Influence of proline-rich inositol polyphosphate 5-phosphatase, on early development of fertilized mouse eggs, *via* inhibition of phosphorylation of Akt

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Abstract

Objectives: Proline-rich inositol polyphosphate 5-phosphatase (PIPP) is one of the signal-modifying enzymes that play pivotal regulatory roles in PI3K signalling pathway. The aim of this study was to determine the role of PIPP in early development of fertilized mouse eggs, *via* inhibition of Akt activity and subsequent downstream signalling events.

Materials and methods: The mRNA transcript levels of endogenous PIPP and Akt1, Akt2, Akt3 were detected in G₁, S, G₂ and M phases of fertilized mouse eggs by RT-PCR. Levels of exogenous PIPP, phosphorylated Akt at Ser473, dephosphorylated cdc2 at Tyr15 and levels of CCNB1, were detected respectively by immunoblotting. Changes in Akt localization were observed by fluoroimmunoassay; meanwhile, changes in activity of Akt and its downstream MPF were detected. Percentages of cells undergoing division were determined by counting, using a dissecting microscope.

Results: PIPP and Akt1 transcripts were detectable in G₁, S, G₂ and M phases of fertilized mouse eggs, but Akt2 and Akt3 were not. We also observed that overexpression of PIPP in fertilized eggs decreased expression of phosphorylated Akt at Ser473 and altered membrane localization of phosphorylated Akt at Ser473 specifically. Furthermore, overexpression of PIPP resulted in decreases in mitosis-phase promoting factor activity, level of dephosphorylated cdc2 at Tyr15 and cleavage rate of fertilized mouse eggs.

Conclusions: Our data suggest, for the first time, that PIPP may affect development of fertilized mouse eggs by inhibition of level of phosphorylated Akt at Ser473 and subsequent inhibition of downstream signal cascades.

Introduction

Proline-rich inositol polyphosphate 5-phosphatase, PIPP, is a novel regulator of phosphoinositide 3-kinase (PI3K) signalling pathway. PIPP hydrolyzes 5-position phosphate of phosphatidylinositol 3,4,5 trisphosphate [PtdIns(3,4,5)P₃] or phosphatidylinositol 4,5 bisphosphate [PtdIns(4,5)P₂] to form PtdIns(3,4)P₂ or PtdIns(4)P, respectively (1–3). Mitchell *et al.* have demonstrated that PIPP may inhibit amplitude of Ser473-Akt phosphorylation by means of hydrolysing PtdIns(3,4,5)P₃ to decrease binding of PtdIns(3,4,5)P₃ and PH domains of Akt in somatic cells (3). Therefore, we postulate that PIPP may also lower the level of phosphorylated Akt at Ser473 in fertilized mouse eggs.

Akt, also called protein kinase B, is a serine/threonine protein kinase and is a downstream factor of PI3K. It is well established that Akt plays an important role in many cell processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration (4–7). There are three isoforms of Akt (1, 2, 3, PKB α , β , γ) and they share high sequence identity and are composed of three functionally distinct regions: an N-terminal pleckstrin homology (PH) domain (amino acids 1–106), a central catalytic domain (amino acids 148–411) and a C-terminal regulatory domain (amino acids 412–480). The PH domain of Akt mediates interactions of Akt with other proteins involved in signal transduction by binding PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂, and then targeting Akt to plasma membranes. Membrane recruitment is a hallmark of Akt activation (8–10). When Akt is in its stable form, it dissociates from the plasma membrane and targets

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substrates located in the cytoplasm and nucleus (8). However, when Akt is phosphorylated at residue Ser473, it is activated and recruited to the cell membrane (8–10). Although it is well established that phosphorylation of Akt at Ser473 is required for plasma membrane localization and that PIPP may inhibit the level of phosphorylation of Akt at Ser473 (8–11), whether PIPP plays a role as negative regulator of Akt in fertilized mammalian eggs remains unexplored.

