

**Signal Transduction:**

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Inositol Pyrophosphates Modulate S Phase Progression after Pheromone-induced Arrest in  
*Saccharomyces cerevisiae*\*

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\*Running title: *Inositol pyrophosphates in cell cycle*

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**Keywords:** inositol pyrophosphates; Plc1; Kcs1; cell cycle

**Background:** Inositol pyrophosphates mediate some effects of activated Plc1.

**Results:** Plc1 and Kcs1 activate early after release from G<sub>1</sub> block and increase the level of InsP<sub>7</sub> and InsP<sub>8</sub>.

**Conclusion:** Plc1- and Kcs1-mediated increase in pyrophosphates is important for progression through S phase.

**Significance:** This appears to be the first evidence for a link between Kcs1-generated pyrophosphates and cell cycle.

#### SUMMARY

Several studies have demonstrated the activation of phosphoinositide-specific phospholipase C (Plc) in nuclei of mammalian cells during synchronous progression through the cell cycle, but the downstream targets of Plc-generated inositol (1,4,5)-trisphosphate are poorly described. Phospholipid signaling in the budding yeast *Saccharomyces cerevisiae* shares similarities with endonuclear phospholipid signaling in mammals, and many recent studies point a role for inositol phosphates, including InsP<sub>5</sub>, InsP<sub>6</sub>, and inositol pyrophosphates, in mediating the action of Plc. In this study, we investigated the changes in inositol phosphate levels in

$\alpha$ -factor-treated *S. cerevisiae*, which allows cells to progress synchronously through the cell cycle after release from a G<sub>1</sub> block. We found an increase in the activity of Plc1 early after release from the block with a concomitant increase in the levels of InsP<sub>7</sub> and InsP<sub>8</sub>. Treatment of cells with the Plc inhibitor U73122 prevented increases in inositol phosphate levels, and blocked progression of cells through S phase after pheromone arrest. The enzymatic activity of Kcs1 *in vitro* and HPLC analysis of [<sup>3</sup>H]inositol-labeled *kcs1* $\Delta$  cells confirmed that Kcs1 is the principal kinase responsible for generation of pyrophosphates in synchronously progressing cells. Analysis of *plc1* $\Delta$ , *kcs1* $\Delta$  and *ddp1* $\Delta$  yeast mutants further confirmed the role that a Plc1- and Kcs1-mediated increase in pyrophosphates may have in progression through S phase. Our data provide genetic, metabolic and biochemical evidence that synthesis of inositol pyrophosphates through activation of Plc1 and Kcs1 plays an important role in the signaling response required for cell cycle progression after mating pheromone arrest.

## INTRODUCTION

In the past 20 years, several studies have demonstrated activation of phosphoinositide-specific phospholipase C (Plc) in nuclei of mammalian cells in response to external stimuli or during synchronous progression through the cell cycle. IGF-mediated increase in the level of nuclear diacylglycerol (DAG) and the activity of Plc- $\beta_1$  isoform occurs independently from the classical pathway at the cell membrane (1-3). In synchronized leukemia cells, the same isoform was found to be activated during the G<sub>1</sub> and G<sub>2</sub>/M phases of the cell cycle (4-6). Plc- $\delta_1$  isoform showed cell-cycle-dependent control of nuclear localization in NIH-3T3 fibroblasts (7), and two peaks of Plc activity were measured in nuclei of regenerating rat liver (8). Although an increase in the level of DAG and inositol trisphosphate (InsP<sub>3</sub>) has been detected in nuclei, there is a paucity of data on how these nuclear messengers regulate nuclear processes and, for some of them, like InsP<sub>3</sub>, there is no proofs that they play the same role in regulation of calcium levels as the classical one at the cell membrane (9). Major progress in understanding the possible functional role of the pathway has been recently made by genetic and biochemical studies in the budding yeast *Saccharomyces cerevisiae*. Phospholipid signaling in yeast shares many similarities with endonuclear phosphoinositide signaling in mammals; they have enzymes to generate nuclear PtdInsP<sub>2</sub> and they have Plc1 encoded by a *PLC1*, which is a homolog of the mammalian Plc- $\delta_1$ , but they lack important elements of the "classical" phosphoinositide signaling as they have no InsP<sub>3</sub> receptor in their genome and do not utilize DAG to activate protein kinase C. However, yeast uses InsP<sub>3</sub> as a precursor for the synthesis of higher inositol phosphates and pyrophosphates, and these products have been proven to regulate several important nuclear events (10). In yeast models, the possible role of higher inositol phosphates in the progression through the cell cycle has not been investigated, but the association of Plc1 with kinetochore has been found to be important for proper chromosome segregation and cell cycle progression (11).

In *S. cerevisiae*, inositol phosphates and pyrophosphates are generated by complex action of four different kinases, as shown in Fig. 1. Ins(1,4,5)P<sub>3</sub> is first phosphorylated by

dual-specificity InsP<sub>3</sub>/InsP<sub>4</sub> 6-/3-kinase Ipk2 into Ins(1,4,5,6)P<sub>4</sub> (InsP<sub>4</sub>) and Ins(1,3,4,5,6)P<sub>5</sub> (InsP<sub>5</sub>), and Ipk1 then phosphorylates Ins(1,3,4,5,6)P<sub>5</sub> into Ins(1,2,3,4,5,6)P<sub>6</sub>. The synthesis of diphosphoinositol phosphates or pyrophosphates in yeast is mediated by two kinases: Kcs1 phosphorylates InsP<sub>5</sub> into 5-PP-InsP<sub>4</sub>, and InsP<sub>6</sub> into 5-PP-InsP<sub>5</sub> (InsP<sub>7</sub>); Vip1 phosphorylates InsP<sub>6</sub> into InsP<sub>7</sub> isomers 1-PP-InsP<sub>5</sub> or 3-PP-InsP<sub>5</sub>. (PP)<sub>2</sub>-InsP<sub>4</sub> (InsP<sub>8</sub>) is generated by either Kcs1-mediated phosphorylation of 1/3-PP-InsP<sub>5</sub> or Vip1-mediated phosphorylation of 5-PP-InsP<sub>5</sub>. The majority of enzymes responsible for synthesis of inositol phosphates can be found in the nucleus, and all of them have been reported to have some role in nuclear processes (10). A major role of Ipk2 and its products, InsP<sub>4</sub> and InsP<sub>5</sub>, is transcriptional regulation by modulation of chromatin remodeling in response to nutrients (12, 13). The yeast Ipk1 and its product, InsP<sub>6</sub>, regulate mRNA export (14) and nonhomologous end joining (15). Kcs1 activity modulates telomere length by generation of 5-PP-InsP<sub>4</sub> (16), and Vip1-generated InsP<sub>7</sub> binds to the cyclin-CDK-CDK inhibitor complex to regulate transcription of the yeast phosphate (Pi)-responsive (PHO) genes (17).

In this study, we investigated changes in inositol phosphate levels in  $\alpha$ -factor-treated *S. cerevisiae*, which allows cells to progress synchronously through the cell cycle after release from the G<sub>1</sub> block. The results of this study show an increase in the activity of Plc1 early after release from the block with a concomitant increase in the level of InsP<sub>7</sub> and InsP<sub>8</sub>. The increase in the level of inositol pyrophosphates was found to be due to the activation of Kcs1, which is necessary for the progression of the cells through the S phase of the cell cycle.

## EXPERIMENTAL PROCEDURES

**Reagents**-Reagents were obtained from the following sources: all media and supplements for yeast growth from Formedium;  $\alpha$ -factor mating pheromone from Zymo Research; U73122 from Calbiochem; glass beads (diameter 425-600  $\mu$ m), RNaseA, proteinase K, bovine brain extract (Type I, Folsch fraction V), InsP<sub>6</sub> and PtdInsP<sub>2</sub> from Sigma; [<sup>3</sup>H]PtdInsP<sub>2</sub> (20 Ci/mmol) and [<sup>32</sup>P]ATP (6,000 Ci/mmol) from Perkin Elmer; [<sup>3</sup>H]inositol (30 Ci/mmol) from Perkin Elmer

or American Radiolabeled Chemicals; Calmodulin-Sepharose 4B from Amersham; InsP<sub>5</sub> from Cell Signaling; and Sytox from Invitrogen. All other chemicals were of analytical grade.

