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The C-terminal domain of PLD2 participates in degradation of protein kinase CKII β subunit in human colorectal carcinoma cells

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Elevated phospholipase D (PLD) expression prevents cell cycle arrest and apoptosis. However, the roles of PLD isoforms in cell proliferation and apoptosis are incompletely understood. Here, we investigated the physiological significance of the interaction between PLD2 and protein kinase CKII (CKII) in HCT116 human colorectal carcinoma cells. PLD2 interacted with the CKIIβ subunit in HCT116 cells. The C-terminal domain (residues 578-933) of PLD2 and the N-terminal domain of CKIIβ were necessary for interaction between the two proteins. PLD2 relocalized CKIIβ to the plasma membrane area. Overexpression of PLD2 reduced CKIIβ protein level, whereas knockdown of PLD2 led to an increase in CKIIβ expression. PLD2-induced CKIIβ reduction was mediated by ubiquitin-dependent degradation. The C-terminal domain of PLD2 was sufficient for CKIIβ degradation as the catalytic activity of PLD2 was not required. Taken together, the results indicate that the C-terminal domain of PLD2 can regulate CKII by accelerating CKIIβ degradation in HCT116 cells. [BMB reports 2011; 44(9): 572-577]

INTRODUCTION

Phospholipase D (PLD) catalyzes hydrolysis of the membrane phospholipid phosphatidylcholine to produce a lipid second messenger, phosphatidic acid, and choline (1, 2). PLD has two isoforms, PLD1 and PLD2. PLD1 has low basal activity and is activated by small G proteins, classical protein kinase C (PKC) isoforms, and phosphatidylinositol 4, 5-bisphosphate (PIP2). In contrast, PLD2 has high basal activity, requires PIP2, and is activated by ARF (ADP-ribosylation factor) and PKC. PLD has been implicated in a variety of physiological cellular functions, such as intracellular protein trafficking, cytoskeletal dynamics, membrane remodeling, and cell proliferation (3). PLD activity was previously shown to increase in response to mitogenic signals and activated oncoproteins such as EGF, insulin, v-Src, and v-Ras. PLD activity and expression are up-regulated in several types of human cancers. Elevated PLD activity has been shown to contribute to cell transformation and survival. In addition, elevated PLD expression prevents cell-cycle arrest and apoptosis (4, 5). However, the roles of PLD isoforms in cell proliferation and apoptosis are not completely understood.

Protein kinase CKII (CKII) is a ubiquitous serine/threonine kinase that catalyzes the phosphorylation of a large number of cytoplasmic and nuclear proteins (6-8). The holoenzyme of CKII is a heterotetramer composed of two catalytic (α and/or α′) subunits and two regulatory (β) subunits. The β subunit stimulates the catalytic activity of the α or α′ subunit, mediating tetramer formation and substrate recognition. Overexpression of the CKII catalytic subunit leads to tumorigenesis in mice overexpressing Myc. Analysis of yeast expressing a temperature-sensitive mutant CKII gene showed that CKII is required for cell cycle progression in both G_1 and G_2/M phases. In addition, recent observations that CKII phosphorylates procaspase-2 or caspase substrate confirmed that CKII prevents apoptosis (9-11). These findings suggest that CKII plays a critical role, not only in cell growth and proliferation, but also in anti-apoptosis. Thus, there must be at least one regulatory mechanism for CKII. Recently, we showed that in non-cancer NIH3T3 cells overexpressing PLD isozymes, CKII activity is down-regulated through the proteasome-dependent degradation of CKIIβ (12). In the present study, we investigated the physiological significance of the interaction between CKII and PLD2 in HCT116 human colorectal carcinoma cells. Our results indicate for the first time that the C-terminal domain of PLD2 modulates CKII by binding to CKIIβ in HCT116 cells.