Previously, we have reported that Akt can phosphorylate cdc25B-S351 (cell division cycle 25 homologue B) and subsequently activate mitosis-phase promoting factor (MPF) to promote cell division of fertilized mouse eggs (12). MPF is a highly conserved complex consisting of a cdc2 kinase and an activating subunit CCNB1 (13–17); prior to mitosis, cdc2/CCNB1 complex remains enzymatically inactive. On entry into M phase, cdc25 dephosphorylates cdc2 on both residues Tyr15 and Thr14, leading to activation of MPF (18,19). Thus, it is likely that G₂/M transition (activation of MPF) is induced by dephosphorylation of cdc2 through cdc25 (20–24). We have previously demonstrated that Akt activity is associated with dephosphorylation of cdc2 and G₂/M transition in fertilized mouse eggs (12). Moreover, PIPP, as one of the newly categorized AKT negative regulators, has been reported to play a critical role in some somatic cells. However, PIPP function in signalling events in development of fertilized mammalian eggs, remains largely unknown.

The fertilized mouse egg is the simplest natural mitotic cycle model in vertebrates that is close to fertilized human eggs, but there have only been limited reports on studying regulatory mechanisms of mitosis of fertilized mouse eggs. We have previously shown that Akt may be involved in regulating G₂/M transition in cells of fertilized mouse eggs (12), therefore, we hypothesize that PIPP might play an important role in their early development by inhibiting phosphorylation level of Akt. To test this hypothesis, in this study we examined the effect of PIPP overexpression on Akt phosphorylation at Ser473, as well as its downstream signalling events, in the early development of fertilized mouse eggs. Our results show that PIPP indeed plays an important role in their early development through influencing the PI3K/Akt signalling pathway.

Materials and methods

Animals

Kunming strain mice were obtained from the Department of Laboratory Animals, China Medical University (CMU). All experiments were performed at CMU

in accordance with NIH Guidelines of USA for Care and Use of Laboratory Animals. Protocols for animal handling and treatment procedures were reviewed and approved by the CMU Animal Care and Use Committee.

Reagents and plasmids

Phospho-Akt1/2/3 (Ser 473) polyclonal antibody, β -actin antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody, phospho-cdc2 at Tyr15 monoclonal antibody, cdc2 monoclonal antibody and CCNB1 antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), enhanced chemiluminescence (ECL) detection kit was from Pierce Biotechnology Inc., Rockford, IL, USA. [γ -³²P]ATP was purchased from Peking YaHui Biotechnology Co., Beijing, China and pEFBOS vectors (FLAG vector) and pEFBOS-PIPP (FLAG-PIPP) plasmids were kind gifts from Professor Christina Mitchell (Monash University, Australia). Other reagents unless otherwise specified, were purchased from Sigma-Aldrich, Shanghai, China.

Collection and culture of mouse GV-intact oocytes and fertilized eggs

GV-intact oocytes were collected from 3-week-old female Kunming mice (24,25) by first immersing ovaries in M2 medium. Follicles were punctured with a fine needle to release cumulus-enclosed oocytes or naturally denuded GV-intact oocytes. GV-intact oocytes were released from attached follicular cells by repeated pipetting with a mouth-operated micropipette, and then collected and stored at -70°C until used.

Fertilized mouse eggs were collected and cultured according to the method described by Hogan and Constantini (26). Female mice (Kunming strain, 4–6 weeks old) were abdominally injected with 10 IU of pregnant mare's serum gonadotropin and 48 h later with 10 IU human chorionic gonadotropin (hCG). A single female was placed with a single male for fertilization. Fertilized mouse eggs were collected with M2 medium the next day (20 h after the hCG injection) from the oviducts of females possessing a vaginal plug. These fertilized eggs were collected by repeated pipetting and counting with a mouth-operated micropipette, and then were cultured in M16 medium under paraffin oil at 37°C in humidified atmosphere of 5% CO₂ in air, until reaching desired cell cycle phases (G₁, 11–20 h after hCG injection; S, 20–26 h after hCG injection; G₂, 26–29 h after hCG injection; M, 29–32 h after hCG injection) (Fig. 1).

Figure 1. Development model of fertilized mouse eggs. G₁ phase: 0–9 h post fertilization (11–20 h after hCG injection); S phase: 9–15 h post fertilization (20–26 h after hCG injection); G₂ phase: 15–18 h post fertilization (26–29 h after hCG injection); M phase: 18–21 h post fertilization (29–30 h after hCG injection).

