

## Overexpression of Phosphatidylinositol Synthase Enhances Growth and G1 Progression in NIH3T3 cells

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Phosphatidylinositol (PI) turnover is thought to play an important role in the regulation of cell growth. PI synthase (PIS, cytidine diphosphate (CDP)-diacylglycerol (DG): *myo*-inositol 3-phosphatidyltransferase, EC 2.7.8.11) acts at the last step in the *de novo* biosynthesis of PI by catalyzing the condensation of CDP-DG and *myo*-inositol. To study the physiological role of PIS, we established murine NIH3T3 fibroblasts that stably overexpress PIS, by transfection with PIS cDNA (NIH-PIS cells). In immunofluorescence assays, the constitutively overexpressed PIS was found to be localized in the endoplasmic reticulum, as previously reported for the native enzyme activity. NIH-PIS cells showed an increase in PI synthesis *in vitro* and *in vivo*, as well as increased cellular levels of PI-4,5-P<sub>2</sub> and PI-3,4,5-P<sub>3</sub>. They also displayed a decrease in their doubling time and accelerated G1 progression. Overexpression of PIS increased cellular levels of the cyclin D1 and E proteins and Akt kinase activity in serum-stimulated quiescent NIH3T3 cells. Moreover, PIS overexpression potentiated the colony formation of NIH3T3 cells in soft agar. These results suggest that PIS accelerates G1 progression and stimulates growth by increasing cellular levels of cyclins D1 and E.

Key words: Phosphatidylinositol synthase — Cell cycle — Cyclin D — Cyclin E — Akt

Phosphatidylinositol (PI) is a minor membrane component representing between 2–12% of the total phospholipids in eukaryotic cells.<sup>1)</sup> It is a precursor of second messengers, including PI phosphates, inositol-polyphosphates, and diacylglycerol (DG). The hydrolysis of PI-4,5-P<sub>2</sub> (PIP<sub>2</sub>) by PI-specific phospholipase C (PI-PLC) is often activated when cells are stimulated by various growth factors or hormones. PIP<sub>2</sub> breakdown leads to the generation of two second messengers: inositol-1,4,5-triphosphate (IP<sub>3</sub>), which is responsible for the release of Ca<sup>2+</sup> from intracellular stores, and DG, which activates several isoforms of protein kinase C.<sup>2–5)</sup> After receptor-triggered hydrolysis of PIP<sub>2</sub>, PI must be resynthesized in order to maintain a constant level of PI in cell membranes, and this requirement is met by phosphatidylinositol synthase (PIS) activity.

PIS (CDP-DG: *myo*-inositol 3-phosphatidyltransferase, EC 2.7.8.11) acts at the last step in the *de novo* biosynthesis of PI by catalyzing the condensation of CDP-DG and *myo*-inositol. PIS appears to be located primarily on the cytoplasmic aspect of the endoplasmic reticulum,<sup>6–8)</sup> and its enzyme activity is detected in plasma membrane preparations.<sup>9–12)</sup>

In yeast, Nikawa *et al.* reported that disruption of the *PIS* gene is lethal. Nonviable spores were observed with characteristic terminal phenotypes, suggesting that the *PIS* gene is essential for progression of the yeast cell cycle.<sup>13)</sup> The rat PIS cDNA has been cloned by functional complementation of a *Saccharomyces cerevisiae* PIS mutant deficient in PIS activity.<sup>14)</sup> The cloned cDNA encodes a protein of 213 amino acids with a calculated molecular weight of 23 613 Da. Lykidis *et al.*<sup>15)</sup> identified the human *PIS* gene in the human expressed sequence-tagged (EST) data base.

Eukaryotic cell-cycle progression is controlled by the sequential activation of cyclin-dependent kinases (CDKs). Cyclin D1/CDK4 or 6, and cyclin E/CDK2 complexes regulate the G<sub>0</sub>-to-S-phase transition during the cell cycle.<sup>16–19)</sup> Cyclin D1/CDK4 activity can be detected in the mid-G<sub>1</sub> phase after stimulation of quiescent cells to enter the cell cycle.<sup>20, 21)</sup> Cyclin E/CDK2 appears later during the G<sub>1</sub> to S transition, suggesting a role for cyclin E in the initiation of DNA synthesis. Activated cyclin D1/CDK4 promotes G<sub>1</sub> progression, at least in part, by phosphorylating the retinoblastoma protein (pRB), thereby inactivating the ability of pRB to act as a transcriptional repressor when it is complexed to the transcription factor, E2F.<sup>22, 23)</sup> In addition, the expression of cyclin E is stimu-

lated when E2F is activated.<sup>24</sup> Phosphorylation of pRB and the release of E2F are correlated with transition across the G1 checkpoint.<sup>25, 26</sup>

We previously found that *src*- and *erbB2*-transformed cells have a higher PIS activity than normal cells and that this enzyme activity is regulated by tyrosine kinase.<sup>27</sup> In quiescent normal rat kidney (NRK) cells, PI synthesis is stimulated by the addition of mitogenic growth factors or serum. On the other hand, a PIS inhibitor,  $\delta$ -hexachloro-cyclohexane, inhibited S phase induction.<sup>28</sup> In previous studies we found that inhibition of PI synthesis by inostamycin<sup>29</sup> blocked cell cycle progression in the G1 phase in NRK cells.<sup>30</sup> Inostamycin also caused a decrease in cellular levels of cyclins D1 and E.<sup>31</sup>

In the present paper, we studied the physiological roles of PI synthesis more directly, by establishing NIH3T3 cells that stably overexpress a FLAG-tagged rat PIS cDNA (NIH-PIS cells), using a cytomegalovirus (CMV) promoter-containing vector.