*Yeast strains, growth condition* - *S. cerevisiae* strains used in this study are isogenic with W303 MATa (*leu2-3,112, his3-11,15, ura3-1, ade2-1, trp1-1, rad5-535, can1-100*). Deletion strains *ddp1::HIS3, kcs1::HIS3, plc1::KANMX* were generated as described previously (16). For labeling experiments, cells were inoculated in synthetic medium without inositol with addition of 5.7 mg/ml ammonium sulphate, 0.82 mg/ml amino acids, 2% glucose, and 5  $\mu$ Ci/ml <sup>3</sup>[H]inositol (labeling medium) and grown to achieve isotopic equilibrium (8-9 divisions) in a shaker incubator at 30 °C and 200 r.p.m. (20). At the end of incubation, cells were collected, counted, resuspended in fresh labeling medium at the concentration of 2.5 x 10<sup>6</sup>/ml, grown into mid-logarithmic phase, and then incubated in the presence of 5  $\mu$ M  $\alpha$ -factor mating pheromone for 6 h (wt and *ddp1* $\Delta$ ) or 24 h (*kcs1* $\Delta$  and *plc1* $\Delta$ ). The G<sub>1</sub>-arrested cells were washed twice with synthetic medium lacking inositol, released into fresh labeling medium, and allowed to progress synchronously through the cell cycle. At the indicated time points, cells were harvested by centrifugation (2,000 x g, 4 °C, 4 min.) For flow cytometric analysis, cells were grown as described above, except that cold inositol was added into the medium at the same concentration as <sup>3</sup>[H]inositol. At indicated time points, 1 ml of cell suspension was harvested, fixed with 9 ml of 90% ethanol and stored at -20 °C.

*Extraction and Analysis of <sup>3</sup>[H]Inositol-labeled Phosphoinositides and Inositol Polyphosphates* - For phosphoinositide analysis, the pellet was resuspended in 0.3 ml of 2:1(v/v) methanol/water containing 0.08 M HCl, 6.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.83 mM EDTA and 0.83 mM EGTA, and vortexed for 30 seconds. Acid-washed glass beads (0.8 g diameter 425-600  $\mu$ m) were added and the slurry was alternately vortexed (30 s) and cooled on ice (30 s) through 10 cycles. Afterwards, 0.47 ml of methanol (containing 0.12 M HCl) and 1.33 ml of chloroform were added, the slurry was vortexed (30 s) and the monophasic mixture was left on ice (15 min). Bovine brain phosphoinositides (10  $\mu$ g, Type I, Folsch fraction V) were added in 2:1 (v/v)

chloroform/methanol, samples were vortexed (30 s) and cooled on ice. After adding 0.4 ml of a mixture of 0.1 M HCl, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 5 mM tetra-n-butyl-ammonium hydrogen sulphate (TEBAS), 2.5 mM EDTA and 2.5 mM EGTA, mixing yielded a two-phase system (separated at 3,000 x g, 5 min, 4 °C) (18). The chloroform-rich lower phase was transferred to a glass vial and dried in vacuo by roto-evaporation, and excess HCl was removed by repeated drying in vacuo with methanol. Lipids were deacylated (monomethylamine reagent, 53 °C, 50 min) and the separation of all the glycerophosphoinositides was achieved using an HPLC high resolution 5  $\mu$ M Partisphere SAX column (Whatman) with a discontinuous gradient up to 1 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> X H<sub>3</sub>PO<sub>4</sub> (pH 3.8) exactly as described in the previous study (19).

For inositol polyphosphate analysis, the pellet was resuspended in 0.2 ml of extraction buffer (1M HClO<sub>4</sub>, 3 mM EDTA and 0.1 mg/ml InsP<sub>6</sub>), glass beads were added and cells were disrupted by vortexing, as described above. After centrifugation for 1 min in microcentrifuge, the soluble extract was transferred to a new tube, 0.2 ml of neutralization buffer (1 M K<sub>2</sub>CO<sub>3</sub> and 3 mM EDTA) was added to achieve pH 6-8, neutralization was allowed overnight at 4 °C, the final volume was adjusted to 0.5 ml, samples were filtered using Spin-X microcentrifuge tubes and stored for HPLC analysis. Separation of all the inositol phosphates was achieved using high resolution 5  $\mu$ M Partisphere SAX column (Whatman) at a flow rate of 1 ml/min, with a gradient generated by mixing buffers A (1 mM EDTA) and B 1.3 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> X H<sub>3</sub>PO<sub>4</sub> (pH 3.8) as follows: 0-10 min, 0% buffer B; 10-75 min, 0-100% buffer B; 75-85 min, 100% buffer B using Ultimate 3000 HPLC system (Dionex) (20).

*Assay of Plc Activity* - Cells were grown in a synthetic media with cold inositol, synchronized and harvested as described above. Pellet was resuspended in 0.2 ml of ice-cold lysis buffer [0.3 M mannitol, 0.1 M KCl, 50 mM Tris-HCl (pH 7.5), 1% NP-40, 50 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EGTA, 1 mM PMSF, 1  $\mu$ g/ml leupeptin and 1  $\mu$ g/ml aprotinin] and cells were disrupted, as described above. The homogenate was centrifuged in microcentrifuge at 14,000 x g, 5 min at 4 °C. The resultant supernatant was

clarified by centrifugation at 90,000 x g, 60 min at 4 °C in a Beckman SW 27 rotor. Plc activity was measured as described by Crljen *et al.* (8). 100 µg of protein were incubated in 0.2 ml assay buffer (100 mM NaCl, 50 mM, HEPES (pH 7.0), 200 µM CaCl<sub>2</sub>, 1 mg/ml bovine serum albumin, 100 µM PtdInsP<sub>2</sub>) and 50,000 cpm of [<sup>3</sup>H]PtdInsP<sub>2</sub> per assay. After 10 min at 37 °C, the reaction was stopped by adding 1 ml of chloroform/methanol (1:1, v/v), followed by 0.25 ml of 2.4 N HCl. After a brief centrifugation, the top phase was removed and the amount of InsP<sub>3</sub> was quantified by liquid scintillation counting.

**Assay of InsP<sub>6</sub> Kinase Activity** - [<sup>32</sup>P]InsP<sub>6</sub> was produced enzymatically in 0.3 ml of buffer [50 mM HEPES (pH 7.5), 1 mM EDTA, 50 mM KCl and 10 mM MgCl<sub>2</sub>] with 30 µl of [<sup>γ</sup><sup>32</sup>P]ATP, 45 µl of GST-*atIpk1* (1mg/ml) and 15 µl of InsP<sub>5</sub> (100 µM). The reaction was run at 37 °C for 60 min, the enzyme was added once again after 30 min and the enzyme was subsequently heat-inactivated at 95 °C for 10 min (21). Yeast *Kcs1-TAP* cells were grown in a synthetic media and synchronized as described above. At indicated time points, cells were harvested and disrupted using glass beads in 1 ml of ice-cold binding buffer [50 mM HEPES (pH 7.4) 150 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl<sub>2</sub>, 2 mM NaF, 1 mM PMSF, 1 µg/ml leupeptin and 1 µg/ml aprotinin]. The homogenate was centrifuged at 14,000 x g for 20 min at 4 °C. Calmodulin-Sepharose 4B (10 µl) was added to the supernatant and incubated at 4 °C for 2 h. The resin was washed twice with binding buffer containing 500 mM NaCl and then twice with 100 mM NaCl (22). The resin was then used to perform InsP<sub>6</sub> kinase assay using 50,000 cpm/assay of [<sup>32</sup>P]InsP<sub>6</sub> in 20 µl of assay buffer [50 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM ATP and 1 µM InsP<sub>6</sub>]. Reaction was run for 30 min at 37 °C and terminated by adding 1 µl of 1 M HCl and placing on ice. After centrifugation for 1 min in microcentrifuge, 5 µl of reaction mixture was spotted onto PEI-TLC plates and developed in a tank equilibrated with buffer containing 1.09 M KH<sub>2</sub>PO<sub>4</sub>/0.72 M K<sub>2</sub>HPO<sub>4</sub>/2.07 M HCl (21). After autoradiography, spots corresponding to InsP<sub>6</sub> and InsP<sub>7</sub> were scraped and counted for radioactivity in a liquid-scintillation counter.