Isolation of mRNA, cDNA synthesis and PCR

Total mRNA was extracted from GV-intact oocytes and fertilized mouse eggs (200 eggs for each sample) at each phase of mitosis, using QuickPrepTM MicromRNA Purification Kit (Amersham Bioscience Co., Shanghai, China) according to manufacturer's instructions. RT-PCR reactions were carried out using RNA PCR Kit (AMV) Ver2.1 (TaKaRa Biotechnology (Dalian) Co., Ltd.) according to manufacturer's instructions. Sequences of primers were designed using PRIMER 5.0 software, and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd., Shanghai, China. Sequences of primers for Akt1, Akt2, Akt3, PIPP and β -actin cDNA amplifications are shown in Table 1. PCR products were separated by electrophoresis in 1% agarose gel and stained with ethidium bromide for visualization.

Immunocytochemical analysis

GV-intact oocytes, fertilized eggs at G₁, S, G₂ or M phase, and fertilized eggs at G₁ phase microinjected with FLAG vector, FLAG-PIPP 1 μ g/ml and/or wortmannin 1 μ M for 10 pL/per egg for 10 h were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature (RT) and permeabilized for 30 min in 0.5% TritonX-100 in PBS at 37 °C. These eggs were stained overnight with phospho-Akt (Ser473) antibody diluted 1:100 at 4 °C. After washing three times in PBS containing 1 mg/ml bovine serum albumin (BSA), eggs were incubated for 2 h at RT in FITC-conjugated goat anti-

rabbit secondary antibody, followed by staining with 2 μ g/ml propidium iodide (PI) for 30 s at RT for chromatin visualization. Immunofluorescence images were obtained using a laser scanning confocal microscope (TCS SP2 AOBS; Leica Microsystems Inc., Exton, PA, USA) with Leica confocal software.

Microinjection and observation of fertilized mouse eggs

Plasmid DNAs (FLAG-PIPP or FLAG vector) were diluted to 1 μ g/ml in 5 mmol/l Tris and 0.5 mmol/l EDTA (pH 7.4; TE) without nuclease contaminant and were microinjected into the fertilized eggs at G₁ phase (18 h after injection of hCG) using a NARISHIGE microinjection system (Japan), and an Olympus model IX-70 inverted microscope with Hoffmann optics, essentially according to methods described by Bornslaeger (27). Typical injection volume was 5% of total cell volume or 10 pL per egg. Eggs in control groups were either not microinjected or microinjected with TE buffer, FLAG vector or wortmannin. Eggs were cultured in M16 medium and collected at indicated time-points after hCG injection, for the further experiments. Percentages of cell division were determined after counting using a dissecting microscope, 30 h after injection of hCG and results were analysed statistically. In addition, microinjected eggs from each group were collected at 26 h for Akt activity analysis, and at 28 h for MPF activity analysis, and western blot analysis for assessing expression of FLAG-PIPP, phospho-Akt at Ser473, dephosphorylation status of phospho-cdc2 at Tyr 15 or expression of CCNB1, and stored in -70 °C until used.

Western blotting

Protein extracts of eggs were prepared by adding approximately 200 eggs or 300 eggs in minimal volume of collection medium to 20 μ L of protein extraction buffer (100 mM NaCl, 20 mM Tris-HCl [pH 7.5], 0.5% Triton X-100, 0.5% NP-40) containing 1 mM phenylmethylsulfonyl fluoride and 1 μ g/ml leupeptin and pepstatin. Laemmli sample buffer was added to protein extracts, and mixtures were boiled for 5 min and resolved on 10% SDS-PAGE gel. For immunoblotting, fractionated proteins were

cDNA (Amplicon size; bp)	Forward 5'–3' primer	Reverse 5'–3' primer	Genbank no.
Akt1 (396)	atgctggacaaggacggg	cacgatgttgcaagaa	NM_009652
Akt2 (672)	aaggatgaagtcgccaca	ccagttgatgctgaggaa	NM_007434
Akt3 (292)	gttggttcagaagagg	tcggtacggcttgata	NM_0117852
PIPP (412)	ctgtccctacccttcgg	gcaaccctatggcaatca	NM_172439
β -actin (337)	gtggcatcatgaaactacat	aacgcagctcagtaacagtc	NM_007393

Table 1. Primer sequences used in PCR analyses

transferred to nitrocellulose membranes which were blocked with 3% BSA in Tris-buffered saline containing 0.05% Tween 20, and probed with primary antibodies in a sealed plastic bag at 4 °C overnight. Primary antibodies against FLAG tag, phospho-Akt1/2/3 (Ser473), β -actin, phospho-cdc2 at Tyr15, cdc2 or CCNB1 were used at 1:400 dilution. Membranes were then incubated in HRP-conjugated second antibody at 1:2000 (Beijing Zhongshan Biotechnology, Beijing, China). Proteins were detected using an ECL detection system.

