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Cell-Cycle-Dependent PC-PLC Regulation by APC/C^{Cdc20}-Mediated Ubiquitin-Proteasome Pathway

Da Fu,[1] Yushui Ma,[2] Wei Wu,[1] Xuchao Zhu,[1] Chengyou Jia,[1] Qianlei Zhao,[1] Chunyi Zhang,[1] and Xing Zhong Wu[1*]

[1]*Department of Biochemistry, Shanghai Medical College, Fudan University, Shanghai 200032, PR China*

[2]*Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, PR China*

ABSTRACT

Phosphatidylcholine-specific phospholipase C (PC-PLC) is involved in the cell signal transduction, cell proliferation, and apoptosis. The mechanism of its action, however, has not been fully understood, particularly, the role of PC-PLC in the cell cycle. In the present study, we found that cell division cycle 20 homolog (Cdc20) and PC-PLC were co-immunoprecipitated reciprocally by either antibody in rat hepatoma cells CBRH-7919 as well as in rat liver tissue. Using confocal microscopy, we found that PC-PLC and Cdc20 were co-localized in the perinuclear endoplasmic reticulum region (the "juxtanuclear quality control" compartment, JUNQ). The expression level and activities of PC-PLC changed in a cell-cycle-dependent manner and were inversely correlated with the expression of Cdc20. Intriguingly, Cdc20 over-expression altered the subcellular localization and distribution of PC-PLC, and caused PC-PLC degradation by the ubiquitin proteasome pathway (UPP). Taken together, our data indicate that PC-PLC regulation in cell cycles is controlled by APC/C^{Cdc20}-mediated UPP. J. Cell. Biochem. 107: 686–696, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: Cdc20; PC-PLC; APC/C; CELL-CYCLE; UPP

Phosphatidylcholine-specific phospholipase C (PC-PLC), the major enzyme in the phosphatidylcholine (PC) cycle, is responsible for the production of phosphocholine (Pcho) and non-PIP2-derived 1,2-Diacylglycerol (DAG), sustaining long-term cellular responses such as activation, proliferation, and differentiation events [Plo et al., 2000; Moreno-Garcia et al., 2005]. DAG is involved in the regulation of several intracellular pathways including the activation of protein kinase C isoforms [Diaz-Laviada et al., 1990; Li et al., 2006]. Although the role of Pcho in signaling is less characterized, it has been shown to promote cell proliferation and transformation [Chung et al., 1997]. PC-PLC activity has been shown to be required for NF-κB activation [Schutze et al., 1992; Lin et al., 2004], and furthermore, bacterial PC-PLC induces a transformed phenotype in transfected NIH3T3 cells [Johansen et al., 1994]. CD38 signaling regulates B lymphocyte activation via a PC-PLC-dependent signaling cascade [Moreno-Garcia et al., 2005].

Although the molecular characterization of mammalian PC-PLC remains unknown, several studies have established a role for PC-PLC in cell signaling. PC-PLC enzyme could play an important role in regulating CD16 expression on membrane and its downstream signal transduction. Analysis of PC-PLC and CD16 distribution in NK cell plasma membrane demonstrates that the proteins are physically associated and partially accumulated in lipid rafts [Cecchetti et al., 2007]. Zamorano et al. [2003] discovered that the activation of PC-PLC seems to be an early event in IL-4 signaling. Furthermore, intracellular pathways regulated by IL-4 may collaborate with PC-PLC to signal STAT6 activation. PC-PLC, which is implicated in mitogen-activated protein kinase (MAPK) activation in tumor necrosis factor-α (TNF-α)-treated immature acute myeloid leukemia cells, plays a role in the TNF-α proliferative effect in immature myeloid cells [Plo et al., 2000]. These studies suggest an active role for PC-PLC in cell signaling.

Abbreviations used: APC/C, anaphase-promoting complex/cyclosome; Cdc20; cell division cycle 20 homolog; EGFP, enhance green fluorescent protein; FITC, fluorescein isothiocyanate; JUNQ, juxtanuclear quality control compartment; PC-PLC, phosphatidylcholine-specific phospholipase C; UPP, ubiquitin proteasome pathway.

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*Correspondence to: Dr. Xing Zhong Wu, MD, PhD, Department of Biochemistry, Shanghai Medical College, Fudan University, 138 Yi Xue Yuan Road, Shanghai 200032, PR China. E-mail: xz_wu@shmu.edu.cn

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A major gap in our knowledge of the hydrolysis mechanism of PC-PLC is due to mammalian PC-PLC not yet being cloned and fully characterized, although direct evidence clearly demonstrates the expression of PC-PLC isoforms in mammalian cells so far described [Johansen et al., 1994; Wu et al., 1997; Preuss et al., 2001; Ramoni et al., 2001; Spadaro et al., 2006]. Clark et al., showed that antibodies against Bacillus cereus phospholipase C prepared in rabbits could be used to purify a PC-PLC from a human monocytic cell line and to analyze this protein specifically by Western blot [Clark et al., 1986; Podo et al., 1996; Ramoni et al. 2001].

Cell division cycle 20 homolog (Cdc20) is an essential cell-cycle regulator required for the completion of mitosis in organisms all of the way from yeast to man [Eytan et al., 2006]. In mitosis, Cdc20 binds to and activates the ubiquitin ligase activity of a large molecular machine called the anaphase-promoting complex/cyclosome (APC/C) and enables the ubiquitination and degradation of specific substrates. Thus, it promotes the onset of anaphase and mitotic exit [Hoyt, 2001; Nakayama and Nakayama, 2006; Thornton et al., 2006]. Cdc20 also serves as an integrator of multiple intracellular signaling cascades that regulate progression through mitosis [Prinz et al., 1998; Yu, 2007]. Ubiquitylated destruction of key cell-cycle proteins is partly coordinated by the activity of APC/C [Lindon, 2008]. The coordinated destruction of cell-cycle proteins is achieved by making them APC/C substrates at specific points in the cell-cycle [Kraft et al., 2005]. The activity of APC/C has been shown to be highly regulated at different levels. To prevent premature activation of APC/C, several inhibitors are also present, such as Emi1/Rca1, Emi2/XErp1, or RASSF1A [Dong et al., 1997; Reimann et al., 2001; Song et al., 2004; Schmidt et al., 2005]. These checkpoints inhibit APC/C activation until all the chromosomes are attached in a bipolar manner [Musacchio and Hardwick, 2002; Yu, 2002]. The fine-tuning of the APC/C activity, by a substrate that is also an inhibitor, is required for the precise coordination and transition through meiosis [Kraft et al., 2003].

