-β-actin antibodies. (D) NIH3T3 cells were treated with 100 μM phosphatidic acid for up to 4 h (left panel) or with 0.1 to 0.5% of 1- or 2-butanol for 20 h (right panel). Protein extracts from the electrophoresed on a 12% (w/v) SDS-polyacrylamide gel, and visualized by Western blotting with anti-CKIIβ or anti-tubulin antibodies.

(K898R) and PLD2 (K758R) were tested for their effect on CKII inhibition. As expected, PLD activity was reduced in these cell extracts (Fig. 2B). However, the CKII activity was also down-regulated in the cell lysates to a degree similar to that seen in the presence of normal PLD activity (Fig. 2C). Furthermore, the inhibition rates of CKII activity in the cells that over-expressed wild-type PLD isozymes were unaffected by treatment with 1-butanol (data not shown). Taken together, these results indicate that the catalytic activity of PLD is not required for the PLD-mediated inhibition of CKII in NIH3T3 cells.

The over-expression of PLD isozymes reduces the protein level of CKIIβ

Next we investigated whether a reduction in CKII activity in PLD-over-expressing cells was due to reduced levels of CKII mRNA or protein. The levels of CKIIα and CKIIβ mRNA in the cells that over-expressed PLD isozymes were unchanged compared to control cells (Fig. 3A). The level of CKIIα protein also was unchanged in the PLD-over-expressing cells, but the protein level of CKIIβ was significantly decreased (Fig. 3B).

To examine whether the over-expression of PLD would promote the destabilization of CKIIβ, PLD1- or PLD2-over-expressing NIH3T3 cells were cultured with 50 μg/ml of the protein synthesis inhibitor cycloheximide. After the addition of cycloheximide, the level of CKIIβ protein was measured at the time points indicated in Fig. 3C. In the cells transfected with the control vector, the levels of CKIIβ protein were unchanged for up to 12 h. However, in the cells that over-expressed PLD1 or PLD2, the expression of CKIIβ protein diminished rapidly after treatment with cycloheximide. Quantification by densitometry revealed that a 12-h treatment with cycloheximide reduced the protein level of CKIIβ by approximately 30% in cells that over-expressed PLD isozymes. The level of β-actin did not change. Thus, these results indicate that the overexpression of PLD isozymes has a significant destabilizing effect on CKIIβ protein.

To investigate whether the catalytic activity of PLD is required for CKIIβ degradation, NIH3T3 cells were treated with 100 μM phosphatidic acid for 1 to 4 h prior to harvest. Western blot analysis revealed that the level of CKIIβ protein was not altered by treatment with phosphatidic acid. Similar results were also obtained by treatment with 1-butanol for 20 h, indicating that the catalytic activity of PLD isozymes is not required for the PLD-mediated degradation of the CKIIβ protein (Fig. 3D).

The over-expression of PLD induces CKIIβ degradation by ubiquitin-proteasome machinery

To investigate the mechanism by which PLD over-expression leads to CKIIβ degradation, PLD-over-expressing NIH3T3 cells were treated with the proteasome inhibitor lactacystin; then the protein level of CKIIβ in cell extracts was analyzed by Western blot. The PLD-induced decrease in CKIIβ protein was almost completely reversed by treatment with proteasome inhibitor when compared with the control cells (Fig. 4A). This finding strongly suggests that the PLD-induced degradation of CKIIβ is mediated by proteasome machinery. Next, we investigated whether the proteasomal degradation of CKIIβ was accompanied by ubiquitination. PLD-over-expressing NIH3T3 cells were treated with or without the proteasome inhibitor MG132; then the CKIIβ protein was immunoprecipitated from these cell extracts. When the immunoprecipitated CKIIβ was probed for ubiquitin with anti-ubiquitin antibody, poly-ubiquitinated CKIIβ accumulated only when the cells were treated with MG132. In addition, the poly-ubiquitinated forms of CKIIβ increased more significantly in the PLD-over-expressing NIH3T3 cells, as compared with the control cells. Similar results were obtained from an immuno-blot reprobed with anti-CKIIβ antibody (Fig. 4B). Taken together, these observations indicate that the effect of PLD over-expression on CKIIβ reduction is mainly due to an increase in CKIIβ ubiquitination and proteosomal degradation.