## MATERIALS AND METHODS

**Materials** Polyclonal antibodies to cyclin D and E were purchased from UBI (Lake Placid, NY). The anti-FLAG antibody was purchased from Sigma (St Louis, MO). The PIP<sub>2</sub> antibody was kindly provided by Mr. Koji Mizuno (Azwell Inc., Osaka). The anti-PI-3,4,5-P<sub>3</sub> antibody was purchased from Alexis Biochemicals (San Diego, CA).

**Cell synchronization** NIH3T3 control and NIH-PIS cells were cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (CS) and then for 72 h in medium containing 0.2% CS. Thereafter, the cells were arrested at the G0 phase, and the quiescent cells were stimulated by the addition of 5% CS.

**Transfection procedures** The FLAG-tagged 704-bp rat PIS cDNA, including the open reading frame, was recloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Cells were transfected with a modified calcium phosphate precipitation technique,<sup>32</sup> with 20  $\mu$ g of plasmid DNA per dish, and the cells were then cultured for 3 weeks in complete growth medium with 800  $\mu$ g/ml of G418 (Invitrogen). Antibiotic-resistant clones were then isolated, expanded and maintained in the selection medium. The levels of expression of PIS in the isolated clones were examined by immunoblot analysis using a monoclonal anti-FLAG antibody.

**PI synthase assay** The cells ( $2 \times 10^6$ ) were suspended in solubilizing buffer (20 mM HEPES [pH 7.2], 40 mM *n*-octylglucoside, 30 mM sodium pyrophosphate, 50 mM sodium chloride, 5 mM  $\beta$ -glycerophosphate, 1 mM EGTA, 1 mM PMSF, 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml leupeptin, 0.25 M sucrose). After sonication, twice for 15 s each time, the extracts were centrifuged at 15 000g for 15 min and the supernatant fraction was used for enzyme assays. The

assay mixture contained 100  $\mu$ M CDP-DG, 1  $\mu$ M [<sup>3</sup>H]-inositol (specific activity 30 Ci/mmol), 2 mM MnCl<sub>2</sub>, 50 mM MgCl<sub>2</sub>, 50 mM Tris-HCl [pH 8.0], and 20–30  $\mu$ g of protein, in a final volume of 50  $\mu$ l. Assay mixtures were incubated for 15 min at 37°C, and the assays were terminated by addition of 180  $\mu$ l of chloroform/methanol/concentrated HCl (1:2:0.02, v/v). Then, 60  $\mu$ l of chloroform and 60  $\mu$ l of 2 M KCl were added, and after vortex mixing, the two phases were separated by centrifugation. The radioactivity in the organic phase was counted with a liquid scintillation counter.

**Incorporation of [<sup>32</sup>P]orthophosphate into phospholipids** NIH-PIS cells ( $1 \times 10^6$ ) or the vector control cells were labeled with <sup>32</sup>Pi (5  $\mu$ Ci/well) in phosphate-free DMEM for 1 h. The cells were harvested and sonicated in buffer A (10 mM HEPES [pH 7.5], 5 mM MgCl<sub>2</sub>, 15 mM KCl, 1 mM PMSF). The protein concentration was adjusted to 5 mg/ml for each sample, in a final volume of 50  $\mu$ l. Then, 50  $\mu$ l of 1 N HCl, 90  $\mu$ l of chloroform, and 180  $\mu$ l of methanol were added. After vortex mixing, 930  $\mu$ l of chloroform/2 M KCl (1:1, v/v) was added, and after vortexing, the phases were separated by centrifugation. The organic phase was evaporated, and the lipid extracts labeled with <sup>32</sup>P were spotted on a silica gel thin-layer chromatography (TLC) plate, which was developed with chloroform/methanol/acetic acid/H<sub>2</sub>O (25:15:4:2). The radioactive spots were identified by autoradiography. Each phospholipid spot was confirmed by comigration of non-radioactive phospholipid.

**Immunocytochemistry by epitope-tagging** NIH-PIS and control cells ( $1 \times 10^4$ ) were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at 4°C, and then treated with 0.1% Triton X-100 for 5 min. Nonspecific staining was blocked by incubating the cells with a blocking solution. After washing, the cells were treated sequentially with the anti-FLAG antibody and then a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ), for 1 h each. The localization of FLAG-tagged PIS was visualized microscopically by use of the Tyramide Signal Amplification method (NEN Life Science Products, Boston, MA).

**Immunocytochemistry with anti-PIP<sub>2</sub> or PIP<sub>3</sub> antibody** NIH-PIS and control cells ( $1 \times 10^4$ ) were fixed with 3% paraformaldehyde in PBS for 15 min at 4°C. Nonspecific staining was blocked by incubating the cells with a blocking solution. After washing, the cells were treated sequentially with an anti-PIP<sub>2</sub> or anti-PIP<sub>3</sub> antibody and then a horseradish peroxidase-conjugated secondary antibody (Calbiochem, San Diego, CA), for 1 h each. The localization of PIP<sub>2</sub> or PIP<sub>3</sub> was visualized microscopically by use of the Tyramide Signal Amplification method.

**Determination of cell doubling time** Cells were plated at a density of  $1 \times 10^4$  in 10.2 mm diameter, 48-well dishes. The numbers of cells per well were counted daily for the

next 7 days, with a Coulter counter. The doubling times were measured during the period of exponential growth.

**Soft agar assay** Cells ( $1 \times 10^4$ ) were seeded into 15.6 mm diameter, 24-well culture dishes in 1 ml of DMEM containing 10% calf serum and 0.4% low melting-point agarose (TaKaRa Biomedicals, Ohtsu), over a layer of 0.53% agarose. Three weeks after seeding, the colonies were photographed.