Cellular quality control networks play a key role in maintaining protein homeostasis. Recently, the experimental data [Kaganovich et al., 2008] implicated that the "juxtanuclear quality control" compartment (JUNQ) is a defined new subcellular compartment, which is normally present in cells. Most ubiquitinated proteins are recognized by the quality control machinery and directed to the JUNQ, a region that concentrates disaggregating chaperones and 26S proteasomes, and is in close proximity to the perinuclear endoplasmic reticulum region involved in soluble endoplasmic reticulum associated protein degradation (ERAD). Our study shows that there is direct evidence demonstrating that PC-PLC is located in the JUNQ in Cdc20-transfected CBRH-7919 cells.

Previous studies, however, have not discussed the interaction between PC-PLC and Cdc20. More limited evidence has been reported on the possible involvement in mammalian cell-cycle of PC-PLC. In the current study, we provide new evidence that PC-PLC interacts with Cdc20 in CBRH-7919 cells. This interaction can alter the intracellular distribution of PC-PLC, mediate the degradation of PC-PLC, and diminish the enzyme activity through ubiquitin proteasome pathway (UPP).

MATERIALS AND METHODS

REAGENTS AND MATERIALS

Unless otherwise noted, chemicals were from Sigma Chemical (St. Louis, MO) and were at least analytical grade. MG132 was from Alexis Biochemicals (San Diego, CA). Trizol reagent was from Dingguo Biochemicals (Beijing, China). Plasmids pcDNA3.1-Cdc20 and pEGFP-Cdc20 were kindly provided by Dr. Somsubhra Nath in Johns Hopkins University School of Medicine (Baltimore, MD) and Dr. Michael Gage in Centre for Ecology (Norwich, UK).

CELL CULTURE

Normal rat liver cell lines (LW3) and African green monkey kidney cell lines (COS-7) were derived from American Type Culture Collection (ATCC). Human breast carcinoma MCF-7, CBRH-7919 (a rat hepatocarcinoma cell line established from *N*-nitrosodiethylamine-induced Wistar rat hepatoma), normal human liver cell line (LO2), and human hepatoma cell line SMMC-7721 were from the Institute of Cell and Biochemistry Research of Chinese Academy of Science. Wistar rats were provided by the Department of Experimental Animals, Fudan University, China. The cell lines were cultured in DMEM or RPMI1640 medium (Gibcol BRL) supplemented with 10% heat-inactivated (56°C, 30 min) newborn calf serum in 5% CO_2 at 37°C.

SYNCHRONIZATION

For synchronization, CBRH-7919 cells were treated with 2 mM thymidine for 18–24 h, released for 8–10 h, then treated with thymidine for 16–18 h. S phase (about 4 h post-release) and mitosis were monitored by Hoechst 33342 (Beyotime, Jiangsu, China) staining [Pines, 1997]. G_2 cells (8 h post-release) were not incorporating Hoechst 33342, and the DNA did not condense. Mitotic cells were collected by shake-off once the cells showed an increase in the mitotic index (about 8–13 h post-release). The adherent cells were washed with PBS and then lysed. This population is not mitotic and is predominantly in G_0. CBRH-7919 isolated cells were replanted in a complete medium and incubated 4 h before harvesting for G_1 cells.

TRANSFECTION

Cdc20 cDNA expressional vector (pcDNA3.1-Cdc20) that expresses Cdc20 and si-Cdc20 vector (si-Cdc20-pSilencer4.1 with neomycin resistance), which produces specific siRNA (si-Cdc20) were used in the experiment. Vectors pcDNA3.1 and siRNA-random were used as the control for pcDNA3.1-Cdc20 and siRNA-Cdc20, respectively. Two sequences: (1) CGGTTTTGATGTG GAGGAA, (2) TCTTGTC-GATTGGAGCTCT were used in the experiment for the hairpin construction of RNAi, but sequence (2) was selected for further study, due to its stronger silence effect. The day before transfection, about 1×10^6 cells were seeded in the media onto a 60 mm dish and incubated for 24 h. The next day, cells were transfected with Sofast® gene transfection reagent kit (Sunma Corp., Xiamen, China) according to the manufacturer's instruction. The transfection efficiency was evaluated by enhance green fluorescent protein (EGFP) expression. Over 60% CBRH-7919 cells expressed the EGFP

protein. The transfected cells were collected for the following experiments after 24 h of incubation.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA (1 μg) was extracted from CBRH-7919 cells in different cell-cycle phases, with Trizol reagent, according to the manufacturer's instruction. This was used as the template for cDNA synthesis. Reverse transcription was then carried out by M-MLV (Toyobo, Japan). Primers used for PCR were as follows: Cdc20 (sense primer: 5′-TGAGGAGTCAGGGATTTGT-3′; antisense primer: 5′-AGATT-TGCCAGGAGTTCG-3′), β-actin (sense primer: 5′-GCCAACCGT-GAAAAGATG-3′; antisense primer: 5′-TGCCGATAGTGATGACCT-3′). For amplification of the reference gene β-actin, the following PCR protocol was applied: 94°C for 2 min, 94°C for 40 s, 54°C for 40 s, 72°C for 60 s with 30 cycles. PCR amplification for Cdc20 was performed by initial denaturation at 94°C for 2 min, followed by 32 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min with a further extension of 72°C for 10 min. The expected sizes were 415 bp (β-actin) and 327 bp (Cdc20). After amplification, PCR products were electrophoresed in 2% agarose gel to confirm that PCR yielded a single product of the expected size. Intensity of the DNA bands was analyzed by software BandScan 4.3, while the β-actin was used as an internal standard.

IMMUNOBLOT

The expression of PC-PLC and Cdc20 was measured by Western blot using GAPDH (glyceraldehydes-3-phosphate) staining as a loading control. Cells were harvested and lysed in a buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, and 1 mM PMSF. Lysate (50–100 μg) was then applied to SDS–PAGE and transferred onto a PVDF membrane. The membrane was then probed with specific primary antibodies followed by rabbit anti-mouse or goat anti-rabbit IgG HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and visualized with enhanced chemiluminescence (ECL). Mouse monoclonal anti-Cdc20 (Santa Cruz Biotechnology, sc-5296) and rabbit polyclonal anti-PC-PLC which was prepared and characterized in our laboratory against Clostridium perfringens PC-PLC [Wu et al., 1997; Wu and Lu, 1998] were used to detect the corresponding proteins.

IMMUNOPRECIPITATION

After pre-cleaning, 300–500 μg of CBRH-7919 cell lysates was respectively incubated with rabbit-anti-PC-PLC or mouse-anti-Cdc20 antibody at 4°C overnight and protein A-Sepharose (Amersham Biosciences, Buckinghamshire, England) or additional 2 h, and then centrifuged to collect the pellets. The precipitates were thoroughly washed, denatured, resolved by 8% SDS–PAGE, and transferred onto PVDF membrane. Then the membrane was respectively probed with rabbit-anti-PC-PLC and mouse-anti-Cdc20 antibodies to detect the presence of PC-PLC and Cdc20 in the complex.