It has been reported that the elevated expression of PLD1 accelerates the degradation of p53 and that this degradation is dependent on mitogen-activated protein (MAP) kinase and

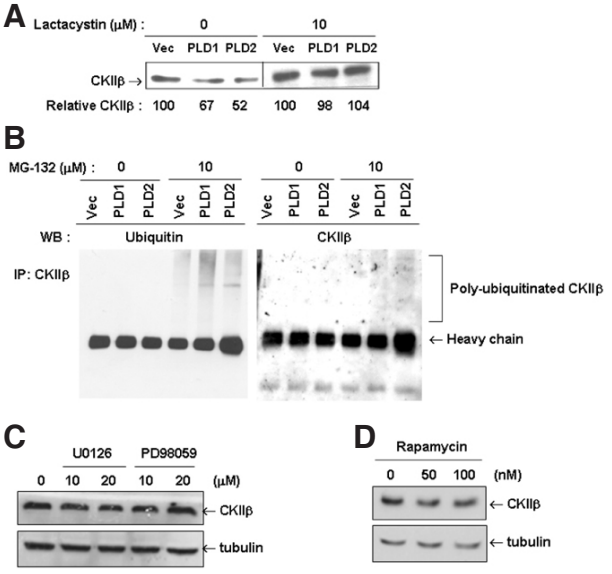

Fig. 4. Ubiquitin-proteasome machinery, but not MAP kinase or mTOR, is involved in PLD-induced CKII degradation. (A) NIH3T3 cells stably expressing the control vector, pcDNA3.1-PLD1, or pcDNA3.1-PLD2 were grown in the presence or absence of 10 μM lactacystin for 24 h. Protein extracts from the cells were electrophoresed on a 12% (w/v) SDS-polyacrylamide gel and visualized by Western blotting with anti-CKIIβ antibody. (B) NIH3T3 cells stably expressing the control vector, pcDNA3.1-PLD1, or pcDNA3.1-PLD2 were grown in the presence or absence of 10 μM MG-132 for 24 h. Cell lysate was immunoprecipitated (IP) with anti-CKIIβ antibody followed by Western blotting (WB) with anti-ubiquitin (left panel) or anti-CKIIβ (right panel) antibodies. (C, D) NIH3T3 cells stably expressing PLD isozymes were treated with U0126 or PD980590 (0 to 20 μM) for 20 h (C) or rapamycin (0 to 100 nM) for 20 h (D). Protein extracts from the cells were electrophoresed on a 12% (w/v) SDS-polyacrylamide gel and visualized by Western blotting with anti-CKIIβ or anti-tubulin antibodies.

mammalian target of rapamycin (mTOR) (Hui et al., 2004). MEK is the kinase that phosphorylates MAP kinase. Therefore, we first investigated the effect of MEK inhibitors (U0126 and PD98059) and the mTOR inhibitor (rapamycin) on CKIIβ degradation in NIH3T3 cells. As shown in Figs. 4C and 4D, U0126, PD98059, and rapamycin all failed to affect the levels of CKIIβ protein in NIH3T3 cells. These results indicate that neither MAP kinase nor mTOR are involved in PLD-induced CKIIβ degradation.

CKIIβ interacts directly with PLD1 and PLD2

To determine whether CKII is associated with both PLD1 and PLD2 in NIH3T3 cells, PLD isozymes were immunoprecipitated from NIH3T3 cell extracts, and the immunoprecipitates were blotted and probed with anti-PLD, anti-CKIIα, or anti-CKIIβ antibodies. Normal IgG immunoprecipitation was used as a control. As shown in Fig. 5A, CKIIα and CKIIβ each co-precipitated with PLD isozymes, indicating that both PLD isozymes interact with CKII in NIH3T3cells. Next, to examine whether PLD isozymes interact with the CKIIβ subunit, MBP-CKIIβ was purified and tested for its ability to form a complex with PLD. Purified MBP-CKIIβ was mixed with extracts from NIH3T3 cells that were stably transfected with pcDNA3.1-PLD1 or pcDNA3.1-PLD2, and the complexes of PLD and MBP-CKIIβ were precipitated with amylose beads. Western blots of the co-precipitates

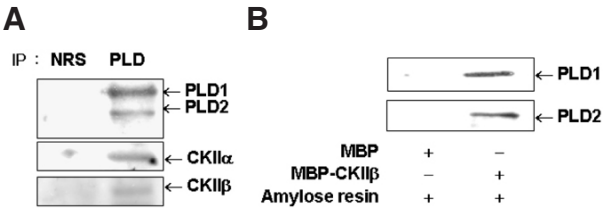

Fig. 5. PLD interacts directly with CKIIβ. (A) Cell lysates from NIH3T3 cells were subjected to immunoprecipitation (IP) with either anti-PLD antibody or normal rabbit serum (NRS). The precipitated proteins were separated by 12% (w/v) SDS-polyacrylamide gel electrophoresis and visualized by Western blotting with anti-PLD, anti-CKIIα, and anti-CKIIβ antibodies. (B) PLD isozymes were precipitated from the cell lysates with amylose resin coated with MBP-CKIIβ. Precipitation with amylose resin coated with MBP was used as a negative control. The immobilized complexes were recovered by an elution step using 20 mM maltose. The proteins were further analyzed by Western blotting with anti-PLD antibody.

probed with anti-PLD antibody revealed that both PLD1 and PLD2 interact with CKIIβ (Fig. 5B).