Assay of MPF activity

MPF kinase activity was measured using histone H1 kinase assay (12,28–31). Five cultured eggs in M16 medium were collected, washed in collection buffer (PBS containing 1 mg/ml polyvinyl alcohol, 5 mM EDTA, 10 mM Na_3VO_4 and 10 mM NaF), and then transferred to an Eppendorf tube containing 5 μl of collection buffer. The Eppendorf tube was immediately stored at -70°C until kinase assay was performed.

Frozen eggs were thawed and subjected to freezing and thawing three times. A total of 25 μl of MPF buffer (54 mM β -glycerophosphate, 14.5 mM p-nitrophenylphosphate, 24 mM 3-(*N*-morpholino)-propanesulfonic acid [MOPS; pH 7.2], 14.5 mM MgCl_2 , 14.5 mM ethyleneglycoltetraacetic acid, 0.12 mM EDTA, 1 mM dithiothreitol [DTT], 2.4 μM PKA inhibitor peptide [PKI], 75 mM genistein [a tyrosine kinase inhibitor], 10 μM ML-9 [a myosin light chain kinase inhibitor], 1 mg/ml histone H1 [type III-s], and 1 mg/l each of leupeptin, aprotonin, pepstatin, chymostatin, and trypsin-chymotrypsin inhibitor) was then added to disrupted cells. Histone H1 kinase reaction was initiated by adding 25 μl of 20 $\mu\text{Ci/ml}$ [γ - ^{32}P]ATP incubated at 30 °C for 10 min. Then 25- μl aliquots were spotted on Whatman p81 paper, and reaction was stopped with 5% H_3PO_4 solution. After thorough washing, radioactivity on the filter paper was counted using a Beckman Scintillation Counter (Beckman, Preston, Maryland, USA).

Parallel incubation was performed to confirm phosphorylation of histone H1. Protein extract from 10 eggs was incubated in 50 μl of MPF reaction buffer containing 50 $\mu\text{Ci/ml}$ [γ - ^{32}P]ATP at 37 °C for 30 min, and reaction was stopped by adding an equal quantity of 2 $\times$ SDS buffer. The reaction was then resolved on 12% SDS-PAGE gel, and incorporation of ^{32}P into histone H1 was visualized by autoradiography.

Assay of Akt activity

Ten eggs cultured in M16 medium were collected and lysed as described above and assayed for 30 min at room temperature in Akt reaction buffer containing 50 mM

Hepes (pH7.5), 10 mM MgCl_2 , 1 mM DTT, 1 μM PKI, 40 $\mu\text{Ci/ml}$ [γ - ^{32}P]ATP, and 0.2 mg/ml histone H2B as substrate. Akt activity was also determined by scintillation counting and autoradiography, as in the MPF activity assay.

Statistical analysis

Data are presented as mean $\pm$ SEM of separate experiments ($n > 3$) and compared by one-way analysis of variance with SPSS 11.5 software (SPSS, Chicago, IL, USA). $P < 0.05$ was taken as significant.

Results

Expressions of Akt1, Akt2 and PIPP mRNA

We have previously reported that Akt may phosphorylate cdc25B-Ser351 and subsequently activate MPF to promote cell division of fertilized mouse eggs. However, expressions of three Akt isoforms and PIPP at mRNA levels in fertilized mouse eggs have not been examined. Therefore, we first examined transcript levels of Akt1, Akt2, Akt3 and PIPP in G₁, S, G₂, and M phases, by RT-PCR. As shown in Fig. 2, Akt1 and PIPP transcripts were detectable in all phases. However, Akt2 transcript was detected only in G₁ phase of these eggs (Fig. 2) and no Akt3 transcripts were detected (data not shown); GV-intact oocytes and β -actin were used as control groups, respectively. The data suggested that Akt1 and PIPP may be necessary for development of fertilized mouse eggs and that Akt2 may play a major role in prophase of DNA synthesis, but Akt3 may not be required for their development.