CBRH-7919 cells were treated in presence or in absence of MG132 (10 μmol/L for 24 h) and transfected with pcDNA3.1-Cdc20 or siRNA-Cdc20 and their control plasmid pcDNA3.1 or siRNA-random, respectively. DMSO was used as the solvent control. CBRH-7919 cells were harvested 24 h after transfection and lysed in the lyses buffer. After pre-cleaning, the lysates were incubated with anti-ubiquitin (Santa Cruz Biotechnology, sc-8017) on a rotation bench for 4 h at 4°C and with 30 μl protein A-Sepharose for additional 1 h at 4°C. The immunoprecipitates were washed with lyses buffer three times, resolved by 8% SDS–PAGE, and then analyzed by Western blot using rabbit-anti-PC-PLC antibody.

IMMUNOFLUORESCENCE

Immunofluorescence was performed according to the method of our previous report [Hu et al., 2008]. Briefly, CBRH-7919 cells were cultured on cover slips and transfected with pEGFP-Cdc20. After 24 h, they were fixed with 3.8% paraformaldehyde and permeabilized with 0.5% Triton X-100/PBS. The cells were blocked with 1% BSA/TBST and stained with anti-PC-PLC antibody, followed by incubation with Rhodamine-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, 111-025-003). Untransfected cells were simultaneously stained with rabbit-anti-PC-PLC antibody and mouse-anti-Cdc20 antibody, followed by incubation with Rhodamine-conjugated anti-rabbit IgG antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Huamei, China). The nuclei were stained with Hoechst 33342. The cells were then observed under a laser scanning confocal microscopy (Nikon, Japan).

MASS SPECTROMETRY AND PEPTIDE SEQUENCE ANALYSIS

Liver samples of normal Wistar rats were lysed in a buffer containing a protease inhibitor cocktail. After pre-cleaning and centrifugation, 0.5 g of lysate protein was incubated with anti-PC-PLC antibody and protein A-Sepharose at 4°C overnight with rotation. After centrifugation, the precipitates were thoroughly washed, denatured, and applied onto SDS–PAGE. Then the gel was stained by Coomassie brilliant blue (R-250). The result of gel staining showed one band at 110 kDa, which was then cut out and sent for MALDI-TOF mass spectrometry analysis by Chinese National Human Genome Center in Shanghai (CHGC).

PC-PLC ASSAY

PC-PLC activity was determined in vitro using the Amplex Red® PC-PLC assay kit (Molecular Probe) according to manufacturer's instruction. In this enzyme-coupled assay, PC-PLC activity was detected using 10-acetyl-3, 7-dihydrophenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2, in a reaction cascade as follows. First, PC-PLC converted exogenous PC (lecithin) to Pcho and DAG. Pcho was hydrolyzed to choline by alkaline phosphatase. Choline was oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2 in the presence of HRP reacted with Amplex Red in a 1:1 stoichiometric ratio to generate resorufin, which is excited at 540 nm and detected at 590 nm using a microplate fluorometer (Fluoromark, Bio-Rad).

STATISTICAL ANALYSIS

All experiments were conducted at least three times. Results were expressed as the means ± SE. Statistical comparison of an average

between two groups was analyzed by Student's *t*-test, and differences with $P < 0.05$ were considered statistical significance.

RESULTS

PC-PLC IN MAMMALIAN CELLS

Previous studies have suggested an expression of PC-PLC isoforms in mammalian cells [Wu et al., 1997; Preuss et al., 2001; Ramoni et al., 2004; Cecchetti et al., 2007]. The activity distribution of PC-PLC among various rat tissues was investigated. Our results showed that the levels of PC-PLC activity varied from 1.51 ± 0.12 to 6.64 ± 0.09 mU/mg protein in different rat tissues (Fig. 1A). PC-PLC activities in LW3, CBRH-7919, LO2, COS-7, MCF-7, and SMMC-7721 were also detected, respectively with a commercial assay kit as described in the Materials and Methods. The activities of PC-PLC were 6.05 ± 0.15, 8.63 ± 0.25, 6.31 ± 0.24, 8.26 ± 0.14, 6.36 ± 0.24, and 3.87 ± 0.69 mU/mg protein, respectively (Fig. 1B). The basal PC-PLC activity levels in hepatoma cells (CBRH-7919, SMMC-7721) were substantially higher than those in normal hepatocytes (LW3, LO2). These differences need further investigation.

PC-PLC was then detected by Western blot using a rabbit polyclonal antibody as described in [Wu et al. 1997; Wu and Lu, 1998] and the result showed one band at 110 kDa (Fig. 1C) in CBRH-7919, SMMC-7721, and MCF-7 cells. Subcellular localization in CBRH-7919 cells by indirect immunofluorescence (Fig. 1D) was investigated. It was observed that the enzyme was spottily located in the cell membrane, and slightly spread into the internal side of the membrane. It was also located in the cytoplasm, indicating that PC-PLC was present in various mammalian cells and rat tissues.

INTERACTION BETWEEN PC-PLC AND CDC20

We sought to identify the mammalian PC-PLC by immunoprecipitation and mass spectrometry analysis. After immunoprecipitation, the precipitate was resolved by SDS–PAGE. Then the band at 110 kDa in gel from rat liver tissue was cut out (Fig. 2A) and analyzed by the MALDI-TOF mass spectrometry (Fig. 2B). Intrigu-