**Western blot analysis** Cells ( $2 \times 10^6$ ) were washed, harvested with PBS, and sonicated in extraction buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol (DTT), 0.1% Tween 20, 10% glycerol, 1 mM PMSF, 10  $\mu$ g/ml leupeptin, 10 mM  $\beta$ -glycerophosphate, 1 mM NaF, 100  $\mu$ M  $\text{Na}_3\text{VO}_4$ ). The lysates were electrophoresed on a 10% polyacrylamide gel, and then electrophoretically transferred to a PVDF membrane. The membrane was blocked with 6% CS (for cyclins D1 and E antibodies) or 5% skim milk (the anti-FLAG antibody), and incubated with a solution containing the primary antibody. After washing, the membrane was incubated with the horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). Protein

bands were visualized with the Western Blot Chemiluminescence Reagent (NEN Life Science Products).

**Cell-cycle analysis** For flow cytometric analysis, the cells were trypsinized, washed twice with PBS, resuspended in 1 ml of PBS, and fixed with 5 ml of 70% ethanol. After sedimentation, the fixed cells were stained with 0.2 mg/ml propidium iodide in the presence of 0.6% NP-40, and then treated for 30 min in the dark with 1 mg/ml RNase. The cell suspension was filtered through a 60- $\mu$ m Spectra nylon mesh filter and analyzed in a flow cytometer (Epics Elite; Coulter, Hialeah, FL) for DNA content. Excitation was carried out with the 488-nm line of an argon ion laser operating at a continuous output of 20 mW.

**Akt kinase assay** The cells ( $1 \times 10^6$ ) were collected, lysed in 100  $\mu$ l of Akt lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 10 mM EDTA, 10% glycerol, 20 mM sodium pyrophosphate, 0.2 mM NaF, 1% Triton X-100, 4 mM  $\text{Na}_3\text{VO}_4$ , 100  $\mu$ g/ml leupeptin, 2 mM PMSF, 15 mM benzamidine). The lysates were then electrophoresed on a 9% polyacrylamide gel, and the gel electrophoretically transferred to a PVDF membrane. The membrane was blocked with 5% skim milk, and incubated with a solut-

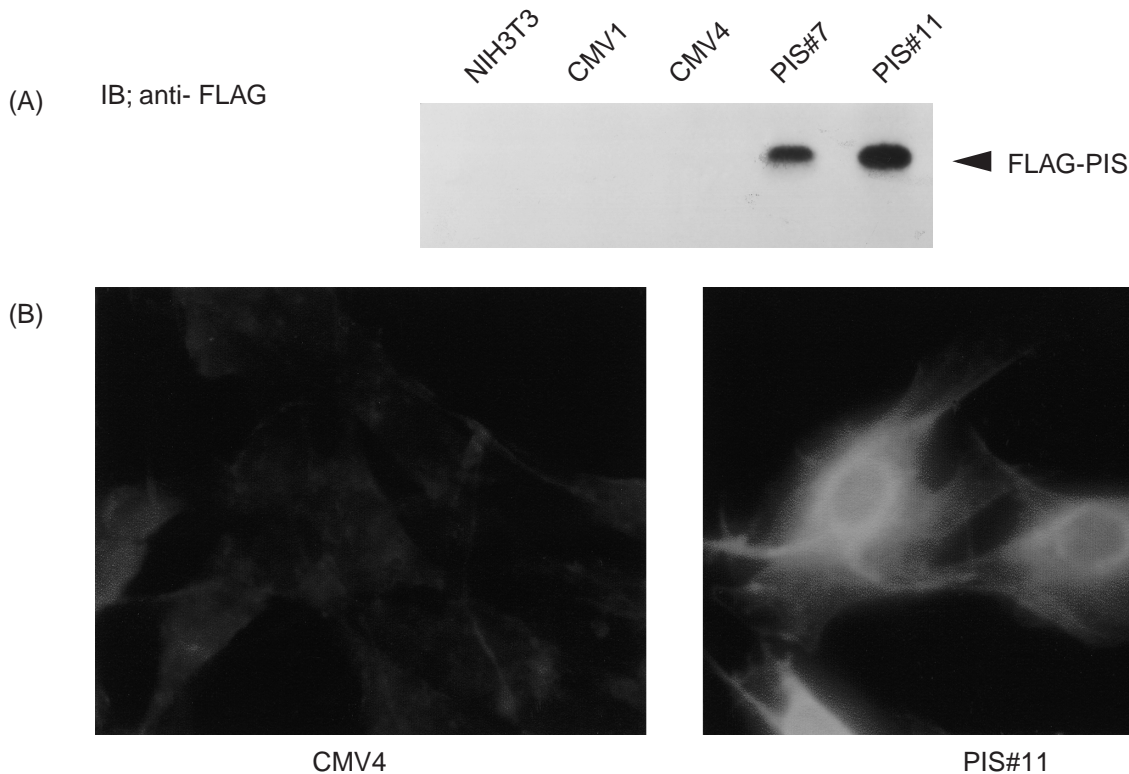


Fig. 1. Stable overexpression of FLAG-tagged rat PIS in NIH3T3 cells. (A) Western blot analysis of the FLAG-tagged PIS protein. Total cellular protein (50  $\mu$ g) from each cell line was subjected to 12.5% SDS-PAGE, and the gel was immunoblotted with the anti-FLAG antibody. (B) Immunohistochemical staining of FLAG-tagged PIS protein in NIH-PIS and CMV4 vector control cells. Immunostaining was performed with anti-FLAG antibody, as described in "Materials and Methods."

ion containing the primary antibody. After washing, the membrane was incubated with the horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). Protein bands were then visualized with the Western Blot Chemiluminescence Reagent (NEN Life Science Products). The activated Akt kinase was detected with the anti-phospho-Akt (Ser 473) antibody (Cell Signaling Technology, Beverly, MA).