Effect of PIPP on phosphorylation status of Akt at Ser473

To determine phosphorylated levels of Akt at Ser473 during G₁, S, G₂ and M phases, samples were taken from

Figure 2. RT-PCR analyses of Akt1, Akt2 and PIPP mRNA expression in fertilized mouse eggs. Line 1: GV-intact oocytes; Line 2: fertilized eggs at G₁ phase; Line 3: fertilized eggs at S phase; Line 4: fertilized eggs at G₂ phase; Line 5: fertilized eggs at M phase. GV-intact oocytes and β -actin were served as controls.

each stage. GV-intact oocytes and β -actin were used as control groups. Immunoblot analysis showed that levels of phosphorylated Akt1/2/3 at Ser473 were higher at four mitotic phases than of GV-intact oocytes (Fig. 3A), suggesting that Akt was phosphorylated.

To determine effects of overexpression of PIPP on phosphorylated Akt at Ser473, fertilized eggs were cultured in M16 medium after microinjection of FLAG-PIPP. Two control groups were non-microinjected or microinjection with FLAG vector only. Cell lysates

Figure 3. Effect of PIPP overexpression on phosphorylation of Akt at Ser473 in fertilized mouse eggs. (A) Expression of phosphorylated Akt at Ser473. (A-a) Immunoblot analysis of phosphorylation of Akt at Ser473. β -actin and GV-intact oocytes were used as control. (A-b) Densitometric analyses of 60 kDa phospho-Akt at Ser473 (pAkt Ser473) expression were reported as phosphorylated Akt at Ser473/ β -actin ratio. Asterisk represents level of significance between experimental groups versus control groups (***) $P < 0.001$. Error bar represents mean $\pm$ SEM of three independent experiments. (B) Expression of exogenous PIPP in fertilized mouse eggs and inhibition of overexpression of PIPP on phosphorylation Akt at Ser473. Fertilized mouse eggs were either not microinjected or microinjected with plasmids DNA (FLAG-vector or FLAG-PIPP). Eggs were immunoblotted with antibodies specific for FLAG tag or phospho-Akt at Ser473. β -actin served as loading control.

were immunoblotted with FLAG tag antibody, phosphorylated Akt at Ser473 site-specific antibody, or β -actin antibody (as control) (Fig. 3B). Compared to untreated eggs and FLAG vector-expressing eggs, overexpression of PIPP at around 130 kDa was observed in fertilized eggs microinjected with FLAG-PIPP (Fig. 3B, upper panel). As molecular weight of FLAG vector (about 1 kDa) is too small to be observed, we ignored its expression. Levels of phosphorylated Akt at Ser473 was distinctly reduced in eggs microinjected with FLAG-PIPP (Fig. 3B, middle panel) compared with eggs non-microinjected or microinjected with FLAG vector only, suggesting that overexpression of PIPP negatively regulated phosphorylation status of Akt at Ser473 in mouse one-cell embryos.

Overexpression of PIPP affects localization of phosphorylated Akt at Ser473

Previous reports have indicated that Akt activity is driven by membrane localization initiated by binding PH domain of Akt to PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂, followed by phosphorylation at residues serine 473 and threonine 308 (3,9,32). Phosphorylation of Thr308 may promote conformation change of hydrophobic and phosphate binding pockets more suitable for the hydrophobic motif (HM) binding to other proteins (8). Thus, membrane localization of Akt requires Ser 473 phosphorylation (8,32,33).

Furthermore, PIPP has been identified as a negative regulator of PI3-kinase signalling by hydrolysing 5-position phosphate of PtdIns(4,5)P₂ and/or PtdIns(3,4,5)P₃ and regulation of phosphorylation of Akt at Ser473 in somatic cells (3). Hence, here we examined the effect of PIPP on localization of phosphorylated Akt at Ser473 in mitotic stages in development of fertilized eggs. Samples were incubated with anti-phospho-Akt (Ser473) specific antibody, and subsequently labelled by secondary FITC-conjugated goat-anti-rabbit antibody (Fig. 4A, left panels, green). DNA was counterstained with PI (Fig. 4A, middle panels, red). Results showed that phosphorylated Akt at Ser473 co-localized with cytomembranes of fertilized eggs during mitotic stages (Fig. 4A-a). When fertilized eggs were treated with FLAG-PIPP or wortmannin, localization of phosphorylated Akt at Ser473 was dissociated from cytomembranes to cytoplasm and nucleus. In the meantime, cell division was delayed (Fig. 54-b, line 3 and 4). As controls, phosphorylated Akt in eggs treated with FLAG vector remained localized at the cytomembrane and cell division of these cells was not affected (Fig. 4A-b, line 2). Untreated GV-intact oocytes were used as further control for inactive Akt (Fig. 4A-b, line 1). Our results suggest that PIPP regulated Akt localization by inhibiting its phosphorylated level.