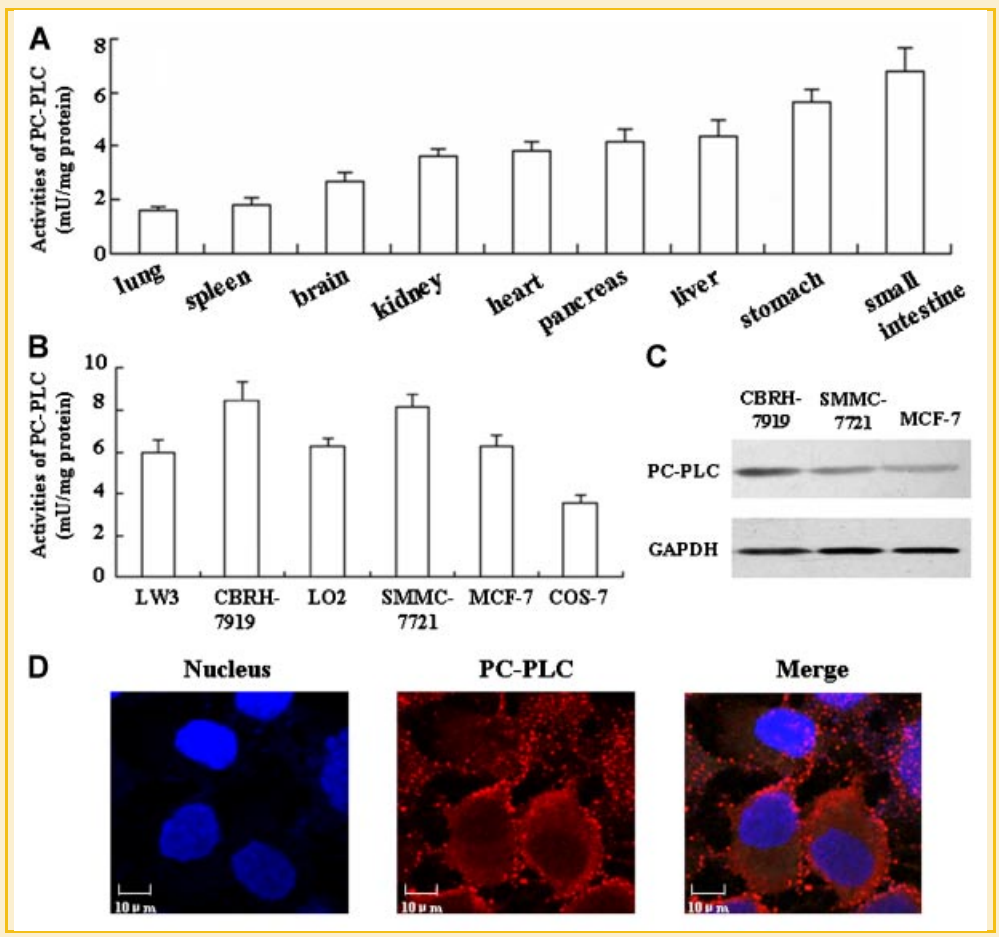

Fig. 1. PC-PLC exists in mammalian cells and rat tissues. A: Observation of PC-PLC activity in normal rat tissues. B: PC-PLC activity was also observed in mammalian cell lines (LW3, CBRH-7919, LO2, COS-7, MCF-7, and SMMC-7721). Total cell extracts from cell lines and homogenate from rat tissues were prepared as the protein source for PC-PLC assay by using the Amplex Red® PC-PLC assay kit. C: PC-PLC expression in CBRH-7919, SMMC-7721, and MCF-7 cells was analyzed by Western blot. D: Subcellular localization of PC-PLC in CBRH-7919 cells. CBRH-7919 cells were cultured on cover slips, fixed, and stained with rabbit-anti-PC-PLC antibody, followed with incubation with Rhodamine-conjugated anti-rabbit antibody (red), and the nucleus was stained with Hoechst 33342 (blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

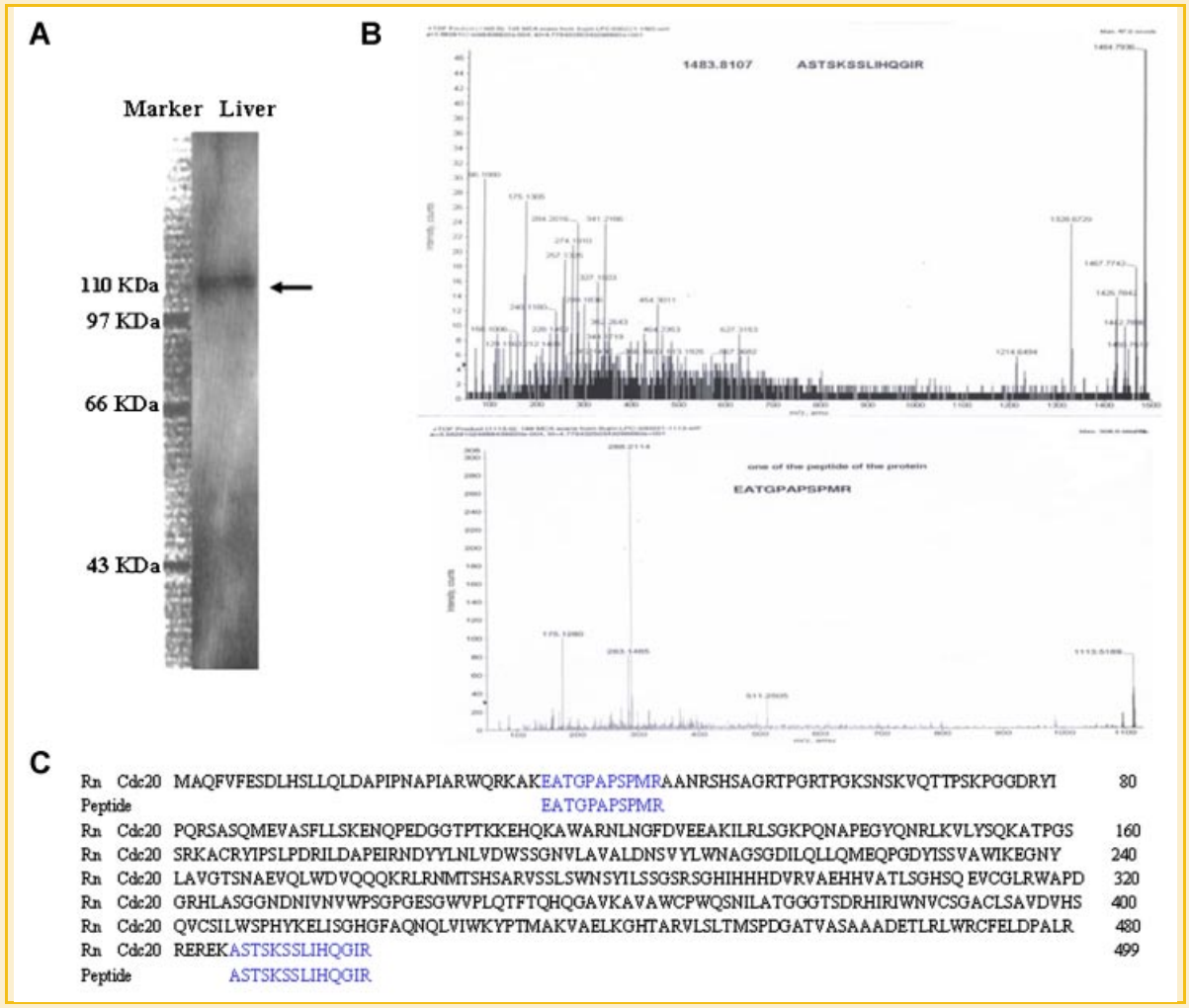

Fig. 2. Cdc20 was identified in the complex of immunoprecipitation. A: PC-PLC protein expression in rat liver was detected by Western blot. The arrow indicated the band at 110 kDa. B: Mass spectrometry analysis of the band. After the homogenate of rat liver tissue was incubated with PC-PLC antibody, co-precipitated, resolved by SDS–PAGE, and stained, the band at 110 kDa was cut out and determined by the MALDI-TOF mass spectrometry. The sequence of the two peptides identified was shown in the figure. C: A BLAST search revealed that the two peptides have homology to the Rattus norvegicus protein Cdc20. Blue = identity; Rn, Rattus norvegicus. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ingly, a sequence of two peptides was identified from the trypsin-digested fragments and was found to match the amino acid sequence of Cdc20 (Fig. 2C), suggesting that Cdc20 was in the complex of immunoprecipitation, and that Cdc20 is associated with PC-PLC. To confirm this, we re-examined immunoprecipitation of the Cdc20-associated PC-PLC activity in CBRH-7919 cells. The results (Fig. 3A) showed that there is an association of Cdc20 with PC-PLC. We also examined whether the products of immunoprecipitation have activity of PC-PLC. Our results showed that the isolated complex had enzymatic activity on PC, but no enzymatic activity in the control (Fig. 3B).