## RESULTS

**Overexpression of PIS in mouse NIH3T3 cells** We attempted to express the rat PIS in mouse NIH3T3 fibroblasts by transfection with a FLAG-tagged PIS cDNA, using the vector pcDNA3. Several G418-resistant colonies were randomly isolated, and we then examined the levels of PIS expression by western blot analysis using an anti-FLAG antibody. We selected the highest PIS-overexpressing clone (PIS#11) and the second highest clone (PIS#7), and we also isolated two vector control clones (CMV1, CMV4) (Fig. 1A). There was no obvious change in the morphology of the PIS#11 or PIS#7 cells. To confirm the levels of PIS expression, we also examined the mRNA levels of PIS in these cells, by northern blot analysis with a rat PIS cDNA. PIS mRNA was not detectable in the CMV4 cells. The PIS#7 cells displayed a moderately intense 704 bp band and the PIS#11 cells a strong 704 bp band (data not shown).

To confirm the localization of exogenous PIS, PIS#11 and PIS#7 cells were fixed with paraformaldehyde and subjected to immunofluorescence microscopy after staining with an anti-FLAG antibody. As shown in Fig. 1B, intense fluorescence was observed in the cytoplasm, presumably in the endoplasmic reticulum and Golgi appara-

tus. The primary structure of the PIS protein indicates that there is a pronounced hydrophobic region containing 60% hydrophobic amino acids, which would be expected to have an affinity for cellular membranes.<sup>4)</sup> In previous studies, PIS activity was reported to be found in the endoplasmic reticulum and Golgi apparatus membrane,<sup>6)</sup> or in the plasma membrane.<sup>9)</sup> In addition, Whatmore *et al.* confirmed that PIS is localized at the endoplasmic reticulum by using an anti-PIS antibody.<sup>33)</sup> Our results are consistent with this localization.

**PIS activity in NIH-PIS cells** We also did *in vitro* assays for PIS activity in extracts of these cells. The PIS activities were approximately 11- and 23-fold higher in the PIS#7 and PIS#11 clones, respectively, than in the control CMV4 cells (Fig. 2A). The effect of PIS overexpression on PI synthesis *in vivo* was determined by labeling the cells with <sup>32</sup>P. The degrees of PI synthesis *in vivo* were about 7.0- and 10.3-fold higher in the PIS#7 and PIS#11 cells than in the CMV4 cells (Fig. 2B). These activities were linearly correlated with the expression levels of PIS.

Since overexpression of PIS increased PI synthesis, we examined the amounts of PIP<sub>2</sub> and PI-3,4,5-P<sub>3</sub> (PIP<sub>3</sub>) in NIH-PIS cells. We performed immunostaining with anti-PIP<sub>2</sub> and anti-PIP<sub>3</sub> antibodies in exponentially growing NIH-PIS cells. As shown in Fig. 3, the amount of PIP<sub>2</sub> increased in NIH-PIS cells, when compared with CMV4 control cells. Similar results were obtained with the anti-PIP<sub>3</sub> antibody (Fig. 3). Thus, overexpression of PIS caused increased cellular levels of PIP<sub>2</sub> and PIP<sub>3</sub>.

**Effect of PIS overexpression on cell proliferation** To study the role of PI synthesis on cell proliferation, we examined the growth properties of these cells. We consistently found that the NIH-PIS cells grew faster than the vector-transfected control cells (CMV4). A representative

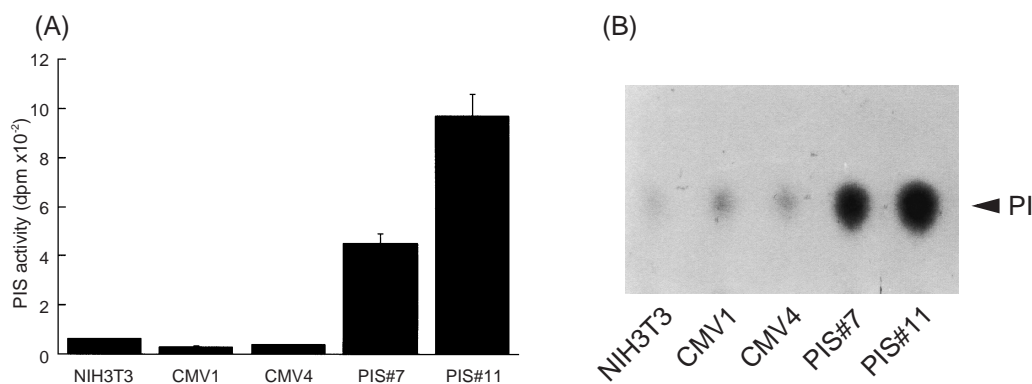


Fig. 2. PIS activity in NIH-PIS cells. (A) PIS activity *in vitro*. The cells were harvested and lysed, and the extracts assayed for PI synthase activities, as described in "Materials and Methods." (B) Cellular PI synthesis in intact NIH-PIS cells. Subconfluent cells were incubated with [<sup>32</sup>P]Pi for 1 h. The radioactive lipids were extracted and analyzed by TLC, as described in "Materials and Methods." Non-radioactive markers for PI; PA, phosphatidic acid, and PC, phosphatidylcholine were visualized with I<sub>2</sub> vapor. Fig. 1B showed only the PI spot. The results are representative of triplicate experiments.