The colocalization of CKIIβ and PLD isozymes in NIH3T3 cells

The finding that CKIIβ interacts with PLD in NIH3T3 cell implies that these two proteins might colocalize to the same cell region. We investigated the intracellular localization of CKIIβ relative to PLD isozymes using immunocytochemical analysis. In the NIH3T3 cells transfected with pcDNA-HA-CKIIβ and the control vector, the CKIIβ expression was detected in both the cytoplasm and the nucleus, but the endogeneous PLD was hardly detected (Fig. 6A). In the cells cotransfected with pcDNA-HA-CKIIβ and pcDNA3.1-PLD1, PLD1 was present primarily in the cytosol, and the subcellular distribution of CKIIβ remained essentially unchanged (Fig. 6B). In the cells that co-expressed HA-CKIIβ and PLD2, however, the subcellular localization of CKIIβ was significantly altered, and CKIIβ and PLD2 were prominently localized in the perinuclear region (Fig. 6C). Differences in localization between PLD1 and PLD2 have been previously documented (Freyberg et al., 2002; Liscovitch et al., 2000), suggesting that these enzymes have separate functions in the cells.

PLD binding inhibits the autophosphorylation of CKIIβ

CKIIβ has three autophosphorylation sites: Ser2, Ser3, and Ser4 (Issinger, 1993; Litchfield, 1993; Pinna, 1990). Because phosphorylation of the autophosphorylation sites on CKIIβ has been reported to be a signal that stabilizes the protein (Zhang et al., 2002), we examined whether PLD binding to CKIIβ inhibits CKIIβ phosphorylation by the phosphotransferase activity of CKIIα. PLD isozymes were immunoprecipitated from NIH3T3 cell extracts that had been transfected with either pcDNA3.1-PLD1 or pcDNA3.1-PLD2 and pre-incubated with CKIIβ; then a phosphorylation reaction was carried out in the presence of MBP-CKIIα and [γ-^{32}P]-GTP. Unlike other protein kinases, CKII can utilize GTP instead of ATP as the phosphate donor (Hathaway and Traugh, 1979). Normal rabbit IgG was used for the control immunoprecipitation. As shown in Fig. 7, PLD preincubation with CKIIβ reduced the autophosphorylation level of CKIIβ when compared with the control immunoprecipitation. These results indicate that PLD binding to CKIIβ inhibits CKIIβ autophosphorylation. However, one cannot exclude the possibility that the autophosphorylation is reduced by any other protein(s) co-immunoprecipitated with PLD.

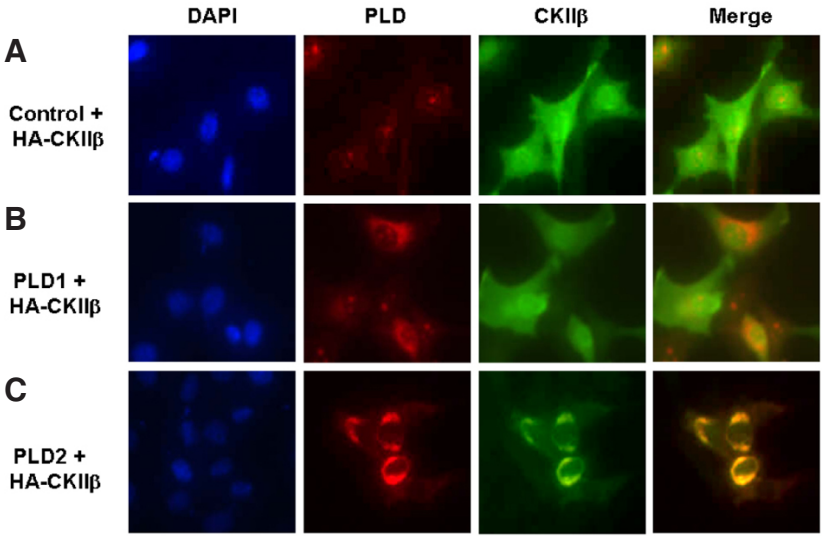

Fig. 6. CKIIβ and PLD colocalize in NIH3T3 cells. NIH3T3 cells were cotransfected with pcDNA3.0-HA-CKIIβ and the control vector pcDNA3.1 (A), pcDNA3.0-HA-CKIIβ and pcDNA3.1-PLD1 (B), or pcDNA3.0-HA-CKIIβ and pcDNA3.1-PLD2 (C) for 48 h. The subcellular distribution of HA-CKIIβ (green) and PLD (red) was assessed by immunocytochemical staining. Cells were counterstained with DAPI to visualize the nuclei (blue). The merged panel is a pseudocolor image generated by combining the FITC (green) and rhodamine (red) channels. All merged images showed a yellow-orange image, indicating the colocalization of CKIIβ and PLD at discrete sites in the cells.