**Flow Cytometric Analysis** - Cells were washed by 50 mM sodium citrate pH 7.4, sonicated, incubated in 50 mM sodium citrate pH 7.4 containing 0.25 mg/ml of RNase A at 50 °C for 1 h, then treated with 1 mg/ml of proteinase K at 50 °C for 1 h, washed and resuspended in 50 mM sodium citrate containing 1 µM Sytox. The DNA fluorescence analyses were performed on at least 10,000 cells for each sample with the FACSCalibur system (Becton Dickinson), and the data were analyzed using CellQuest and ModFit software (Becton Dickinson).

**Statistical Evaluation** - The data are shown as means ± S.E.M. For statistical analysis, the Student's *t* test for unpaired samples at the level of significance of 0.05 was used.

## RESULTS

Yeast cells were grown in synthetic medium containing 0.2 µM inositol with an average cell doubling time of 3 hours. To allow synchronous progression through the cell cycle, logarithmically growing cells were first incubated in the presence of  $\alpha$ -factor for 6 h, and then washed and released into fresh medium. An aliquot of cells was harvested at indicated times, stained with Sytox, and analyzed by FACS. As shown in Fig. 2A, G<sub>1</sub>-arrested cells returned to a cycling state with the majority of cells completing S phase within 2 h after reseeded. To test for the possible *de novo* synthesis of inositol phospholipids and phosphates, a pulse experiment was performed by adding fresh medium containing [<sup>3</sup>H]-labeled inositol to logarithmically growing cells or cells that were washed and released from  $\alpha$ -factor block. The results demonstrated a significant increase in the incorporation of radiolabel into both PtdInsP<sub>2</sub> (Fig. 2B) and InsP<sub>6</sub> (Fig. 2C) 30-120 min after release from the block.

To further test for the possible changes in the level of inositol phospholipids and phosphates, yeast cells were first cultured for 8-9 cell divisions in medium containing [<sup>3</sup>H]inositol, then synchronized by G<sub>1</sub>-arrest, released into fresh labeling medium and allowed to progress synchronously through the cell cycle. Comparison of HPLC elution profiles of inositol phosphates at times 0 (Fig. 3A) and 30 minutes after release from the block (Fig. 3B) indicated an increase in the level of InsP<sub>3</sub>, a decrease in the level of InsP<sub>6</sub> (not shown), an increase in two peaks eluted

after  $\text{InsP}_6$  that corresponded to  $\text{InsP}_7$  and  $\text{InsP}_8$ , and no changes in the level of  $\text{InsP}_4$  and  $\text{InsP}_5$ . Time-course analysis of the level of  $\text{InsP}_3$  in synchronized cells revealed a significant increase in the level as early as 15 min after the release from the block, the peak level was measured at 30 min and the level returned to the basal level in  $G_1$ -arrested cells at 90 min after release (Fig. 3C). As shown in Fig. 3D, time-course analysis of the level of  $\text{PtdInsP}_2$  mirrored the changes in the level of  $\text{InsP}_3$ , and both changes corresponded well with the time-dependent increase in the activity of  $\text{Plc1}$  when measured in an assay that uses exogenous  $[^3\text{H}]\text{PtdInsP}_2$  as a substrate (Fig. 3E).

To confirm that observed changes in the level of  $\text{PtdInsP}_2$  and inositol phosphates were initiated by an increase in the activity of  $\text{Plc1}$ , labeled cells were synchronized in  $G_1$  with  $\alpha$ -factor, washed and released into fresh medium containing the commercially available  $\text{Plc}$  inhibitor U73122. Comparison of HPLC elution profiles of inositol phosphates at times 0 (Fig. 4A) and 30 minutes after release from the block in the presence of U73122 (Fig. 4B) confirmed that the presence of  $\text{Plc}$  inhibitor completely abolished all peaks eluted, except for the peak corresponding to  $\text{InsP}_6$ . Furthermore, the presence of  $\text{Plc}$  inhibitor prevented time-dependent changes in the level of  $\text{PtdInsP}_2$  (results not shown), decreased the level of  $\text{InsP}_3$  (Fig. 4C),  $\text{InsP}_7$  (Fig. 4E), and  $\text{InsP}_8$  (Fig. 4F) as early as 15 min after release, and completely abolished the peaks at 30-120 min. Regarding  $\text{InsP}_6$ , the time-course analysis showed that the presence of U73122 caused a progressive decline in the level of  $\text{InsP}_6$  which reached zero at 120 min after release (Fig. 4D). It is important to note that 10  $\mu\text{M}$  U73122 completely inhibited activity of  $\text{Plc1}$  when assayed using exogenous  $[^3\text{H}]\text{PtdInsP}_2$  as a substrate (results not shown).

To test for the role of  $\text{Plc1}$  activation in the progression through the cell cycle, FACS analysis was performed on wild-type cells arrested by  $\alpha$ -factor and released into medium in the presence of U73122 or vehicle (control). As shown in Fig. 5, the presence of  $\text{Plc}$  inhibitor completely prevented the progression through the S phase. A similar block in the progression through the cell cycle was observed in *plc1 $\Delta$*  mutants, in which no inositol phosphates could be detected by HPLC (results not shown).

Time-dependent changes in the level of inositol phosphates were further investigated. As shown in Fig. 6, the time-dependent decrease in the levels of  $\text{InsP}_6$  (Fig. 6A) was mirrored by an increase in the level of both  $\text{InsP}_7$  (Fig. 6B) and  $\text{InsP}_8$  (Fig. 6C). In *S. cerevisiae*, *Kcs1* is one of the two kinases responsible for the phosphorylation of  $\text{InsP}_6$  and generation of pyrophosphates (10). To test whether an increase in the level of  $\text{InsP}_7$  and  $\text{InsP}_8$  was due to the activity of *Kcs1*, endogenous TAP-*Kcs1* was purified from yeast cells harvested at different time points after release from the block, and assayed for its ability to phosphorylate exogenous  $[^{32}\text{P}]\text{InsP}_6$ . As shown in Fig. 6D, an increase in the activity of *Kcs1 in vitro* paralleled the observed changes in the level of pyrophosphates.

The labeling experiments of the present study were performed by using synthetic medium containing only radiolabeled inositol (5  $\mu\text{Ci/ml}$ ), which results in a final concentration of inositol (0.2  $\mu\text{M}$ ) that is significantly lower than 11  $\mu\text{M}$  inositol concentration in complete standard synthetic medium. To rule out the possibility that observed changes in the level of inositol phosphates were influenced by lack of inositol in labeling media, two additional sets of experiments were performed using different labeling protocols. First, cells were labeled using synthetic medium containing only radiolabeled inositol, but at a concentration (50  $\mu\text{Ci/ml}$  of  $[^3\text{H}]\text{inositol}$ ) that increases the final concentration to 2  $\mu\text{M}$ . In a second protocol, cells were labeled using standard synthetic complete medium (containing 11  $\mu\text{M}$  unlabeled inositol) that was supplemented with 10  $\mu\text{Ci/ml}$  of  $[^3\text{H}]\text{inositol}$ , which results in a total inositol concentration of 11.4  $\mu\text{M}$  (16). Again, labeled cells were arrested with  $\alpha$ -factor, released into fresh labeling medium, and allowed to progress synchronously through the cell cycle. As shown in Fig. 7, the time-course changes in the levels of  $\text{InsP}_3$ ,  $\text{InsP}_6$ ,  $\text{InsP}_7$  and  $\text{InsP}_8$  for both labeling protocols follow similar pattern when compared with time-course changes of the level of inositol phosphates extracted from the cells that were labeled using synthetic medium containing only 5  $\mu\text{Ci/ml}$  of  $[^3\text{H}]\text{inositol}$  (Fig. 3C, and Fig. 6A-C). Furthermore, the proportion of radioactivity in each of inositol phosphates, for each time points, using different labeling

protocols is similar (1:4.3:0.6). Similar to results obtained using labeling media containing 5  $\mu\text{Ci/ml}$  of  $^3\text{H}$ inositol (Fig. 3A and B), no time-dependent changes in the level of  $\text{InsP}_4$  and  $\text{InsP}_5$  were observed using additional labeling protocols, but still the proportions of radioactivity in each of the inositol phosphates were similar to the ones observed above (1:4.3:0.6) (results not shown). Therefore, although the amount of radioactivity which can be determined in each of inositol phosphates varies because of different amounts of labeled and unlabeled inositol used in labeling media, it can be concluded that cells reach isotopic equilibrium in all labeling conditions tested, including the one using only 5  $\mu\text{Ci/ml}$  of  $^3\text{H}$ inositol in labeling medium, as has been suggested by Azevedo and Saiardi (20).