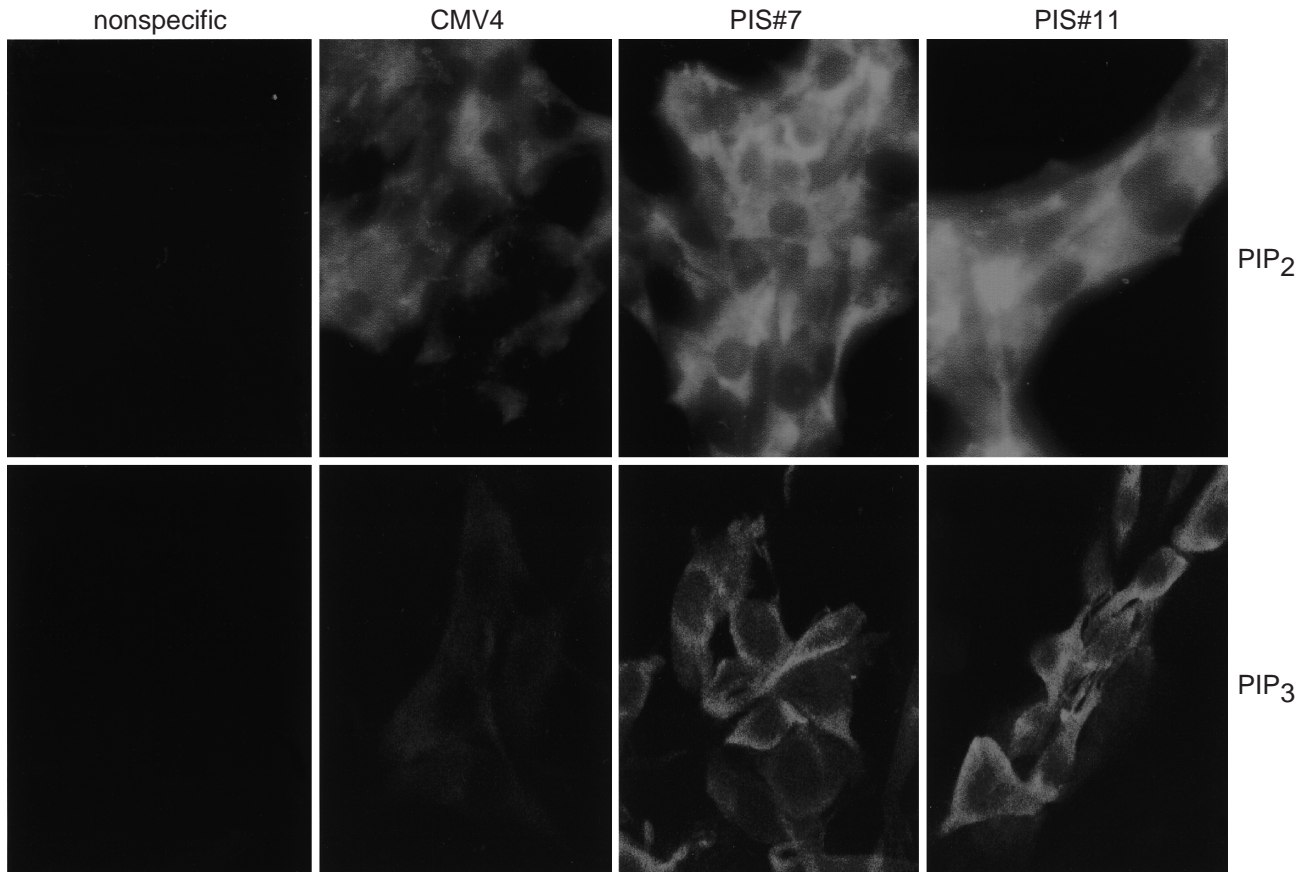


Fig. 3. Immunohistochemical staining of PIP<sub>2</sub> and PIP<sub>3</sub> in NIH-PIS and CMV4 vector control cells. Immunostaining was performed with anti-PIP<sub>2</sub> and PIP<sub>3</sub> antibodies, as described in “Materials and Methods.”

growth curve for each cell line is shown in Fig. 4. The cell doubling times of PIS#7 and PIS#11, during the exponential phase of growth, were  $19.5 \pm 1.0$  and  $17.5 \pm 0.9$  h, respectively, whereas that of the control cell line was  $23.6 \pm 0.8$  h.

In addition, the saturation densities of the PIS#11 and PIS#7 cells were increased by about 1.5-fold, when compared with that of the control CMV cells. We next examined these cells for anchorage-independent growth. The control CMV4 cells failed to form colonies in the medium containing 0.4% agar and 10% CS. However, under the same conditions the PIS#7 and PIS#11 cells formed small colonies in 0.4% agar (Fig. 5). Therefore, the increase in PI synthesis enhances the cell proliferation, in both monolayer cultures and agar suspension.

**PIS overexpression enhances PI synthesis and accelerates G1 progression** Based on results obtained with a PIS inhibitor, we previously suggested that PI synthesis might be involved in regulation of G1 progression in NRK cells,<sup>30</sup> and also in NIH3T3 cells (data not shown). There-

fore, we examined whether PIS overexpression enhanced PI synthesis and accelerated G1 progression. The cell lines were arrested at the G0 phase by serum starvation, and then stimulated to enter the cell cycle by the re-addition of serum. Assays for PI synthesis were then done at subsequent time points. The serum-starved cells were incubated with <sup>32</sup>P phosphorus for 1 h or cells were labeled for 1 h beginning at either 0.5, 1 or 3 h after the addition of serum (Fig. 6A). No significant synthesis of PI was detected in the serum-starved CMV4 control or PIS#7 or PIS#11 cells. However, at all 3 time points after the addition of serum, PI synthesis was increased about 3.1-fold in the PIS#7 cells and about 3.8-fold in the PIS#11 cells (Fig. 6A). Phosphatidylcholine and phosphatidic acid also increased slightly in the cells that overexpressed PIS, as shown in Fig. 6A, possibly through the action of phospholipase D or as a result of the overexpression of PIS.