Figure 4. Effects of PIPP on distribution of phosphorylated Akt at Ser473 and Akt activity in fertilized mouse eggs. (A) Expression of phospho-Akt at Ser473 was fixed and detected with pAkt Ser473 antibody and then treated with goat anti-rabbit fluorescein isothiocyanate (FITC)-labelled antibody (green, left). Nuclei were stained with propidium iodide (PI, red, middle). Panels (right) showed mergers of FITC (green) and PI (red). Scale bar = 75 μ m. (A-a) Localization of phospho-Akt Ser473 during G₁, S, G₂ or M phases in fertilized mouse eggs. (A-b) Localization of phospho-Akt Ser473 in GV-intact oocytes or in fertilized eggs microinjected with FLAG vector, FLAG-PIPP or wortmannin. (B) Variation in Akt activities in GV-intact oocytes, untreated fertilized eggs, or fertilized eggs treated with FLAG vector, FLAG-PIPP or wortmannin.

Overexpression of PIPP affects Akt activity

To investigate effects of PIPP on Akt activity, we measured Akt activities using histone H2B as substrate. Fertilized eggs were either not microinjected or microinjected with FLAG vector, FLAG-PIPP, or wortmannin. Untreated GV-intact oocytes were used as further control for inactive Akt. Results showed that Akt activities in non-microinjected fertilized eggs or fertilized eggs microinjected with FLAG vector were significantly higher than that in GV-intact oocytes or fertilized eggs microinjected with FLAG-PIPP or wortmannin ($***P < 0.001$) (Fig. 4B), suggesting that PIPP plays an important role in regulating Akt activity during early development of fertilized mouse eggs.

Overexpression of PIPP affects MPF activity and dephosphorylation of *cdc2* at Tyr15

The inhibitory effect of FLAG-PIPP on Akt activity in fertilized eggs suggests a role for PIPP in regulating fertilized mouse embryonic cleavage, by influencing Akt downstream signal cascades. First, to determine whether overexpression of PIPP may suppress MPF activity (H1 kinase activity) in early development of fertilized eggs were collected at 28 h after hCG injection for assay of H1 kinase activity. The results showed that MPF activities in eggs treated with FLAG-PIPP or Wortmannin were maintained notably at a relatively lower level than that in untreated eggs, or eggs treated with TE buffer or FLAG vectors ($***P < 0.001$; Fig. 5A).

Figure 5. Effects of PIPP on mitotic division of fertilized mouse eggs. (A) Effect of PIPP on MPF activity during G₂/M transition of fertilized mouse eggs. MPF activity was determined with histone H1 as substrate. Eggs were lysed and examined by scintillation counting and autoradiography. Each value was expressed as mean $\pm$ SEM from three independent experiments. Significant difference between experimental groups versus control groups is indicated by asterisk (***) $P < 0.001$). (B) Effect of PIPP on phospho-cdc2 at Tyr15 during G₂/M transition of fertilized mouse eggs. (B-a) Immunoblot analysis of dephosphorylation status of phospho-cdc2 at Tyr15 with the anti-phospho-cdc2 Tyr15 antibody (upper panel). Unphosphorylated cdc2 was detected using anti-cdc-2 antibody (lower panel). (B-b) Densitometric analyses of phospho-cdc2 at Tyr15 expression were reported as phospho-cdc2 at Tyr15/Total Cdc2 ratio). Significant difference between experimental groups versus control groups is indicated by asterisk (***) $P < 0.001$. Error bars represent mean $\pm$ SEM of three independent experiments. (C) Expression of CCNB1 in fertilized mouse eggs. Eggs were untreated, treated with TE buffer, FLAG vector, FLAG-PIPP or wortmannin. (D) Division rate in cultured fertilized mouse eggs after FLAG-PIPP microinjection. Percentage of division was calculated after counting under a dissecting microscope 30 h after injection of hCG in female mice. Data were pooled from three experiments and are expressed as mean $\pm$ SEM. Significant difference between experimental groups versus control groups is indicated by asterisk (***) $P < 0.001$.