To further assess the physiological role of Kcs1-generated pyrophosphates in cell cycle progression, yeast strains with mutations in enzymes involved in pyrophosphate metabolism were investigated. HPLC analysis of  $^3\text{H}$ inositol labeled *kcs1* $\Delta$  strain cells revealed no peaks eluted after  $\text{InsP}_6$  in  $\alpha$ -factor arrested (Fig. 8A) or cells collected 30 minutes after release from the block (Fig. 8B), confirming that Kcs1 participates in mediating changes observed in pyrophosphates in synchronously progressing cells. In contrast, the lack of Kcs1 activity had no effects on Plc1 activation as the level of  $\text{InsP}_3$  (Fig. 8A and B) and time-dependent changes of  $\text{InsP}_3$  (Fig. 8C) in cells released from the block was similar to those observed in wild-type cells. The basal level of  $\text{InsP}_6$  measured in *kcs1* $\Delta$  strain was similar to the one in wild-type cells suggesting that the deletion of *KCS1* does not alter  $\text{InsP}_6$  levels, as previously described (16). In contrast to wild-type cells, the time-course analysis of the level of  $\text{InsP}_6$  in synchronized cells showed no significant decrease at any time point after release from the block (Fig. 8D).

*DDP1* encodes a diphosphoinositol-polyphosphate phosphatase that dephosphorylates both  $\text{InsP}_8$  and  $\text{InsP}_7$  down to  $\text{InsP}_6$  (23); disruption of the *DDP1* gene in yeast strain *ddp1* $\Delta$  increases the level of pyrophosphates (16). When labeled *ddp1* $\Delta$  cells were arrested by the presence of  $\alpha$ -factor, HPLC analysis revealed an increase in the basal level of  $\text{InsP}_7$  (Fig. 9A) in comparison to wild type cells (Fig. 3A). At 30 min after

release from the block, the increase in the peaks corresponding to pyrophosphates (Fig. 9B) was more prominent than the increase in wild type cells (Fig. 3B). Similar to wild type cells, the time-dependent decline in the level of  $\text{InsP}_6$  (Fig. 9D) was mirrored by an increase in both  $\text{InsP}_7$  (Fig. 9E) and  $\text{InsP}_8$  (Fig. 9F).

Both mutant yeast strains were subjected to FACS analysis. As shown in Fig. 10, *kcs1* $\Delta$  cells remain arrested in  $G_1$  phase up to 120 min after being washed from  $\alpha$ -factor, and start to show some progression into S phase at 180 min after release. In contrast, majority of  $G_1$ -arrested and released *ddp1* $\Delta$  cells reached  $G_2/M$  phase earlier than wild type cells.

## DISCUSSION

Although a search of the literature reveals many studies related to phosphoinositide signaling in regulation of the cell cycle, there are only few studies that report cell-cycle associated changes in the levels of higher inositol phosphates or pyrophosphates, and all of these have been performed in mammalian cells (24, 25). The route of synthesis of higher inositol phosphates and pyrophosphates in mammalian cells is much more complicated than the pathway operating in yeast cells; e.g., there are at least three different mammalian isoforms of inositol hexakisphosphate kinases instead of two in yeasts, and four mammalian diphosphoinositol-polyphosphate phosphatases instead of a single Ddp1 in yeasts (26). However, the common theme between yeasts and mammals is that the synthesis of higher inositol phosphates starts with  $\text{InsP}_3$  being generated by a Plc-mediated hydrolysis of  $\text{PtdInsP}_2$ .

The results of our study demonstrate an early activation of Plc1 in yeast cells allowed to progress synchronously through S phase after the release from  $G_1$  block. A significant delay in cell cycle progression of deletion mutants of the *PLC1* gene (that make no  $\text{InsP}_{3-8}$ ) as well as wild type cells released from the block in the presence of a Plc-inhibitor suggests that Plc1 activation has a functional role in progression through S phase during mating pheromone signalling. In *S. cerevisiae* there is only one Plc1 encoded by the gene *PLC1* (9, 10). In mammalian cells, at least 13 Plc isoforms have been identified, and many of them, including Plc- $\beta$ , - $\gamma$ , - $\delta$  and - $\zeta$ , have been found to be activated in nuclei of

cells at a particular phase of the cell cycle (27). A sole Plc1 in yeasts corresponds best to the mammalian Plc- $\delta$ 1, and this is the isoform that has been most consistently found to be associated with nuclei of mammalian cells somewhere at the beginning of S phase. In regenerating rat liver, Plc- $\delta$ 1 participated in the increase in the Plc-activity in nuclei at 20 h after hepatectomy (8). In synchronized NIH3T3 cells, the level of Plc- $\delta$ 1 in nuclei increased at the G<sub>1</sub>/S boundary (7), and “knockdown” of Plc- $\delta$ 1 in rat C6 glioma cells significantly decreased proliferation and delayed the progression through S phase (28). Enhanced degradation of cyclin E levels in mammalian cells (28) has been suggested to mediate Plc role in the cell cycle control, but it is not clear how the enzyme might interact with the cell cycle machinery to regulate progression through different phases of the cell cycle (9, 27).

Data from the present study support the hypothesis that at least some of the effects of cell cycle-associated activation of Plc1 are due to the generation of higher inositol phosphates. The kinetics of the increase in the levels of PP-InsP<sub>5</sub> and (PP)<sub>2</sub>-InsP<sub>4</sub> corresponds well with the time frame of the increase in the activity of Plc1. There are very few previous reports of specific stimuli that acutely increase the level of pyrophosphates, but a 20-25% decrease in InsP<sub>6</sub> levels 30 minutes after release from the  $\alpha$ -factor block is similar to the one observed previously following 30-min exposure of U2-OS cells to 200 mM sorbitol (29). In the present study, the total radioactivity in the inositol pyrophosphate region of the chromatograph (i.e. [<sup>3</sup>H]InsP<sub>7</sub> plus [<sup>3</sup>H]InsP<sub>8</sub>) equals half of the change in InsP<sub>6</sub> level, suggesting that at least half of the decrease in InsP<sub>6</sub> level could be ascribed to phosphorylation into pyrophosphates. In contrast to sorbitol stimulated cells, in which the Vip1-mediated increase of (PP)<sub>2</sub>-InsP<sub>4</sub> was near quantitatively balanced by a corresponding decrease in PP-InsP<sub>5</sub> levels (29), the level of both pyrophosphates increased during the progression through the S phase. Again, a significant delay in cell cycle progression in yeast mutants lacking Kcs1, which is clearly responsible for the generation of pyrophosphates in synchronized cells, as well as accelerated progression through S phase of yeast strains lacking the principal pyrophosphates phosphatase Ddp1, suggest the

possible role of pyrophosphates in the progression through the S phase.

It is unclear what might be the possible link between the Kcs1-mediated increase in diphosphoinositol polyphosphates and the progression of yeast cells through S phase of the cell cycle. The *KCS1* gene was first identified in a screen for suppression of temperature sensitivity of mutation of the protein kinase C gene (30), and was later found to have kinase activity responsible for the phosphorylation of InsP<sub>5</sub> into PP-InsP<sub>4</sub> and InsP<sub>6</sub> into PP-InsP<sub>5</sub> (31, 32). Loss-of-function studies have implicated pyrophosphates in regulation of DNA hyper-recombination (33) and Kcs1-generated PP-InsP<sub>4</sub> as a negative regulator of telomere length (16, 34), but it is unclear whether pyrophosphates directly regulate these processes. The most selective putative intracellular receptor for pyrophosphates is Pho80/Pho85/Pho81 cyclin-dependent kinase/cyclin kinase inhibitor complex (17) and that provides a link to the regulation of cytoskeletal dynamics and cell cycle as the human homolog of Pho85 is Cdk5 (35). However, InsP<sub>7</sub>, which has been isolated as a cellular component that directly regulates the complex in response to phosphate starvation is a distinct stereoisomer, 1/3-PP-InsP<sub>5</sub>, generated by Vip1 kinase, but not Kcs1-generated 5-PP-InsP<sub>5</sub> (17). Previous analyses of yeast Kcs1 mutants suggested a role for the kinase in vacuole biogenesis (32, 36), cell wall integrity (36), and proper endocytic trafficking (37), and this may also provide a clue to the possible function of Kcs-generated pyrophosphates in cell cycle progression, as the process of bud formation during progression of yeast cells through S phase is certainly associated with a significant change in the cell wall morphology that might include phospholipid signaling (38). A novel possible mode of pyrophosphate-mediated signaling has been recently proposed that includes a direct transfer of phosphate groups from PP-InsPs to protein substrates in a kinase-independent manner (39). For Kcs1 mutants, the analysis revealed a decrease in the phosphorylation of some nucleolar proteins, including nucleolin (39). However, studies in mammalian cells ruled out the possibility that nucleolin serves as a molecular target for inositol pyrophosphates response to osmotic stress *in vivo* (40).