We then examined the effects of serum restimulation of serum-starved CMV4 control cells and the NIH-PIS cells, with respect to cell cycle progression (Fig. 6B). As

expected, at time 0 both types of cells (about 88%) were mostly in G0/G1 and only a small fraction was in S or G2/M. After serum stimulation, at 12 h 21.5% of the CMV4 cells were in S phase but 27% of the PIS#7 cells and 34.2% of the PIS#11 cells were in S phase (Fig. 6B). In addition, the NIH-PIS cells also entered the G2/M phase more rapidly than the CMV4 cells, since with the CMV4 cells the maximum percent of cells in G2/M occurred at about 20 h, but with the PIS#7 and PIS#11 cells it occurred at about 16 h (Fig. 6B). Therefore, after

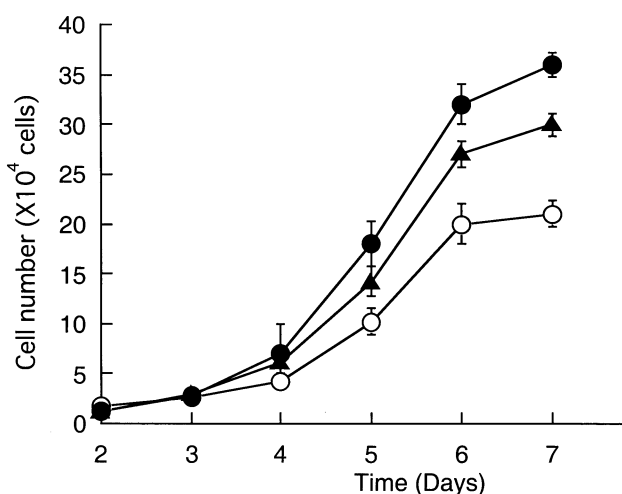


Fig. 4. Growth properties of NIH-PIS cells in monolayer culture. PIS#11 (●), PIS#7 (▲), and CMV4 (○) cells were grown in medium containing 5% calf serum (CS), and cell numbers were then determined. Each point is the mean of triplicate determinations. Similar results were obtained in three independent experiments.

serum stimulation the NIH-PIS cells progress through the cell cycle more rapidly than the control CMV4 cells. This is consistent with our finding that non-synchronized exponentially dividing NIH-PIS cells also have a shorter doubling time (Fig. 4).

**PIS overexpression increases expression of G1 cyclins**

Cyclin D1 and cyclin E are known to be rate-limiting factors in the G1 progression of the cell cycle. Therefore, we examined the effects of PIS overexpression on cyclin D1 and E expression levels, after serum stimulation of serum-starved cells. Cyclin D1 was detected at 4 h after serum addition in the CMV4, PIS#7 and PIS#11 cells, but the levels were higher and more persistent in the NIH-PIS cells. Cyclin E was not increased in the CMV4 cells until 8 h, but in the NIH-PIS cells it increased within 4 h and its level was higher, particularly in the PIS#11 cells, than in the control CMV4 cells (Fig. 6C). These results suggest that the shortened G1 phase noted in Fig. 6B, associated with increased expression of PIS and enhancement of PI synthesis (Fig. 6A) may be due to increased expression of cyclins D1 and E.

**PIS overexpression increases Akt kinase activation**

To examine possible downstream effectors of PIS overexpression that may be relevant to cyclin D1 expression and enhanced G1 progression, we focused on the PI3-kinase/Akt pathway since it is known to regulate the transcription,<sup>34)</sup> translation,<sup>35)</sup> and stability of cyclin D1.<sup>36)</sup> Immunostaining with the anti-PIP<sub>2</sub> and anti-PIP<sub>3</sub> antibodies indicated that cellular levels of PIP<sub>2</sub> and PIP<sub>3</sub> were higher in PIS#11 cells than in CMV4 cells (Fig. 3). Therefore, PIS overexpression probably increases cellular levels of PI-3-P, PI-3,4-P<sub>2</sub>, and PI-3,4,5-P<sub>3</sub> which are known to activate Akt kinase activity.<sup>37)</sup> Phosphorylation of Akt is associated with activation of its kinase activity.<sup>38)</sup> Therefore,

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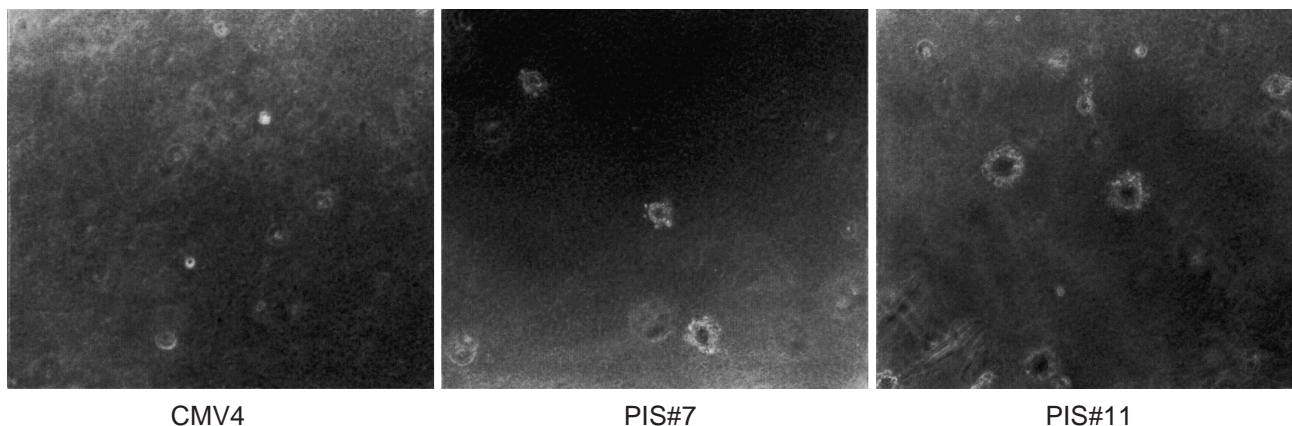


Fig. 5. Anchorage-independent growth of NIH-PIS cells. Cells were seeded into 0.4% soft agar suspension. Three weeks after seeding, each plate was photographed. The results are representative of triplicate assays. The CMV4 cells displayed only single cells, but the NIH-PIS cells displayed numerous small colonies.

we examined Akt kinase activation in the control and NIH-PIS cells using a phospho-Akt specific antibody, in western blot analysis (Fig. 7). Following the addition of serum to serum-starved cells an increase in phospho-Akt was detected within 15 min in both the CMV4 control and

PIS#7 and PIS#11 cells. However, the levels were about 3.5-fold higher in the PIS#11 cells (Fig. 7). Therefore, PIS overexpression might enhance Akt activation, which could contribute to the increased expression of cyclin D1 in the NIH-PIS cells.