Second, to investigate whether overexpression of PIPP may suppress dephosphorylation of cdc2 at Tyr15 in fertilized mouse eggs, we used pTyr15 of cdc2 antibody to detect phosphorylation status of cdc2 at Tyr15 in M phase, by microinjecting them with FLAG-PIPP or wortmannin. Eggs were non-microinjected, microinjected with TE buffer or FLAG vector as control groups. Eggs were collected

at 28 h after hCG injection and immunoblotted using specific phospho-cdc2 Tyr15 antibody; cdc2 antibody was used as control. As anticipated, no significant difference was noted in the three control groups ($P > 0.05$). However, in groups microinjected with FLAG-PIPP or wortmannin, phosphorylation levels of cdc2 at Tyr15 were relatively higher than those of control groups (***) $P < 0.001$;

Fig. 5B), suggesting that PIPP overexpression may delay G_2/M transition by blocking dephosphorylation level of phosphorylated cdc2 at Tyr15, which in turn lead to mitotic delay of these fertilized eggs. In addition, we also examined expression of CCNB1 during G_2/M transition in various groups of eggs (non-microinjected, microinjected with TE buffer, FLAG vector, FLAG-PIPP, or with wortmannin). No significant difference in CCNB1 expression among these groups was observed (Fig. 5C).

Overexpression of PIPP affected egg cell division

To examine whether PIPP would affect the mitotic cell cycle, fertilized mouse eggs were cultured in M16 medium in G_1 phase for 10 h after eggs were non-microinjected, microinjected with TE buffer, FLAG vector, FLAG-PIPP, or wortmannin. Eggs were non-microinjected, microinjected with TE buffer or FLAG vector as control groups. In three control groups, in the region of 56% eggs reached the two-cell stage 30 h after hCG injection; there was no significant difference among these control groups ($P > 0.05$). However, only <11% of eggs that had been microinjected with FLAG-PIPP or wortmannin reached two-cell stages 30 h after hCG injection ($***P < 0.001$, Fig. 5D). These results demonstrate that overexpression of PIPP may interfere with the time course of cell division of fertilized mouse eggs, suggesting that PIPP may indirectly block early cell division by inhibiting Akt activity in these early embryos. Together, these results strongly suggest that PIPP functions as a negative regulator at G_2/M transition by inhibiting Akt phosphorylation and subsequent downstream signalling events.

Discussion

The relatively uncharacterized PIPP possesses N- and C-terminal proline-rich domains, a central 5-phosphatase domain and a SKICH domain. The 5-phosphatase domain encoded by amino acids 424–729 may hydrolyze 5-position phosphate from $\text{PtdIns}(3,4,5)\text{P}_3$ and/or $\text{PtdIns}(4,5)\text{P}_2$ forming $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(4)\text{P}$, respectively. Catalytically inactive PIPP in this 5-phosphatase domain was made from mutating histidine 557 within the PIPP sequence to alanine (3). The SKICH domain from amino acids 736 to 816 mediates PIPP localization to plasma membrane ruffles (Fig. 6). Although PIPP has been shown to have a wide tissue distribution, including in brain, heart, kidney, stomach, small intestine and lung, little is known about its function in fertilized eggs (1,3). Previous reports indicate that PI3K is activated by stimulation of growth factors and then PI3K phosphorylates $\text{PtdIns}(4,5)\text{P}_2$ at D-3 position of the inositol ring to generate $\text{PtdIns}(3,4,5)\text{P}_3$ (34,35). $\text{PtdIns}(3,4,5)\text{P}_3$ may bind

Figure 6. Domain organization of the mammalian proline-rich inositol polyphosphate 5-phosphatases (PIPP) and protein kinase B (Akt). The proline-rich domain of PIPP may hydrolyze 5-position phosphates of $\text{PtdIns}(3,4,5)\text{P}_3$ and/or $\text{PtdIns}(4,5)\text{P}_2$ forming $\text{PtdIns}(3,4)\text{P}_2$ and/or $\text{PtdIns}(4)\text{P}$. The PH domain of Akt may bind $\text{PtdIns}(3,4,5)\text{P}_3$.

the PH domain of Akt and meanwhile, Akt becomes activated which facilitates glucose uptake. In somatic cells, $\text{PtdIns}(3,4,5)\text{P}_3$ is rapidly dephosphorylated by lipid phosphatases including PIPP. However, the role of 5-phosphatases as a suppressor has not been elucidated in fertilized mammalian eggs (36–38).