More detailed insight on the association between Cdc20 and PC-PLC in rat CBRH-7919 cells was obtained by dual fluorescence CLSM evidence pertaining to the co-localization of these proteins. As shown in Figure 3C, both PC-PLC and Cdc20 were co-localized in the perinuclear endoplasmic reticulum region (or the ''juxtanuclear quality control'' compartment, JUNQ). A similar effect was found in CBRH-7919 cells after transfection of pEGFP-Cdc20 plasmid (Fig. 3D). These findings further support the association of Cdc20 with PC-PLC in the cellular mechanisms responsible for target cells.

PC-PLC AND CDC20 IN DIFFERENT CELL-CYCLE PHASES

Cdc20 is an essential cell-cycle regulator. Since the data above indicated an association of Cdc20 with PC-PLC, we assume that PC-PLC may be involved in cell-cycle. The cell-cycle profile of PC-PLC expression levels and activities was examined in rat CBRH-7919 cells. After cells were synchronized, CBRH-7919 cells were collected at the following cell-cycle phases: G_0, G_1, S, G_2, and M (obtained via mitotic shake-off, which does not depend on the spindle damage checkpoint arrest). As expected from previous work [Fang et al., 1998; Eytan et al., 2006; Fry and Yamano, 2006; Yu, 2007; Lindon, 2008], Cdc20 protein levels increased to a peak in G_2, remained high in M phase, and decreased somewhat in G_1 and G_0 phase cells

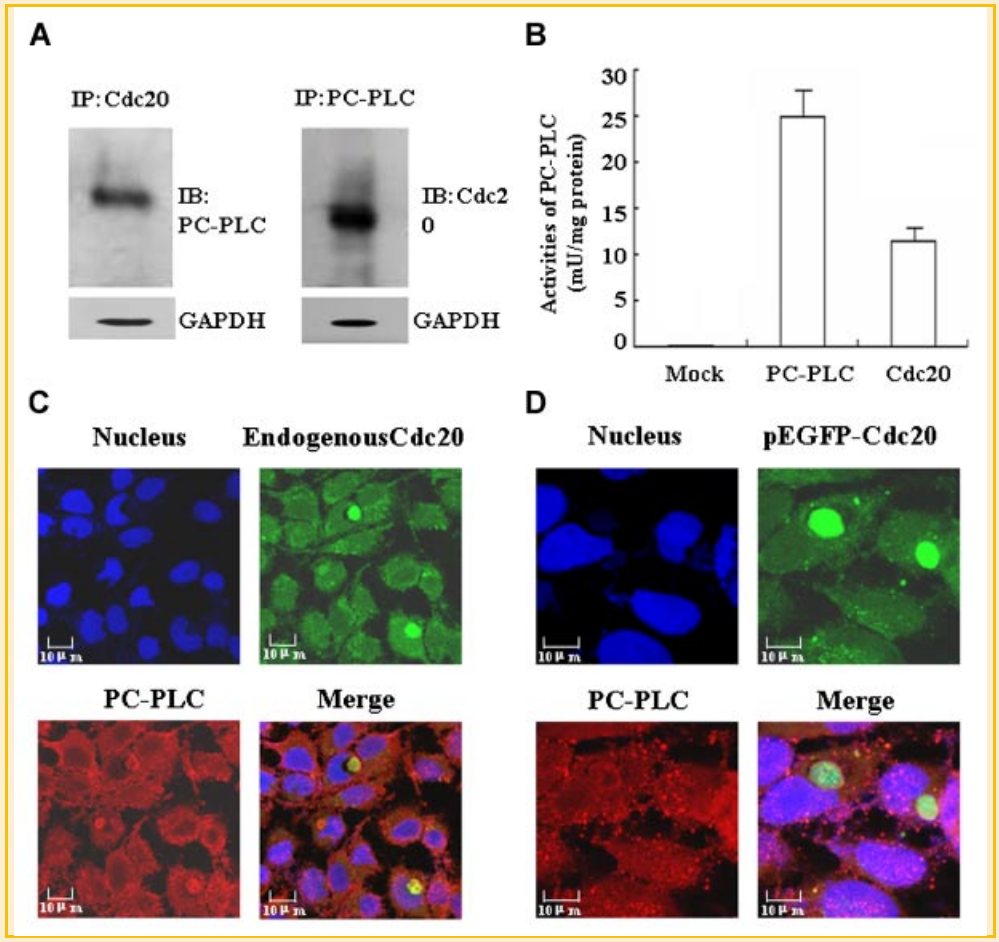

Fig. 3. The interaction between PC-PLC and Cdc20. A: Co-precipitation of PC-PLC and Cdc20. CBRH-7919 cell lysates were subjected to immunoprecipitation with a rabbit-anti-PC-PLC antibody, and analyzed by Western blot for Cdc20. GAPDH staining was used as the total protein control (Left panel). CBRH-7919 cell lysates were subjected to immunoprecipitation with a mouse-anti-Cdc20 antibody, and analyzed by Western blot for PC-PLC (Right panel). B: Precipitates were analyzed by the Amplex Red® PC-PLC assay kit for measurement of PC-PLC activity after CBRH-7919 cell lysates were subjected to immunoprecipitation using rabbit-anti-PC-PLC antibody or mouse-anti-Cdc20 antibody, respectively. C: The interaction of endogenous Cdc20 with PC-PLC. CBRH-7919 cells were simultaneously stained with rabbit-anti-PC-PLC antibody and mouse-anti-Cdc20 antibody, followed with incubation with Rhodamine-conjugated anti-rabbit antibody (red) and FITC-conjugated anti-mouse IgG antibody (green). The interaction was viewed with fluorescence merging (yellow). D: The interaction of exogenous Cdc20 with PC-PLC. CBRH-7919 cells were cultured on cover slips and transfected with plasmid pEGFP-Cdc20. The cells were then stained with anti-PC-PLC antibody, followed with incubation with Rhodamine-conjugated anti-rabbit antibody, and the nucleus was stained with Hoechst 33342 (blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 4A,C). Conversely, PC-PLC levels and activities were high in G_1 phase cells, gradually lower across the cell-cycle, and lowest in both G_2 and M phase cells (Fig. 4A,B). Data suggested that there is an inverse correlation between PC-PLC protein level/activity and the expression of Cdc20.