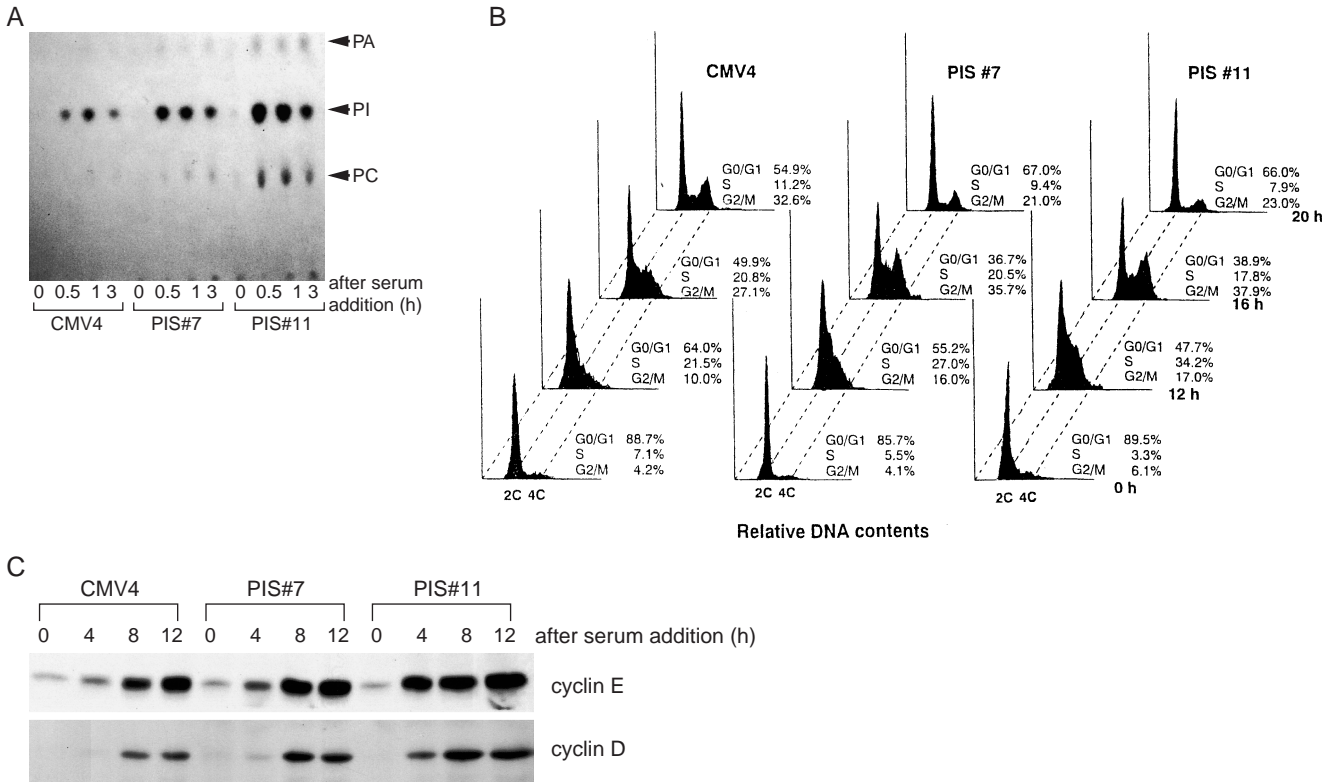


Fig. 6. Effects of PIS overexpression on PI synthesis and cell-cycle progression. (A) Induction of PI synthesis in serum-stimulated NIH-PIS cells. Quiescent serum-starved PIS#11, PIS#7 and CMV4 cell lines were stimulated with 5% CS at time 0, and at the indicated subsequent times the cells were labeled with [<sup>32</sup>P]Pi for 1 h. Radioactive lipids were then extracted and analyzed by TLC, as described in "Materials and Methods." Lipid standards were visualized by use of I<sub>2</sub> vapor. The results are representative of triplicate experiments. PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine. (B) Flow cytometric analysis of NIH-PIS cells. Quiescent PIS#11, PIS#7, and CMV4 cell lines were stimulated with 5% CS, and then, at the indicated times, were collected and analyzed for DNA content by flow cytometry. Values in each panel represent % of the cells in the indicated phases of the cell cycle. (C) Effect of PIS overexpression on the expression of G1 cyclins. Quiescent PIS#11, PIS#7, and CMV4 cell lines were stimulated with 5% CS, cell extracts were obtained at the indicated subsequent times, and 50 μg protein from each extract was subjected to 10% SDS-PAGE and immunoblotted with anti-cyclin D and anti-cyclin E antibodies.

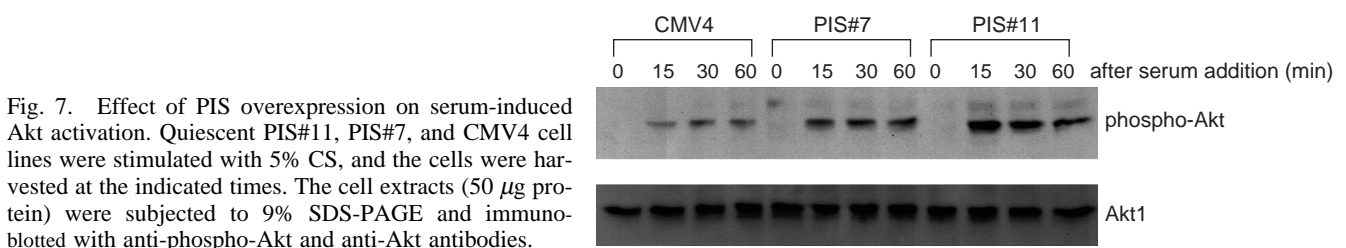


Fig. 7. Effect of PIS overexpression on serum-induced Akt activation. Quiescent PIS#11, PIS#7, and CMV4 cell lines were stimulated with 5% CS, and the cells were harvested at the indicated times. The cell extracts (50 μg protein) were subjected to 9% SDS-PAGE and immunoblotted with anti-phospho-Akt and anti-Akt antibodies.