RESULTS AND DISCUSSION

Interaction between PLD2 and CKIIβ in HCT116 cells

To determine whether or not CKII is associated with PLD2 in

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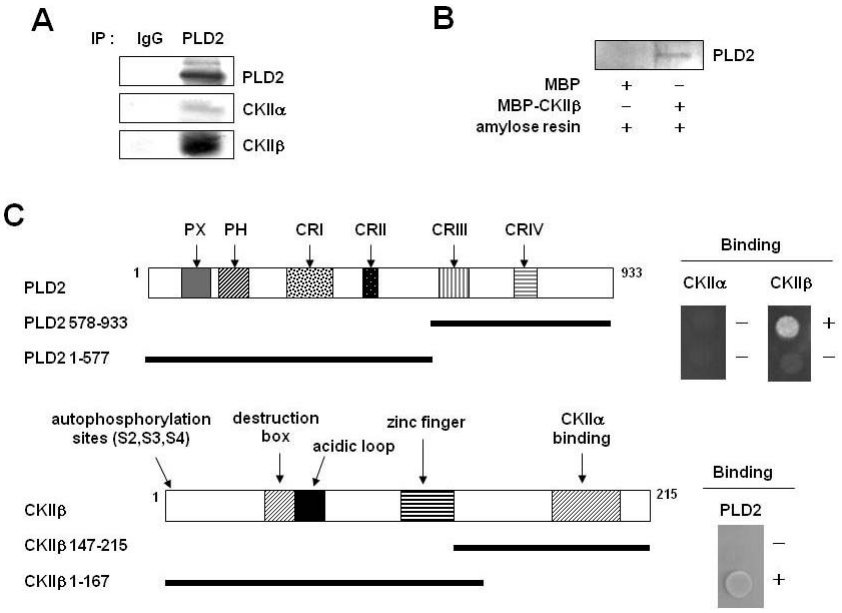

Fig. 1. Interaction of the C-terminal domain of PLD2 with the N-terminal domain of CKIIβ. (A & B) HCT116 cell extracts were subjected to immunoprecipitation (IP) with either anti-PLD antibody or normal IgG (A). The cell lysates were also used for the precipitation of PLD2 with amylose resin coated with either MBP-CKIIβ or MBP (control) (B). The precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by Western blotting. (C) The yeast reporter strain was co-transformed with pGADGH-PLD2 deletion constructs and pGBT9-CKIIα or -CKIIβ (upper panel) or with pGBT9-CKIIβ deletion constructs and pGADGH-PLD2$^{578\text{-}933}$ (bottom panel). All constructs were also tested against the empty expression vector pGBT9 and pGADGH to control for autonomous activation of the hybrid proteins. PX, phox domain; PH, pleckstrin homology domain; CR, conserved region.

HCT116 cells, PLD2 was immunoprecipitated from HCT116 cell extracts. Normal IgG immunoprecipitation was used as a control. As shown in Fig. 1A, CKIIα and CKIIβ co-precipitated with PLD2, indicating that PLD2 interacts with CKII enzyme in HCT116 cells. Next, to determine whether or not PLD2 interacts directly with the CKIIβ subunit, we attempted to form a complex between MBP-CKIIβ and PLD2. When MBP-CKIIβ was mixed with cell extract, PLD2 bound to MBP-CKIIβ (Fig. 1B). To identify the regions of PLD2 and CKIIβ that participate in this interaction, we used a yeast two-hybrid system. PLD2 did not bind directly to CKIIα, but the C-terminal domain (amino acids 578-933) of PLD2 interacted with CKIIβ. CKIIβ interacted with PLD2 when amino acids 1-167 were present, but not when amino acids 147-215 were present (Fig. 1C). These results suggest that the C-terminal domain of PLD2 and the N-terminal domain of CKIIβ are necessary for the interaction between PLD2 and CKIIβ.

Co-localization of CKIIβ and PLD2 in HCT116 cells

The finding that CKIIβ associated with PLD2 implies that CKIIβ and PLD2 might co-localize to the same cell region. We investigated the intracellular localization of CKIIβ relative to that of PLD2 using immunocytochemical analysis. In HCT116 cells transfected with pcDNA-HA-CKIIβ and control vector, CKIIβ expression (green) was detected in both the cytoplasm and nucleus. However, in cells co-expressing HA-CKIIβ and PLD2, the subcellular localization of CKIIβ was significantly altered; both CKIIβ and PLD2 were prominently localized to the plasma membrane region, indicating the co-localization of CKIIβ and PLD2 at discrete sites in the cells (Fig. 2).

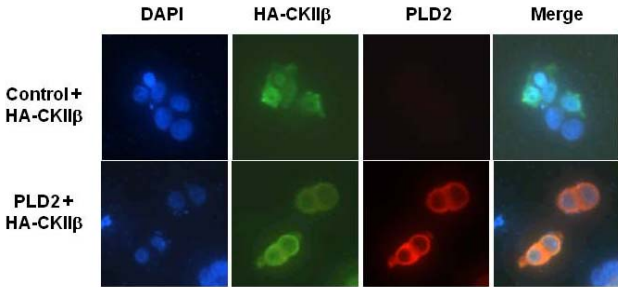

Fig. 2. Co-localization of PLD2 and CKIIβ in HCT116 cells. HCT116 cells were seeded on Nunc chamber slides and co-transfected with pcDNA3.0-HA-CKIIβ and the control vector pcDNA3.1 (upper panel) or pcDNA3.0-HA- CKIIβ and pcDNA3.1-PLD2 (bottom panel). The subcellular distributions of HA-CKIIβ (FITC) and PLD2 (rhodamine) were 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), rhodamine (red), and DAPI (blue) channels.