PC-PLC DEGRADATION MEDIATION BY CDC20

First identified as a protein essential for cell-cycle progression in budding yeast, Cdc20 has since emerged as a major mitotic activator of the APC/C, a large cell-cycle degradation machine. As such, Cdc20 appears to bridge the interaction between APC/C and its substrates. Since PC-PLC can interact with Cdc20, it may be responsible for the decrease of PC-PLC activity in certain cell-cycles. This degradation could potentially have been due to APC/C^{cdc20}. In order to obtain more detailed information on PC-PLC expression affected by Cdc20, this experiment was performed in order to examine whether Cdc20 expression could mediate the degradation of PC-PLC. Also, the silence of Cdc20 by siRNA could upregulate the enzyme activities and protein levels in CBRH-7919 cells. A downregulation of PC-PLC expression was observed after pcDNA3.1-Cdc20 transfection (Fig. 5A,B) as compared to control ($P < 0.05$). The enzyme activity of PC-PLC was also significantly decreased ($P < 0.05$; Fig. 5C). A similar effect was found in both COS-7 and LW3 cells (Fig. 5D). The observed alterations in PC-PLC enzyme activity were fully reversible in siRNA-Cdc20-transfected cells (Fig. 5). These results provided in vitro evidence to support the hypothesis that regulation of certain mitotic checkpoint proteins plays an important part in PC-PLC regulation. These experiments also indicated a possible involvement in the mammalian cell-cycle of PC-PLC.

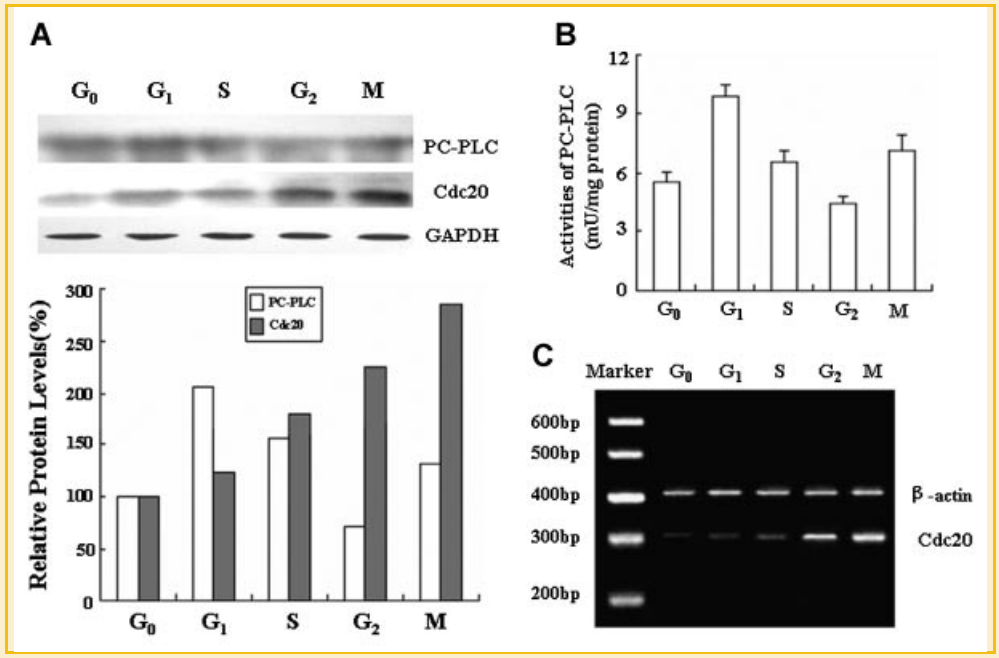

Fig. 4. The expression of PC-PLC and Cdc20 in different cell-cycle phases. A: The protein level of PC-PLC and Cdc20 in different cell-cycle phases. Total cell extracts were prepared, resolved by SDS–PAGE, and blotted for the measurement after synchronization of cell-cycle. The protein expression of PC-PLC and Cdc20 in CBRH-7919 cells was compared among different cell-cycle phases. B: Observation of PC-PLC activity in different cell-cycle phases. Total extracts from CBRH-7919 cells in different cell-cycle phases were prepared after synchronization of cell-cycles and the activity of PC-PLC measured by using the Amplex Red® PC-PLC assay kit. C: Cdc20 mRNA expression in CBRH-7919 cells with different cell-cycles was measured by RT-PCR.

PC-PLC TRANSPOSITION BY CDC20 IN CBRH-7919 CELLS

In CBRH-7919 cells, PC-PLC was spottily located on the membrane and slightly spread in the internal side of the membrane and in cytoplasm (Fig. 1D). The PC-PLC was distinctly located in the "juxtanuclear quality control" compartment, or JUNQ (Fig. 3D), where PC-PLC and Cdc20 were co-localized with the perivacuolar peripheral inclusion. In CBRH-7919 cells transfected with exogenous Cdc20, the signal was enhanced in the JUNQ compartment, where PC-PLC was co-localized Cdc20. In cells with low expression of Cdc20, PC-PLC was mainly on the membrane and slightly spread in the cytoplasm. This new finding strongly suggests that the major mitotic activator of APC/C, Cdc20 is able to induce (or mediate) translocation of PC-PLC across the plasma membrane. This has possible implications not only on cell biochemistry (impairment of PC-PLC activity, and consequent production of intra- and extracellular Pcho and accumulation of neutral lipids), but also on cell-cycle mechanisms, which facilitate temporal and spatial regulation of APC/C^{Cdc20}.

UBIQUITINATION OF PC-PLC BY CDC20

To investigate whether PC-PLC fluctuation in the cell-cycle phases was regulated by APC/C^{Cdc20}-mediated ubiquitin-proteasomal degradation, the degradation profile of PC-PLC was examined. In agreement with the protein expression and enzyme activity of PC-PLC, the level of ubiquitinated PC-PLC was increased by Cdc20 transfection. The observed alterations in ubiquitinated PC-PLC were fully reversible in siRNA-Cdc20-transfected cells. To investigate the effect of proteasome inhibitor MG132 on the ubiquitination of PC-PLC, cells were treated with MG132 (10 μmol/L) for 24 h. Our results indicated that the addition of MG132 induced accumulation of ubiquitinated PC-PLC (Fig. 6). Therefore, it was concluded that Cdc20 mediated degradation of PC-PLC and diminished the enzyme activity of UPP.