Regardless of detailed mechanisms, our findings provide the first evidence for time-dependent increase in the activity of Plc1 and an increase in the level of pyrophosphates in yeast cells synchronized by  $\alpha$ -factor arrest

and release. In synchronized yeast cells, the increase in the level of InsP<sub>7</sub> and InsP<sub>8</sub> is due to the activation of Kcs1, which is necessary for the progression of the cells through S phase of the cell cycle.

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## FOOTNOTES

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**Abbreviations:** Plc, phospholipase C; DAG, diacylglycerol; PtdInsP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; InsP<sub>3</sub>, inositol trisphosphate; InsP<sub>4</sub>, inositol tetrakisphosphate; InsP<sub>5</sub>, inositol pentakisphosphate; InsP<sub>6</sub>, inositol hexakisphosphate; InsP<sub>7</sub> (also referred as PP-InsP<sub>5</sub>), diphosphoinositol pentakisphosphate; InsP<sub>8</sub> [also referred as (PP)<sub>2</sub>-InsP<sub>4</sub>], bisphosphoinositol tetrakisphosphate; PP-InsP<sub>4</sub>, diphosphoinositol tetrakisphosphate, HPLC, high performance liquid chromatography; TAP, tandem affinity purification; wt, wild type.

## FIGURE LEGENDS

**Fig. 1. The pathway involved in the synthesis of inositol phosphates in *S. cerevisiae*.** InsP<sub>3</sub> serves as a substrate for the inositol kinase Ipk2, which phosphorylates both the D-6 and D-3 position hydroxyl groups to produce InsP<sub>5</sub>. InsP<sub>5</sub> is phosphorylated by Ipk1 at 2-hydroxyl to generate InsP<sub>6</sub>. The synthesis of diphosphoinositol phosphates is mediated by two kinases: Kcs1 phosphorylates InsP<sub>5</sub> into 5-PP-InsP<sub>4</sub>, and InsP<sub>6</sub> into 5-PP-InsP<sub>5</sub> (InsP<sub>7</sub>); Vip1 phosphorylates InsP<sub>6</sub> into InsP<sub>7</sub> isomers 1/3-PP-InsP<sub>5</sub>. (PP)<sub>2</sub>-IP<sub>4</sub> (InsP<sub>8</sub>) is generated by either Kcs1-mediated phosphorylation of 1/3-PP-InsP<sub>5</sub> or Vip1-mediated phosphorylation of 5-PP-InsP<sub>5</sub>.

**Fig. 2. Release of the wild-type yeast cells from  $\alpha$ -factor block stimulates incorporation of [<sup>3</sup>H]inositol into PtdInsP<sub>2</sub> and InsP<sub>6</sub>.** Cells were synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium, harvested at the indicated times, stained with Sytox and assessed for cell cycle distribution by flow cytometric analysis as described in Experimental Procedures. (A) Representative histogram. Logarithmic phase (*open bars*) or cells released from  $\alpha$ -factor block (*black bars*) were pulse-labeled with 50  $\mu$ Ci/ml [<sup>3</sup>H]inositol for indicated times, harvested, and analyzed for radiolabel incorporation into PtdInsP<sub>2</sub> (B) and InsP<sub>6</sub> (C) by HPLC as described in Experimental Procedures. The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to logarithmic phase cells.

**Fig. 3. Release of the wild-type yeast cells from  $\alpha$ -factor block stimulates Plc activity.** Cells were labeled, synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium, and harvested at the indicated times. Inositol lipids and phosphates were analyzed by HPLC, while Plc-activity was assayed using PtdInsP<sub>2</sub> as substrate as described in Experimental Procedures. HPLC elution profile of inositol polyphosphates at time 0 (A) or 30 min after the release of the cells from  $\alpha$ -factor block (B). Time-course analysis of the level of InsP<sub>3</sub> (C) and PtdInsP<sub>2</sub> (D). Plc-activity (E) after the release of the cells from  $\alpha$ -factor block. The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to the time 0.

**Fig. 4. The effect of the Plc inhibitor (U73122) on the level of inositol phosphates in the wild type yeast cells released from  $\alpha$ -factor block.** Cells were labeled, synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium containing 10  $\mu$ M U73122, harvested at the indicated times, and inositol phosphates were analyzed by HPLC as described in Experimental Procedures. HPLC elution profile of inositol phosphates at time 0 (A) or 30 min after the release of the cells from  $\alpha$ -factor block (B). Time-course of changes in the level of InsP<sub>3</sub> (C), InsP<sub>6</sub> (D), InsP<sub>7</sub> (E) and InsP<sub>8</sub> (F) after the release of the cells from  $\alpha$ -factor block. The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to the time 0.

**Fig. 5. Plc inhibitor (U73122) treated wild-type cells and *plc1Δ* cells do not progress through the S phase of the cell cycle.** Cells were synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium or medium containing 10  $\mu$ M U73122, harvested at the indicated times, stained with Sytox and assessed for cell cycle distribution by flow cytometric analysis as described in Experimental Procedures. (A) Representative histograms. (B) Time-course of changes in the percentage of the cells in G<sub>1</sub>, S and G<sub>2</sub>/M phase. Wild-type cells (*open bars*), wild-type cells treated with inhibitor (*black bars*) and *plc1Δ* cells (*gray bars*). The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to wild-type.

**Fig. 6. Release of the wild-type yeast cells from  $\alpha$ -factor block stimulates Kcs1 activity.** Cells were labeled, synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium, and harvested at the indicated times. Inositol phosphates were analyzed by HPLC, while Kcs1 activity was assayed using InsP<sub>6</sub> as the substrate as described in Experimental Procedures. Time-course of changes in the level of InsP<sub>6</sub> (A), InsP<sub>7</sub> (B) and InsP<sub>8</sub> (C) and Kcs1 activity (D) after the release of the cells from  $\alpha$ -factor block. The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to the time 0.

**Fig. 7. The effect of different labeling protocols on the level of inositol phosphates in the wild-type yeast cells released from  $\alpha$ -factor block.** Cells were labeled using synthetic medium containing 50  $\mu$ Ci/mL <sup>3</sup>[H]inositol (upper panel) or standard synthetic complete medium containing 11  $\mu$ M unlabeled inositol, supplemented with 10  $\mu$ Ci/mL of <sup>3</sup>[H]inositol (lower panel). Cells were synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium, harvested at the indicated times, and inositol phosphates were analyzed by HPLC as described in Experimental Procedures. Time-course of changes in the level of InsP<sub>3</sub> (A and E), InsP<sub>6</sub> (B and F), InsP<sub>7</sub> (C and G) and InsP<sub>8</sub> (D and H) after the release of the cells from  $\alpha$ -factor block. The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to the time 0.

**Fig. 8. Changes in inositol phosphate levels in *kcs1Δ* cells following the release from  $\alpha$ -factor block.** Cells were labeled, synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium, harvested at the indicated times and inositol phosphates were analyzed by HPLC as described in Experimental Procedures. HPLC elution profile of inositol polyphosphates at time 0 (A) or 30 min after the release of the cells from  $\alpha$ -factor block (B). Time-course of changes in the level of InsP<sub>3</sub> (C) and InsP<sub>6</sub> (D) after the release of the cells from  $\alpha$ -factor block. The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to the time 0.