DISCUSSION

In the current study we investigated the physiological significance of the interaction between PLD isozymes and CKII. The over-expression of either PLD1 or PLD2 caused a decrease in the level of CKII activity in mouse fibroblast NIH3T3 cells. Neither the treatment of NIH3T3 cells with 1-butanol nor the over-expression of the dominant negative mutants of PLD abolished the PLD-induced suppression of CKII activity, indicating that the catalytic activity of PLD is unnecessary for CKII inactivation. Reverse-transcription-PCR and Western blotting analysis revealed that the mRNA levels of the CKII subunits did not change but that the protein levels of the CKIIβ subunit decreased in the PLD-over-expressing NIH3T3 cells. In addition, our data indicated that a reduction in the CKII activity in the PLD-over-expressing NIH3T3 cells was due mainly to a destabilization of the CKIIβ protein, as CKIIβ protein disappeared more rapidly when PLD-over-expressing cells were treated with the translation inhibitor cycloheximide. It is well known that CKIIβ stimulates the catalytic activity of CKIIβ through the formation of CKII heterotetramer (Issinger, 1993; Litchfield, 1993; Pinna, 1990). Hence, the reduction in CKIIβ protein would lead to a decrease in overall CKII activity in the PLD-over-expressing NIH3T3 cells.

It has been reported that the over-expression of PLD1 in 3Y1 rat fibroblast cells facilitates the ubiquitination and proteasomal degradation of p53. The ability of PLD1 to suppress p53 stabilization was blocked by the inhibition of MAP kinase and mTOR (Hui et al., 2004). In the present study, we observed that the ubiquitin proteasome machinery was also involved in the PLD-induced CKIIβ degradation. The PLD-mediated reduction in CKIIβ protein was nearly reversed by treatment with a proteasome inhibitor and poly-ubiquitination of CKIIβ increased significantly in the PLD-over-expressing NIH3T3 cells. However, neither MAP kinase nor mTOR were involved in the PLD-mediated CKIIβ degradation. Since it has been proposed that PLD activity is required for the activation of both MAP kinase and mTOR (Shen et al., 2001), these data are consistent with our conclusion that the catalytic activity of PLD is unnecessary for the PLD-mediated CKII inhibition.

Other researchers have demonstrated that the presence of CKIIβ enhances the amount of CKIIβ bound to PLD1 and the phosphorylation of PLD1 by recombinant CKIIα (Ganley et al., 2001). Here, we were able to confirm the interaction of PLD isozymes with CKIIβ and the colocalization of PLD and CKIIβ in NIH3T3 cells. In addition, our results indicated that PLD preincubation with CKIIβ reduced autophosphorylation of CKIIβ. It has been reported that the N-terminal region of CKIIβ has autophosphorylation sites that are important for CKIIβ stabilization (Zhang et al., 2002). Based on these results, we suggest that PLD binds to the N-terminal region of CKIIβ and enhances the destabilization of the CKIIβ protein by preventing CKIIβ autophosphorylation. In conclusion, the present study proposes one possible inhibition mechanism for CKII.

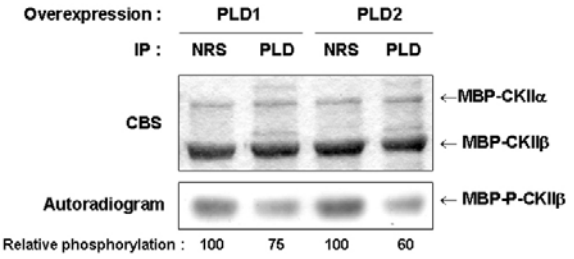

Fig. 7. PLD binding inhibits CKIIβ autophosphorylation. Cell lysate (1.5 mg) from NIH3T3 cells that were transfected with the pcDNA3.1-PLD1 or pcDNA3.1-PLD2 was pre-cleared, and the supernatant was used for PLD immunoprecipitation (IP) as described in "Materials and Methods". As a control, normal rabbit IgG (NRS) was used in the precipitation. Immunoprecipitated proteins were incubated with 200 ng of MBP-CKIIβ for 2 h at 4°C and then subjected to a phosphorylation reaction containing 100 ng MBP-CKIIα and [γ-^{32}P]GTP. Proteins were separated on a 10% (w/v) SDS-polyacrylamide gel and visualized by Coomassie blue staining (CBS, upper panel) and autoradiography (bottom panel). P-CKIIβ, phosphorylated CKIIβ.

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