DISCUSSION

Hydrolysis of PC, the major phospholipid in eukaryotic cell membranes, involves the use of distinct classes of phospholipase, including phospholipase A_2 (PLA_2), C (PLC), and D (PLD) [Chen et al., 1997; Li et al., 2006]. Limited evidence correlates with the possible involvement of PC-PLC, the major enzyme of the PC cycle, in the mammalian cell-cycle. This holds responsibility for the production of Pcho and non-PIP2-derived DAG, sustaining long-term cellular responses such as activation, proliferation, and differentiation events [Plo et al., 2000; Moreno-Garcia et al., 2005]. PC-PLC catalyzes the production of Pcho and DAG in a number of cells, which are involved in long-term cellular responses and considered to be responsible for protein kinase C activation [Clark et al., 1986; Podo et al., 1996; Monick et al., 1999]. However, no PC-PLC binding partners have currently been identified from mammalian sources, nor has the mechanism by which the enzyme is involved in cellular response been elucidated.

To address the distribution of PC-PLC in mammalian tissues, we analyzed the enzyme activity in various rat tissues. Our results showed that the activity of PC-PLC is mainly distributed in the

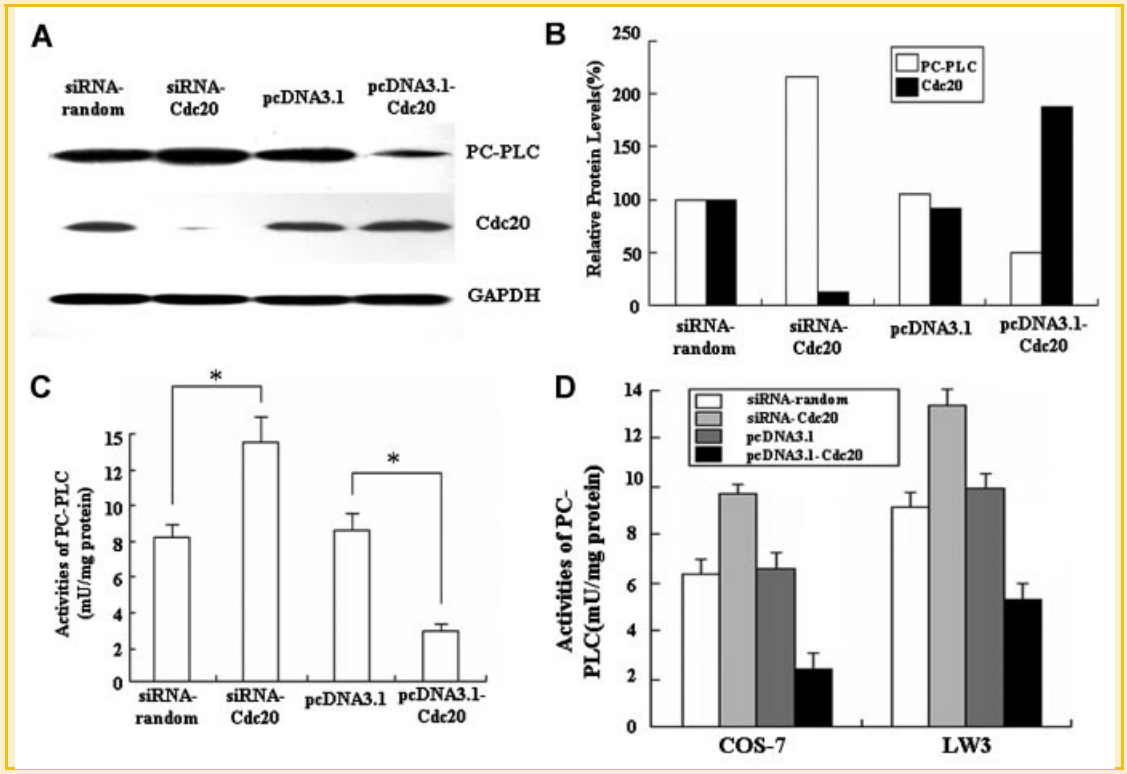

Fig. 5. Observation of Cdc20 effect on PC-PLC expression and activity. A: Regulation of PC-PLC protein expression. CBRH-7919 cells were transfected with pcDNA3.1-Cdc20 or siRNA-Cdc20 (24 h), while vector pcDNA3.1 or siRNA-random as the control, respectively. The expression of PC-PLC and Cdc20 was measured by Western blot. GAPDH was used as a loading control. B: Density analysis of the bands of PC-PLC and Cdc20. Bands in A were scanned with a densimeter and compared in gray scale. C: Regulation of PC-PLC activity. Total cell extracts were prepared 24 h after transfection and PC-PLC activity was determined by using the Amplex Red® PC-PLC assay kit. D: Regulation of PC-PLC activity in COS-7 and LW3 cells. After harvest, total cell extracts from the transfectants were prepared and PC-PLC activity was assayed. Cdc20-related regulation of PC-PLC activity was observed in COS-7 and LW3 cells.

digestive system, including the intestines, stomach, and liver (Fig. 1A). PC-PLC activity in different cell lines was also detected by the assay kit (Fig. 1B) and the results showed that PC-PLC is present in different cell lines and rat tissues (Fig. 1).

PC-PLC hydrolyzes the phosphate bond on PC, yielding diacylglycerol and phosphorylcholine. In recent years, PC-PLC has been reported to be associated with a growing number of critical signal transduction mechanisms in eukaryotic cells [Cheng et al., 1997; Nofer et al., 2000; Zhang et al., 2001]. In mammalian systems, the product DAG is involved in powerful signal transduction cascades and has been implicated in transformation, proliferation, and inflammation [Exton, 1990; Nishizuka, 1992; Rhee and Bae, 1997; Tschaikowsky et al., 1998]. However, it is unclear how the potential mitotic functions of PC-PLC contribute to cytodieresis. These issues need to be resolved by additional experiments. Therefore, we tried to identify the mammalian PC-PLC and its potential role in cell signaling. Occasionally, we identified cell division cycle 20 (Cdc20) in the complex precipitated by PC-PLC antibody. It was found that Cdc20 and PC-PLC could be co-precipitated by antibodies against either Cdc20 or PC-PLC in rat hepatoma cells CBRH-7919 and rat liver tissue. Through confocal microscopic investigation, PC-PLC and Cdc20 were further found to be co-localized in the perinuclear endoplasmic reticulum region (JUNQ).