**Fig. 9. Changes in inositol phosphate levels in *ddp1Δ* cells following the release from  $\alpha$ -factor block.** Cells were labeled, synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium, harvested at the indicated times and inositol phosphates were analyzed by HPLC as described in Experimental Procedures. HPLC elution profile of inositol polyphosphates at time 0 (A) or 30 min after the release of the cells from  $\alpha$ -factor block (B). Time-course of changes in the level of InsP<sub>3</sub> (C), InsP<sub>6</sub> (D), InsP<sub>7</sub> (E) and InsP<sub>8</sub> (F) after the release of the cells from  $\alpha$ -factor block. The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to the time 0.

**Fig. 10. Progression of *kcs1Δ* and *ddp1Δ* cells through the cell cycle following the release from  $\alpha$ -factor block.** Cells were synchronized in G<sub>1</sub> with  $\alpha$ -factor, released into fresh medium, harvested at the indicated times, stained with Sytox and assessed for cell cycle distribution by flow cytometric analysis as described in Experimental Procedures. (A) Representative histograms. (B) Time-course of changes in the percentage of the cells in G<sub>1</sub>, S and G<sub>2</sub>/M phase. Wild-type cells (*open bars*), *kcs1Δ* cells (*black bars*) and *ddp1Δ* cells (*gray bars*). The results are means  $\pm$  S.E.M. for three different experiments. \*  $p < 0.05$  (Student's *t* test) with respect to wild-type.

Fig. 1.

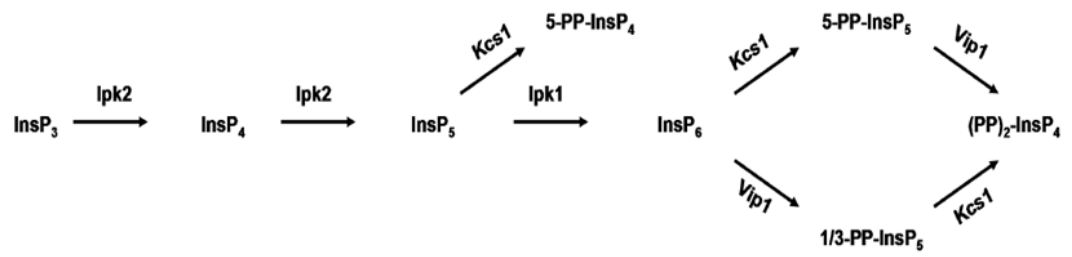
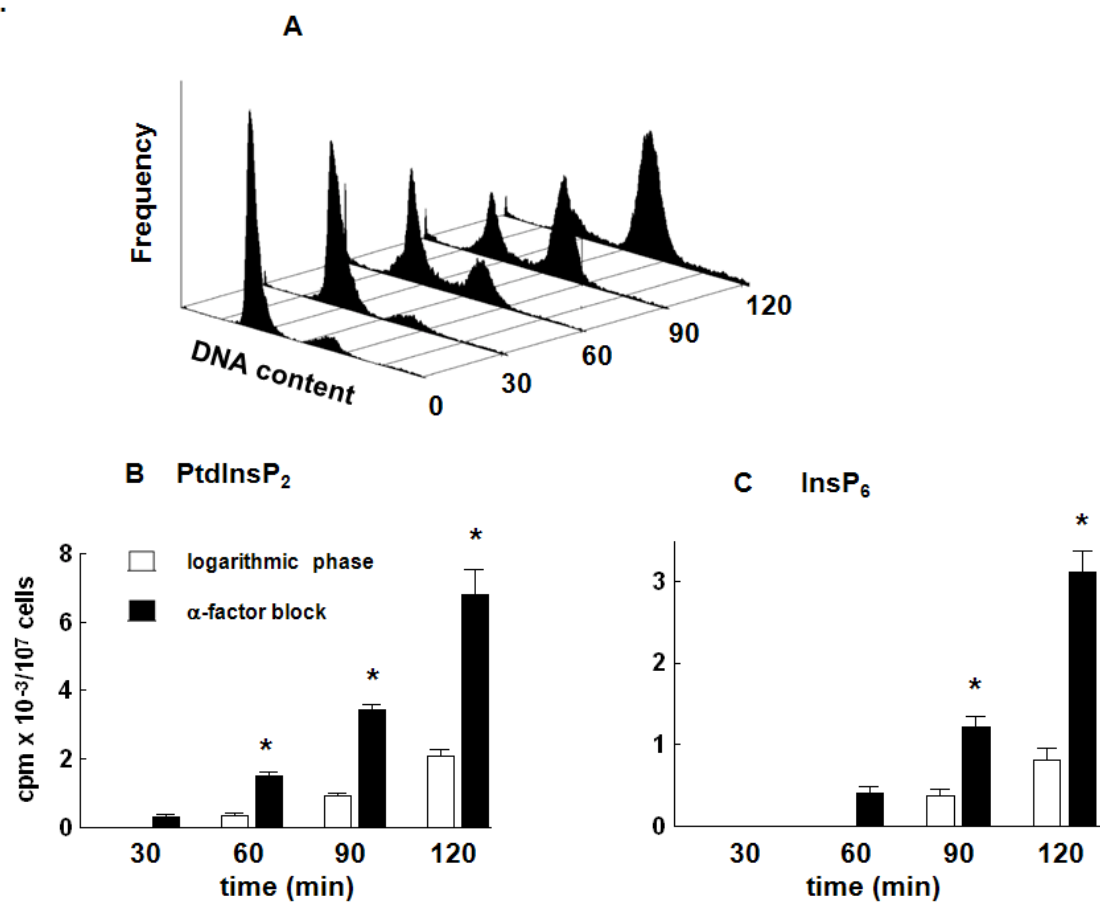


Fig. 2.