## DISCUSSION

We previously found that an inhibitor of PIS, inostamycin, induced a G1 arrest of the cell cycle and decreased cellular levels of cyclins D1 and E in NRK cells<sup>31)</sup> and NIH3T3 cells (unpublished studies). These results suggested that PI synthesis can regulate G1 progression through the expression of these two G1 cyclins. To clarify further the physiological roles of PI synthesis, we transfected a PIS cDNA into NIH3T3 cells and isolated clones PIS#7 and PIS#11 that stably overexpress moderate and high levels of PIS, respectively, when compared to the parental NIH3T3 or CMV4 vector control cells (Fig. 1A). Immunofluorescence studies indicated that the overexpressed PIS was distributed throughout the cytoplasm (Fig. 1B), which is consistent with previous evidence that PIS has a hydrophobic region<sup>14)</sup> and is associated with the endoplasmic reticulum and Golgi.<sup>6,8)</sup> Thus, the NIH-PIS cells should be useful in studies on the physiological roles of PIS.

We found that PIS overexpression enhanced the rate of PI synthesis, both *in vivo* and *in vitro*, although the increase in *in vitro* PIS synthesis was greater than that *in vivo* (Fig. 2, A and B). Imai and Gershengorn reported that *de novo* PI synthesis is inhibited by cellular PI.<sup>9)</sup> In addition, the PI transfer protein is known to carry PI and phosphatidyl choline (PC) from the Golgi complex to the plasma membrane.<sup>39)</sup> Therefore, in the NIH-PIS cells PI synthesis *in vivo* may be limited by the cellular level of PI and the PI transfer protein and/or other factors.

Lykidis *et al.*<sup>15)</sup> investigated whether CDP-DG synthetase (cytidine triphosphate (CTP): phosphatidate cytidyltransferase, EC 2.7.7.41) and PIS regulate the cellular content of PI. They found that transient expression of either CDP-DG synthetase or PIS, alone or in combination, did not enhance the rate of PI biosynthesis. They suggested, therefore, that the levels of these two enzymes are not critical determinants of cellular PI content, although they could regulate the rate of PI turnover rather than the rate of *de novo* PI synthesis. However, in the present study we did find an increase of *in vivo* PI synthesis (Fig. 2B). The differences between the results of Lykidis *et al.*<sup>15)</sup> and the present study may reflect the fact that they used a transient transfection procedure, whereas we used cells that stably overexpress PIS.

We also found that stable overexpression of PIS in NIH3T3 cells induced earlier PI synthesis (Fig. 6A), accelerated cell cycle progression (Fig. 6B), and enhanced expression of cyclin D1 and cyclin E (Fig. 6C), following serum stimulation of serum-starved cells. It is known that pRB is phosphorylated by cyclin D1/CDK4 or cyclin D1/CDK6 and that this phosphorylation causes functional inactivation of pRB and activation of the transcription factor E2F.<sup>26)</sup> E2F-binding sites are present in the cyclin E

promoter region and this promoter is activated by E2F.<sup>40,41)</sup> Furthermore, serum-induced expression of cyclin E is higher in cells constitutively overexpressing cyclin D1.<sup>42)</sup> Therefore, the enhancement of cyclin E expression in NIH-PIS cells (Fig. 6C) could be secondary to the increase in cyclin D1. Presumably, the acceleration of G1 progression in the NIH-PIS cells is due, at least in part, to the increased expression of cyclins D1 and E.

We found that the NIH-PIS cells displayed enhanced growth in both monolayer culture (Fig. 4) and in soft agar (Fig. 5). Jiang *et al.* found that stable overexpression of cyclin D1 in fibroblasts shortened the G1 phase, enhanced cell growth in monolayer and soft agar cultures, and also enhanced tumorigenesis in mice.<sup>43)</sup> Therefore, increased PIS expression could play a role in cell transformation through the increased expression of cyclin D1, and possibly other growth-enhancing effects.

How does PIS enhance cyclin D1 expression? Since the overexpression of PIS in NIH3T3 cells increased PI synthesis (Fig. 2, A and B), and the amounts of PI-4,5-P<sub>2</sub> and PI-3,4,5-P<sub>3</sub> (Fig. 3), it is possible that PIS overexpression also enhances cellular levels of PI-3-P, PI-3,4-P<sub>2</sub>, and PI-3,4,5-P<sub>3</sub>, which, together with serum, cause increased activation of Akt kinase. The PI3-kinase/Akt pathway is known to regulate the transcriptional activity of cyclin D1<sup>34)</sup> and Muise-Helmericks *et al.*<sup>35)</sup> found that the translation of cyclin D1 is also regulated by PI3-kinase and Akt. Furthermore, Diehl *et al.*<sup>36)</sup> found that glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) phosphorylates cyclin D1 at threonine 286 and thus targets it for proteasomal degradation. Therefore, phosphorylation of GSK-3 $\beta$  by Akt, which inactivates GSK-3 $\beta$ , can cause cyclin D1 accumulation. Since we found that PIS overexpression enhanced Akt kinase activation (Fig. 7), this could explain the increased expression of the cyclin D1 protein in our NIH-PIS cells. On the other hand, further studies are required to determine whether increased expression of PIS alters other downstream events that play a role in the expression of cyclins D1 and E and the altered growth properties of our NIH-PIS cells.

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