PLD2 overexpression stimulates proteasome-dependent degradation of CKIIβ in HCT116 cells

To investigate the physiological significance of the interaction between PLD2 and CKIIβ, we analyzed CKII expression in PLD2-overexpressing HCT116 cells. As shown in Fig. 3A, the protein level of endogenous CKIIβ, but not CKIIα, decreased. The protein level of exogenously expressed HA-CKIIβ also decreased in PLD2-overexpressing cells (Fig. 3B). Quantification by densitometry revealed that PLD2 overexpression reduced the protein level of CKIIβ by approximately 50-60% in HCT-116 cells. To confirm the role of PLD2 in CKIIβ reduction, we knocked down PLD2 in HCT116 cells using PLD2 siRNA. As shown in Fig. 3C, knockdown of PLD2 resulted in an increase in CKIIβ protein expression. We then examined whether or not the reduction in CKIIβ protein expression in PLD2-overexpressing cells was due to reduced levels of CKIIβ mRNA. The amount of CKIIβ mRNA remained unchanged in the cells overexpressing PLD2 (Fig. 3D). To determine whether or not CKIIβ degradation PLD2 activity is required for CKIIβ degradation, HCT116 cells were treated with phosphatidic acid or 1-butanol, an inhibitor of PLD-mediated phosphatidic acid production. As shown in Fig. 3E, the level of CKIIβ protein was not altered by