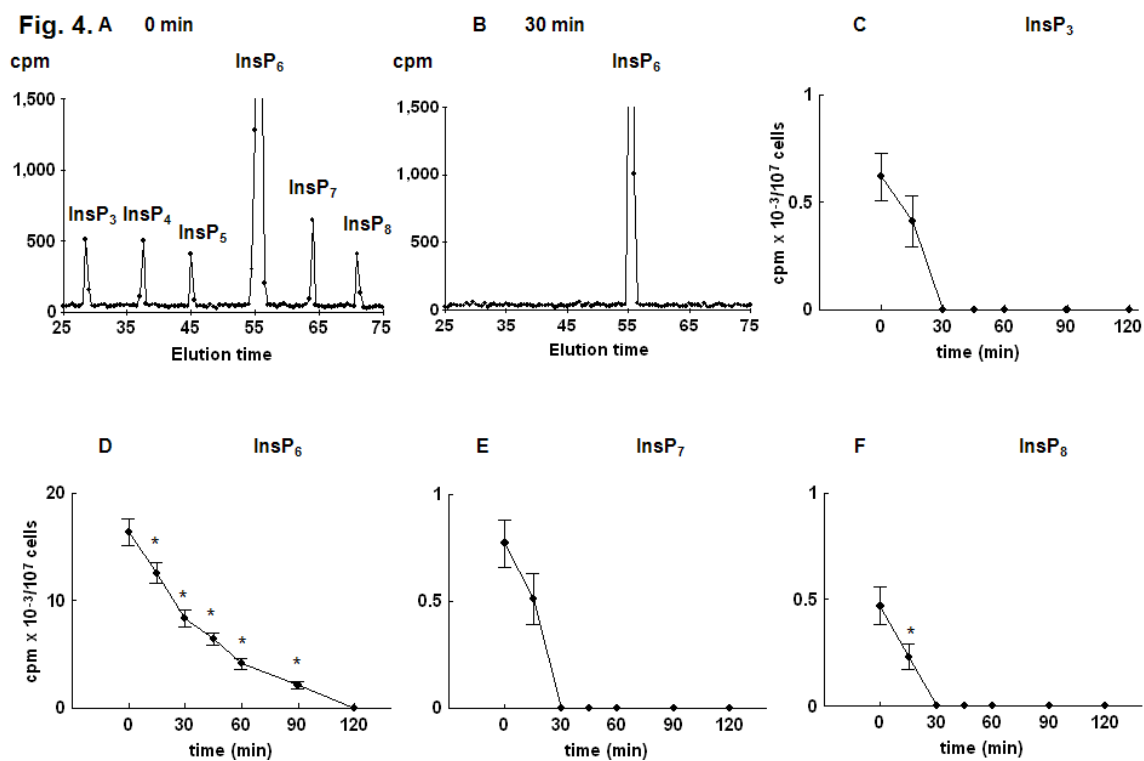
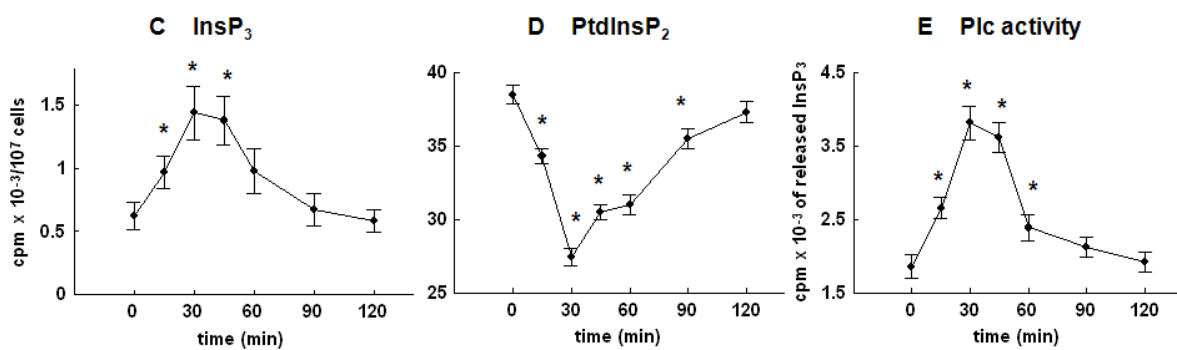
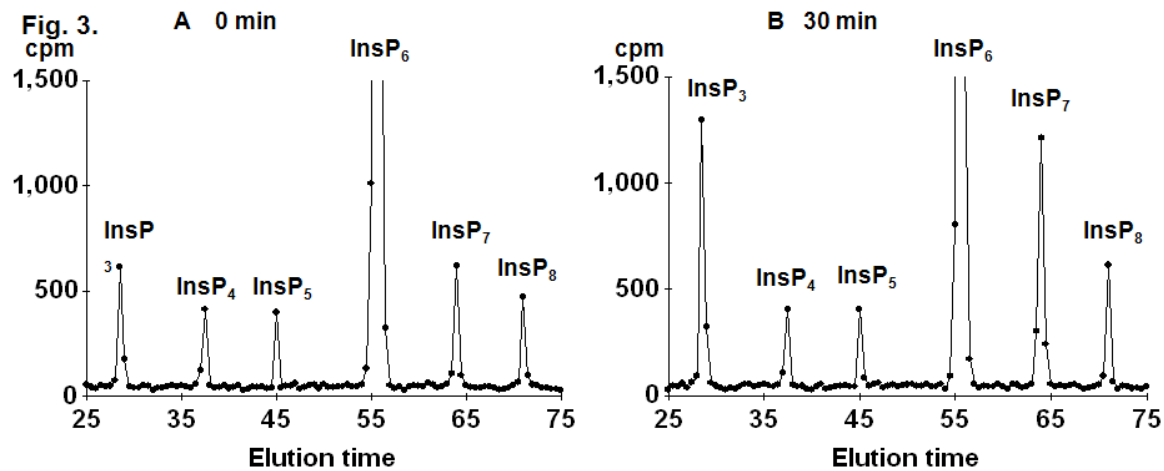


Fig. 5.