In this study, we detected Akt1, Akt2, Akt3, PIPP mRNA expression and expressive change of phosphorylated Akt1/2/3 at Ser473 in the first round of mitosis of the fertilized mouse eggs. To the best of our knowledge, these observations have not been reported previously. Furthermore, we microinjected FLAG-PIPP or FLAG vector into the eggs in G_1 phase and harvested them during transition of G_2/M . We chose anti-FLAG tag antibody to detect expression of FLAG-PIPP after microinjecting the eggs with FLAG-PIPP or FLAG vector. Molecular weight of PIPP is 108 kDa and we observed expression of FLAG-PIPP of around 130 kDa in these eggs microinjected with FLAG-PIPP (Fig. 3B, upper panel). Mochizuki and Ooms have also observed this band of around 130 kDa (2,3); moreover, Mochizuki and Takenawa have reported that this was due to phosphorylation of PIPP (2). Our data indicate that exogenous PIPP may be expressed in fertilized eggs and overexpression of PIPP may inhibit phosphorylation of Akt (Fig. 3B, middle panel). Thus, we speculated that PIPP may interfere with the mitotic process of our fertilized mouse eggs *via* an Akt pathway.

A previous report of our team has shown that Akt is one of the key promoting factors for the first round of mitosis of fertilized mouse eggs; Akt overexpression promotes their (12). In this study, we further demonstrated that Akt1 and PIPP transcripts were detected in G_1 , S, G_2 and M phase, but Akt2 transcripts were expressed only in G_1 and no Akt3 transcript was found in all phases

examined (data not shown). These results suggested that as signalling molecules, Akt1 and PIPP may be involved in proliferation and differentiation of early development of fertilized mouse eggs.

As PIPP has only recently been recognized as a regulator in somatic cells, its roles in many aspects of cell function have not yet been fully explored. Mitchell *et al.* have demonstrated that PIPP may affect PtdIns(3,4,5)P₃ and phosphorylation of Akt at Ser473 in somatic cells (3). In the study described here, we examined effects of overexpression of PIPP on phosphorylation of Akt in fertilized mouse eggs. We found that overexpression of PIPP reduced the level of phosphorylated Akt at Ser473, changed localization of phosphorylated Akt at Ser473 and decreased Akt activity and our observations are consistent with previous reports on Akt activity and the role of PIPP in somatic cells (1–3,8–10). Our results suggest that PIPP plays a negative regulatory role in Akt activity and may regulate Akt downstream signalling cascades in the early development of these fertilized eggs.

Early embryonic cell division is characterized by a rapid succession of interphase and mitotic states in many species and it is well known that MPF is a critical regulator of early embryo development in fertilized mouse eggs (27–29,39–43). Our previous reports indicated that Akt may phosphorylate cdc25B at S351 and activate initiation of MPF activation by regulating phosphorylation status of cdc2 at Tyr15 to promote cell division in early mammalian embryos (12). To investigate whether overexpression of PIPP could affect mitosis of fertilized mouse eggs by regulation of Akt activity, we microinjected FLAG-PIPP or FLAG vector into fertilized mouse eggs. Our data are consistent with our hypothesis that overexpression of PIPP inhibits transition of G₂/M by suppressing MPF activity, dephosphorylation status of cdc2 at Tyr15 and cell division.

As a regulatory protein, PIPP may restrict the PI3K signalling pathway by suppressing Akt activation in somatic cells (1–3). This notion is supported by our observation that PIPP down-regulates expression of Akt and its downstream signalling proteins in fertilized mouse eggs and our results provide new evidence that PIPP functions as a negative regulator for mitosis there. Our experiments revealed, for the first time, a connection between PIPP, Akt-Ser473, MPF, Cdc2-Tyr15, and cleavage rate in our specimens. One of the implications of our finding is that PIPP may serve as a novel biomarker for early development of fertilized mouse eggs.

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