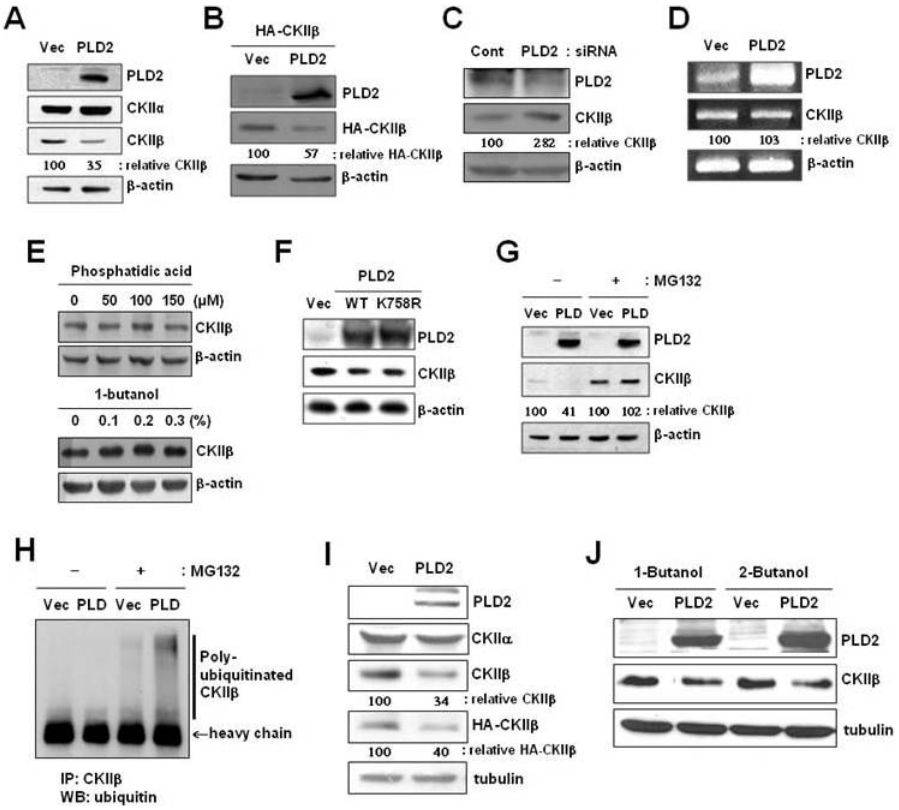

Fig. 3. Induction of CKIIβ degradation by PLD2 overexpresssion in HCT116 and HEK293 cells. (A) HCT116 cells stably transfected with the control vector (Vec, lane 1) or pcDNA3.1-PLD2 (lane 2) were lysed, electrophoresed on an SDS-polyacrylamide gel, and visualized by Western blotting. (B) Cells transiently expressing the control vector (Vec) and pcDNA3.1HA-CKIIβ (lane 1) or pcDNA3.1-PLD2 and pcDNA3.1HA-CKIIβ (lane 2) were lysed, electrophoresed, and visualized by Western blotting. (C) Cells were transfected with control (lane 1) or PLD2 (lane 2) siRNAs, lysed, electrophoresed, and visualized by Western blotting. (D) Total RNA was extracted from cells stably expressing the control vector (lane 1) or pcDNA3.1-PLD2 (lane 2) and reverse-transcribed using CKIIβ-specific primers and reverse transcriptase. (E) Lysates from cells treated for 20 h with phosphatidic acid (upper panel) or 1-butanol (bottom panel) were electrophoresed and visualized by Western blotting. (F) Cells expressing wild-type (WT) or catalytically negative mutant (K758R) PLD2 were lysed, electrophoresed, and visualized by Western blotting. (G) HCT116 cells expressing the control vector (Vec) or pcDNA3.1-PLD2 (PLD) were treated without or with 10 μM MG132 for 24 h. The cells were lysed, electrophoresed, and visualized by Western blotting with anti-CKIIβ antibody. (H) Cells expressing the control vector (Vec) or pcDNA3.1-PLD2 (PLD) were grown in the presence or absence of 10 μM MG132. Cell lysates were immunoprecipitated (IP) with anti-CKIIβ antibody followed by Western blotting (WB) with anti-ubiquitin antibody. (I) HEK293 cells transiently expressing the control pcDNA3.1 vector and pcDNA3.1HA-CKIIβ (lane 1) or pcDNA3.1-PLD2 and pcDNA3.1HA-CKIIβ (lane 2) were lysed, electrophoresed, and visualized by Western blotting. (J) HEK293 cells stably expressing the control vector (lanes 1 and 3) or pcDNA3.1-PLD2 (lanes 2 and 4) were treated for 20 h with 0.3% 1-butanol (lanes 1 and 2) or 2-butanol (lanes 3 and 4), lysed, electrophoresed, and visualized by Western blotting.

treatment with phosphatidic acid or 1-butanol, indicating that PLD2 activity is not required for PLD2-mediated degradation of CKIIβ. To again confirm that PLD2 activity is not linked to CKIIβ degradation, the catalytically inactive mutant of PLD2 (K758R) was tested for its effect on CKIIβ degradation. Despite PLD2 activity being reduced in this cell extract (data not shown), CKIIβ protein was down-regulated to a level similar to that induced by wild-type PLD2 overexpression (Fig. 3F).

We recently demonstrated that reduction of CKIIβ protein expression in PLD-overexpressing NIH3T3 cells is due to protein destabilization, as these cells display rapidly decreased CKIIβ protein expression upon treatment with the translation inhibitor cycloheximide (12). Thus, we next investigated whether or not degradation of CKIIβ in HCT116 cells is accompanied by ubiquitination. PLD2-overexpressing HCT116 cells were treated with the proteasome inhibitor MG132, after which the protein level of CKIIβ was analyzed by immunoblotting. The PLD2-induced decrease in CKIIβ protein expression was almost completely reversed by treatment with MG132 when compared with control cells (Fig. 3G). In addition, when CKIIβ protein was immunoprecipitated from the HCT116 cell extracts and the immunoprecipitates were probed for ubiquitin, poly-ubiquitinated CKIIβ accumulated only when the cells were treated with MG132. The poly-ubiquitinated forms of CKIIβ increased to a more significant degree in the PLD2-overexpressing HCT116 cells than control cells (Fig. 3H). These data indicate that the effect of PLD2 overexpression on CKIIβ reduction is mainly due to an increase in CKIIβ ubiquitination and proteosomal degradation.

Next, we examined the ability of PLD2 to regulate CKIIβ protein expression in HEK293 cells. HEK293 cells were transfected with plasmids encoding PLD2 and HA-CKIIβ. The protein levels of endogenous CKIIβ and exogenously expressed HA-CKIIβ decreased in PLD2-overexpressing HEK293 cells (Fig. 3I). The level of CKIIβ protein was not recovered by treatment with 1-butanol. A secondary alcohol, 2-butanol, was used as a control (Fig. 3J).