Cdc20 is an essential cell-cycle regulator required for the completion of mitosis in organisms from yeast to human beings, and contains a WD40 repeat domain at its C terminus, that mediates protein–protein interaction. In mitosis, Cdc20 binds to and activates the ubiquitin ligase activity of APC/C and enables the ubiquitination and degradation of securin and cyclin B [Arvand et al., 1998; Bastians et al., 1998], thus promoting the onset of anaphase and mitotic exit. APC/C^{Cdc20} is temporally and spatially regulated during the somatic and embryonic cell-cycle by numerous mechanisms, including the spindle checkpoint and the cytostatic factor (CSF). Therefore, Cdc20 serves as an integrator of multiple intracellular signaling cascades that regulate progression through mitosis [Yu, 2007]. As such, Cdc20 appears to bridge the interactions between APC/C and its substrates [Camasses et al., 2003]. Our findings suggest that in mammalian cells, there is an interaction between Cdc20 and PC-PLC. Through this interaction, Cdc20 can alter the intracellular distribution of PC-PLC, mediate the degradation of PC-PLC, and diminish the enzyme activity by Cdc20-mediated UPP. We proposed that Cdc20 combines with PC-PLC, then mediates ubiquitination and degradation of PC-PLC. Therefore, PC-PLC may be a new substrate of APC/C^{Cdc20}. Further work is needed to be done to understand the mechanism by which PC-PLC contributes to spindle checkpoint.

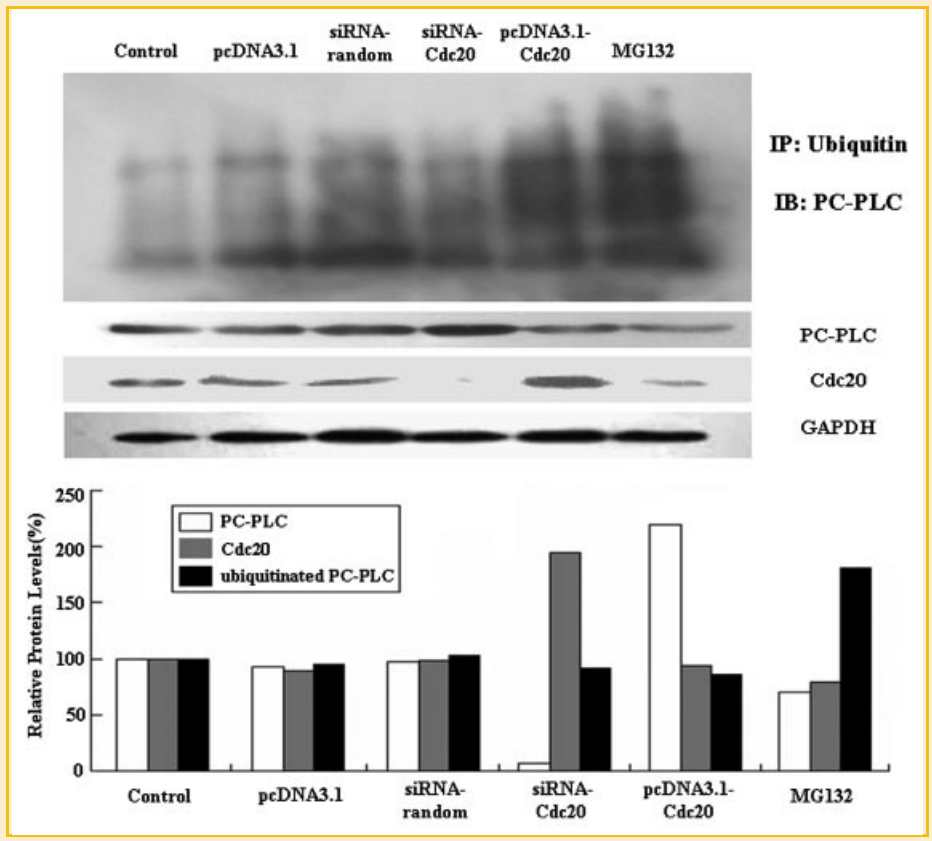

Fig. 6. Cdc20 induction of ubiquitylation-dependent degradation of PC-PLC. CBRH-7919 cells were treated in absence or presence of MG132 (10 μmol/L, for 24 h) and transfected with pcDNA3.1-Cdc20 or siRNA-Cdc20 (24 h). Vectors pcDNA3.1 and siRNA-random were used as their control, respectively. DMSO with the equal volume to MG132 group was used as a solvent control. The expression of PC-PLC and Cdc20 was measured by Western blot, respectively. GAPDH staining was as a loading control. Precipitates were analyzed by Western blot for the measurement of ubiquitinated PC-PLC by using rabbit-anti-PC-PLC antibody after the cell lysates were subjected to immunoprecipitation by anti-ubiquitin antibody.

APC/C is a large (1.5 MDa) and multi-subunit ubiquitin ligase that controls two key events in mitosis: sister chromatid separation and inactivation of cyclin-dependent kinases (Cdks) via ubiquitylation [Bharadwaj and Yu, 2004]. The activity of APC/C has been shown to be highly regulated at different levels. In addition to phosphorylation, the activity of APC/C is controlled by the recruitment of Fizzy family activators comprising two related WD40 repeat proteins, Fizzy/Cdc20 and Fizzy-related/Cdh1. This checkpoint inhibits APC/C activation until all the chromosomes are attached in a bipolar manner [Musacchio and Hardwick, 2002; Yu, 2002]. Similarly, PC-PLC binding to Cdc20 may block the formation of APC/C^{Cdc20} complex and subsequently affect the activity of the APC/C^{Cdc20} complex. The fine-tuning of the APC/C activity by a substrate that is also an inhibitor, is required for the precise coordination and transition through meiosis [Kraft et al., 2003]. From our results, we think that PC-PLC interacts with Cdc20 and then binds to APC/C, and hence make a bold hypothesis that PC-PLC may be both a substrate and an inhibitor of APC/C. Information for further investigating the role of PC-PLC in cell-cycle regulation should be provided.

Once misfolded, proteins will be recognized and ubiquitinated by the quality control machinery, which directs them to the JUNQ. JUNQ is formed in an indentation of the nucleus, is closely associated with the nucleus, and may be flanked by proliferations of the nuclear membrane. Soluble ubiquitinated misfolded proteins accumulate in a juxtanuclear compartment where proteasomes are concentrated. The perinuclear JUNQ compartment acts as a major site of proteasome concentration and misfolded protein degradation. In this study we observed the ubiquitination of PC-PLC was regulated by Cdc20. This was an interesting result, given that the likely signal transduction pathway employed in cell-cycle PC-PLC, as an APC/C regulator directly or indirectly restricting APC/C activity, is itself APC/C substrates. An increase in APC/C activity is expected to cause a decrease in the levels of APC/C inhibitors, leading to further activation of APC/C [Fry and Yamano, 2006]. This mutually antagonistic relationship between APC/C and its regulators ensures the abrupt and irreversible transitions during mitosis [Nasmyth, 2005].

Taken together, our findings suggest that in mammalian cells there is an interaction between Cdc20 and PC-PLC. Cdc20 can alter the intracellular distribution of PC-PLC, mediate the degradation of PC-PLC, and diminish the enzyme activity by Cdc20-mediated UPP. We propose that Cdc20 combines with PC-PLC to mediate ubiquitination and degradation of PC-PLC.

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