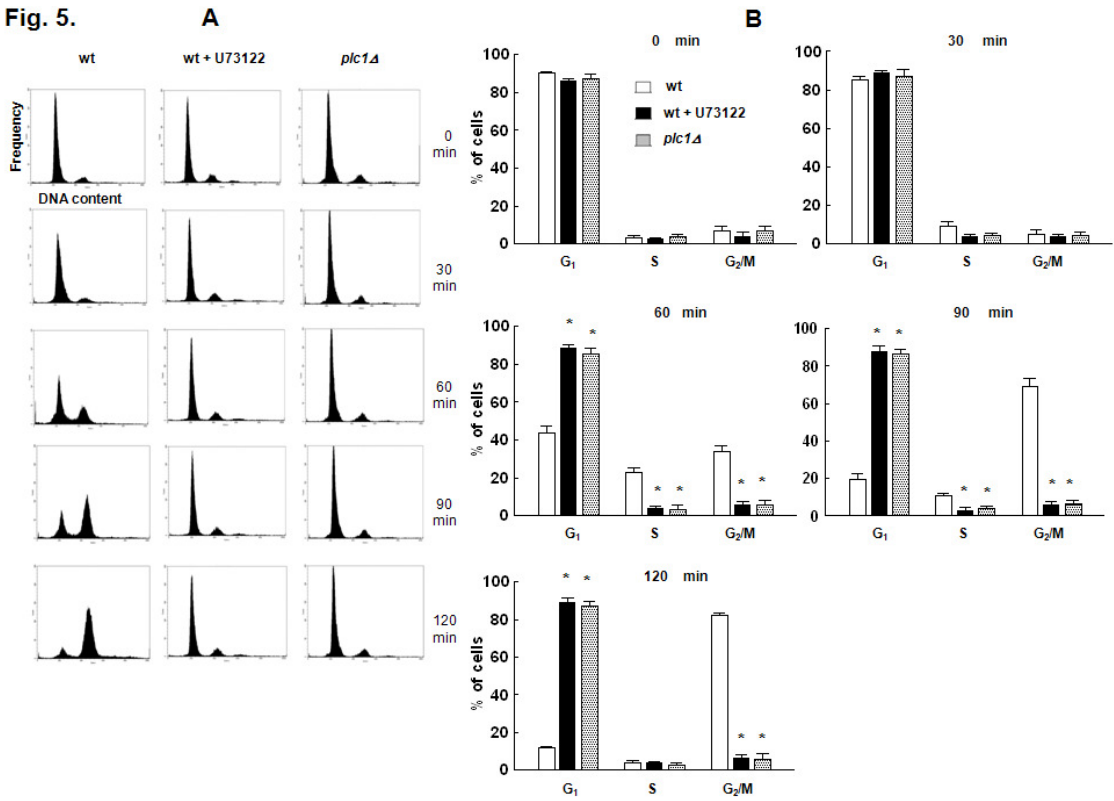
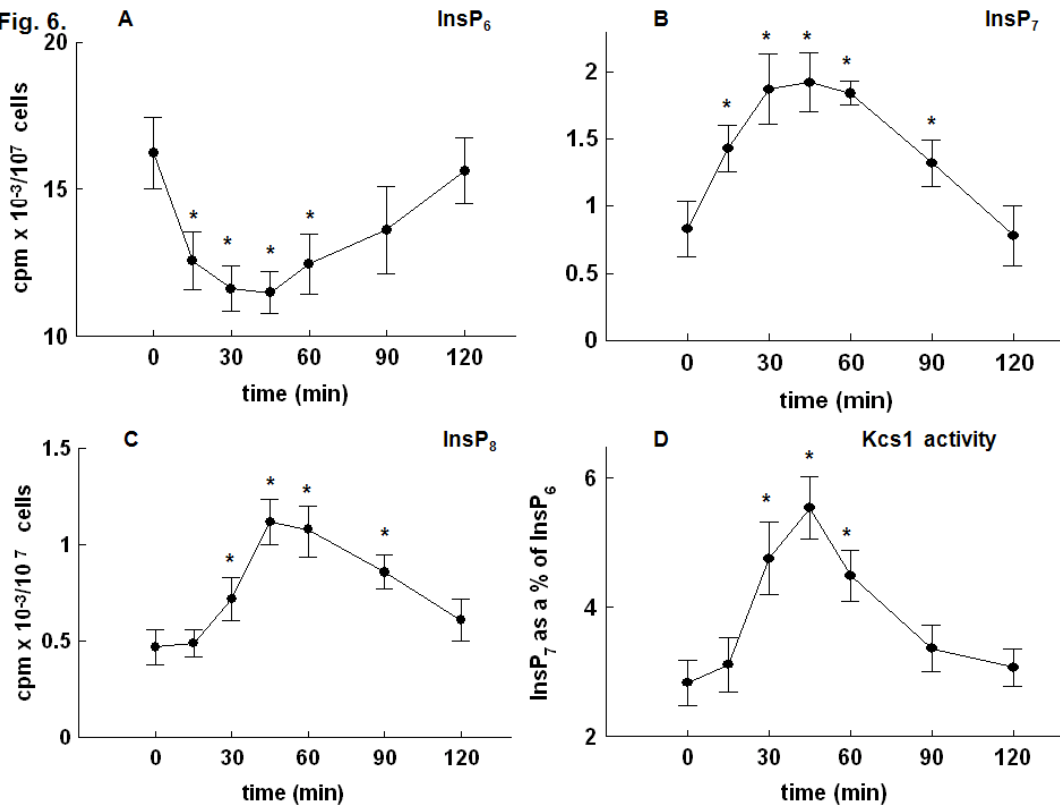
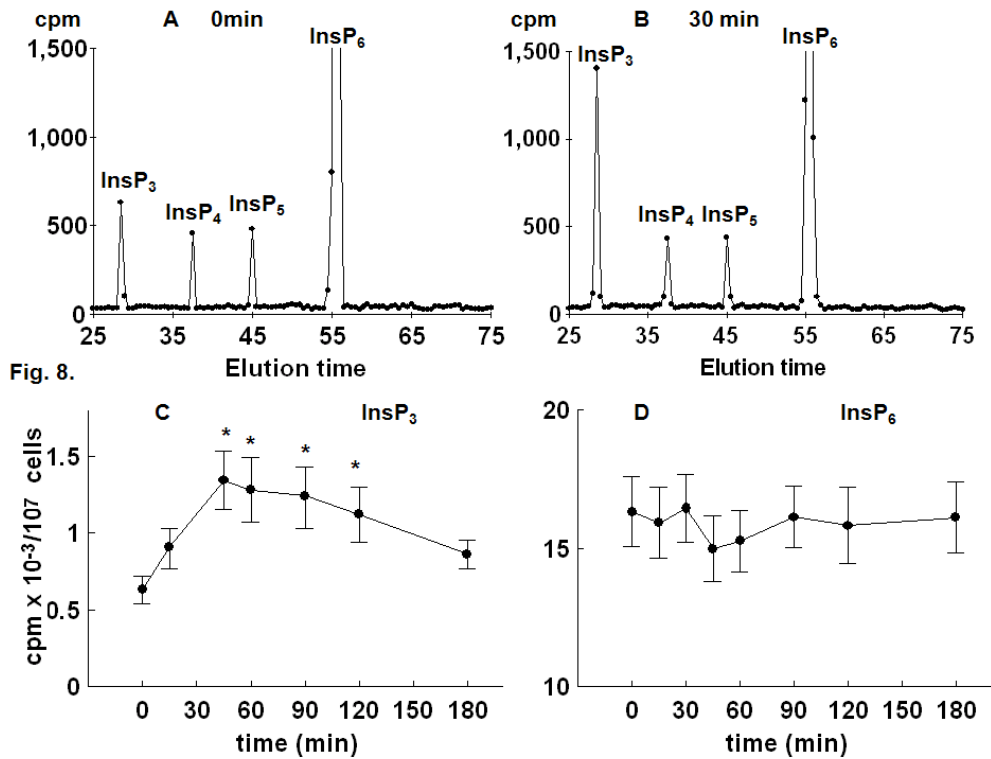
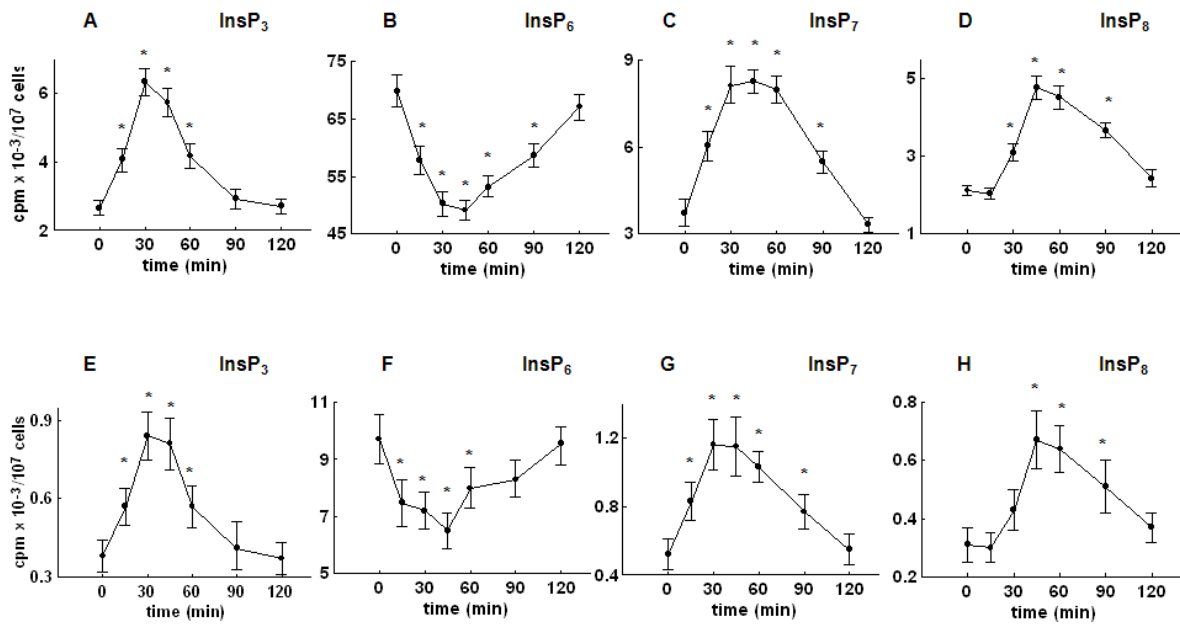


Fig. 6.



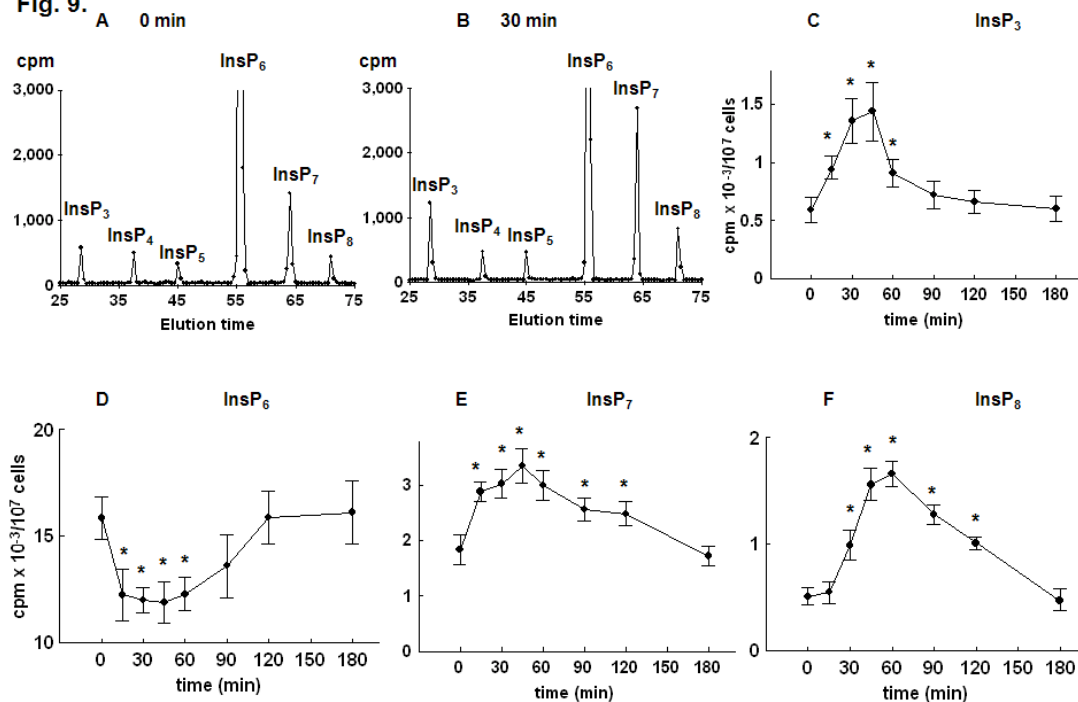
**Fig. 7.**



**Fig. 8.**



**Fig. 9.**



**Fig. 10.**