C-terminal domain of PLD2 is involved in degradation of CKIIβ in HCT116 cells

To map the subregion within PLD2 that is required to stimulate CKIIβ degradation, different regions of PLD2 were expressed in HCT116 cells. PLD2 caused a reduction in CKIIβ protein expression when amino acids 1-875 were present, but not when the C-terminus was deleted to amino acid 579. Deletion of 577 amino acids from the N-terminal end of PLD2 did not abolish its ability to induce CKIIβ degradation. The residues 578-933 of PLD2 stimulated CKIIβ degradation more significantly, probably due to increased protein number and/or accessible protein structure (Fig. 4A). Furthermore, the C-terminal domain (residues 578-933) of PLD2 increased the poly-ubiquitinated forms of CKIIβ in HCT116 cells (Fig. 4B). Taken together, these results indicate that residues 578-875 of PLD2, a region containing CRIII and CRIV, are involved in ubiquitin-dependent CKIIβ degradation (Fig. 4C).

In summary, this study showed for the first time that PLD2 is involved in destabilization of CKIIβ in HCT116 cells. The PLD2-mediated reduction in CKIIβ protein expression was almost completely reversed by treatment with the proteasome inhibitor MG132. The level of poly-ubiquitinated CKIIβ increased more significantly in PLD2-overexpressing HCT116 cells, suggesting that PLD2-induced destabilization of CKIIβ is mediated by the ubiquitin proteasome machinery. CKIIβ degradation occurred even in the presence of a catalytically in-

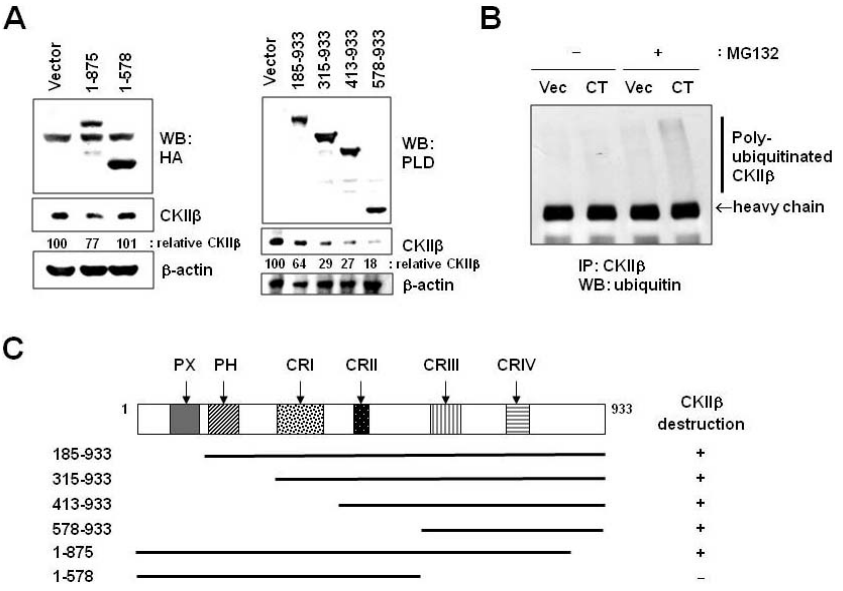

Fig. 4. Involvement of the C-terminal region of PLD2 in CKIIβ degradation in HCT116 cells. (A) Cells expressing different fragments of PLD2 were lysed, electrophoresed, and visualized by Western blotting. (B) Cells expressing the control vector (Vec) or pcDNA3.1-PLD2/578-933 (CT) were grown in the presence or absence of 10 μM MG-132. Cell lysates were immunoprecipitated with anti-CKIIβ antibody followed by Western blotting with anti-ubiquitin antibody. (C) Diagrams summarizing the results. PX, phox domain; PH, pleckstrin homology domain; CR, conserved region.

active mutant PLD2, and neither phosphatidic acid nor 1-butanol altered CKIIβ stability in cells. It was previously shown that the region containing CRI to CRIV is critical for PLD activity (13). Our current results show that PLD2 amino acids 578-933, a region that lacking PX, CRI, or CRII, is sufficient for CKIIβ degradation. Taken together, our data and those of others reveal that the catalytic activity of PLD2 is unnecessary for CKIIβ degradation. What is the mechanism for CKIIβ degradation by PLD2? In this study, we found that the PLD2 578-933 region is the CKIIβ-binding domain. CKIIβ is localized throughout the cell; however, it translocates to the plasma membrane when PLD2 is overexpressed. Therefore, our study suggests that CKIIβ binding to PLD2 might induce nuclear export followed by proteasome-dependent degradation of CKIIβ. However, we cannot exclude the possibility that PLD2 578-933 binds to the N-terminal region of CKIIβ and enhances destabilization by modulating CKIIβ phosphorylation (12). The N-terminal region of CKIIβ has autophosphorylation sites that might be involved in CKIIβ stabilization (14).

MATERIALS AND METHODS

Antibodies and enzymes

Polyclonal anti-CKIIα and monoclonal anti-CKIIβ antibodies were obtained from Calbiochem (Darmstadt, Germany). Polyclonal anti-PLD antibody was raised against the C-terminal peptide of PLD (15). Polyclonal anti-ubiquitin, anti-β-actin, and anti-tubulin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-HA antibody was from Roche (Basel, Switzerland). Rhodamine-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG were from Invitrogen (Carlsbad, CA). CKII α and β subunits tagged with maltose-binding protein (MBP) were expressed in *E. coli* and purified as described previously (16).

Cell culture and preparation of cell extract

HCT116 and HEK293 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 PLD2-overexpressing cell lines, cells were transfected with the pcDNA3.1-PLD2 construct or pcDNA3.1-CKII by Lipofectamine (Bethesda Research Laboratories, Bethesda, MD), as described by the manufacturer. For preparation of cell extract, 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.

Plasmid constructions

PLD2 deletion mutants were generated by a polymerase chain reaction (PCR)-based cloning strategy. The four primers used to generate the PLD2 deletions were as follows: 1) 5'-ATGGATCCATACCCCTACCTGCTTC-3', 2) 5'-CGAATTCCTATGTCCACACTTCTAGGG-3', 3) 5'-ACTAGTGATGACGGCGACCCCT-3', 4) 5'-CGAATTCGTATGTGGGAGTCTTGTACTT-3'. The pGADGH-PLD2$^{578\text{-}934}$ construct was generated using primers 1 and 2. The pGADGH-PLD2$^{1\text{-}578}$ construct was generated using primers 3 and 4. All PCR products were digested with *Bam*HI and *Eco*RI (or *Spe*I and *Eco*RI) and cloned into pGADGH.

RNA interference

The PLD2 siRNA used was 5'-AAGAGGUGGCUGGUGGUGAAGdTdT. The negative control siRNA was 5'-GCUCAGAUCAAUACGGAGAdTdT. Cells were transfected with siRNAs using Lipofectamine (Invitrogen, Carlsbad, CA) as described by the manufacturer. At 5 h after transfection, the medium was changed and the cells grown for another 2 days before being harvested.

Reverse Transcription-PCR

Total RNA was extracted from HCT116 cells. RNA was reverse-transcribed using CKIIβ-specific primers and reverse transcriptase (Takara, Japan), and the resulting cDNAs were PCR-amplified. Primers for β-actin RNA were used to standardize the amount of RNA in each sample. PCR products were resolved on a 1.5% agarose gel.

Immunoprecipitation, MBP pull-down, and yeast two-hybrid assays

For immunoprecipitation, cell lysates were pre-cleared with normal IgG and protein A sepharose (Amersham Biosciences, Piscataway, NJ) for 1 h at 4°C. The supernatant was then incubated with anti-CKIIβ or anti-PLD antibody and protein A sepharose by mixing for 12 h at 4°C. An MBP pull-down assay was performed by incubating amylose-agarose beads with MBP-CKIIβ and cell lysates that were transfected with pcDNA3.1-PLD2 in 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. Then, the beads were collected by centrifugation and washed three times with phosphate-buffered saline (PBS). Yeast two-hybrid assay was carried out as described (17).

Immuno-blotting and immunocytochemical staining

For immune-blotting, proteins were separated on polyacrylamide gels in the presence of SDS, and then transferred electrophoretically to a 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, Piscataway, NJ). Some membranes were stripped in stripping buffer [2% SDS, 100 mM β-mercaptoethanol, and 50

mM Tris-HCl (pH 7.0)] 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. For immunocytochemical staining, HCT116 cells were seeded on four-well micro-chamber slides (Nunc, Naperville, IL) and transfected the next day with pcDNA3.0-HA-CKIIβ and pcDNA3.1-PLD2. Forty-eight hours after transfection, the cells were fixed with 4% 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). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR), and fluorescence signals were detected with a Carl Zeiss Axioplan 2 microscope.

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