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Protein tyrosine phosphorylation activates rat splenic type II phosphatidylinositol 4-kinase in vitro

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Abstract Regulation of phosphatidylinositol 4-kinase (PtdIns 4-kinase) by protein tyrosine phosphorylation has been indirect and the effects of phosphorylation are debatable. Rat splenic type II PtdIns 4-kinase was phosphorylated in vitro with protein tyrosine kinases from Con A stimulated splenic lymphocytes. Stoichiometric analysis showed one mole of phosphate was incorporated per mole of PtdIns 4-kinase. Tyrosine phosphorylation increased the enzyme activity by 3-fold. Kinetic analysis showed a reduction in K_m for PtdIns and an increase in V_{max}. Dephosphorylation with protein phosphotyrosine phosphatase abolished the activation of PtdIns 4-kinase while protein phosphatase 2A had no effect. Protein tyrosine phosphorylation and activation of PtdIns 4-kinase appear to be tissue specific.

Key words: T cell activation; Signal transduction; Protein phosphatase

1. Introduction

Agonist induced phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) hydrolysis generates the second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. These second messengers in turn regulate calcium homeostasis and activate protein kinase C, respectively, thereby initiating a cascade of biochemical events that result in secretion, motility, cell division and differentiation [1–4]. In addition to their role in signal transduction, phosphorylated derivatives of phosphatidylinositol are shown to regulate protein kinases, actin polymerization and GTPase activating proteins [5–7].

Following breakdown, PtdIns(4,5)P_2 is rapidly resynthesized by sequential phosphorylation of PtdIns by PtdIns 4-kinase and PtdIns 4-phosphate 5-kinase(s). PtdIns 4-kinase mediates the first committed step in PtdIns(4,5)P_2 biosynthesis [8]. Biochemical characterization of PtdIns 4-kinases suggests that at least two types (type II and type III) of PtdIns 4-kinase exist in mammalian cells while molecular cloning of PtdIns 4-kinase cDNA sequences suggests the presence of more isoforms [8–10]. The type II PtdIns 4-kinase activity changes rapidly in response to external stimuli implicating a physiological function in signal transduction [11–13]. However, the molecular mechanisms that regulate the enzyme are largely undefined.

Circumstantial evidence suggests that type II PtdIns 4-kinase is regulated by phosphorylation. The enzyme was phosphorylated on serine and tyrosine residues in response to EGF treatment in A431 cells and was immunoprecipitated with anti-phosphotyrosine and/or anti-EGF-receptor antibodies [14,15]. However, the immunoprecipitated EGF receptor did not phosphorylate PtdIns 4-kinase on tyrosine residues but phosphorylated at serine in in vitro assays. These results suggest that PtdIns 4-kinase was not a substrate for EGF-receptor kinase, and a serine kinase may be associated with either PtdIns 4-kinase or EGF receptor. Further, the effect of tyrosine phosphorylation on PtdIns 4-kinase was controversial. Earlier studies had suggested a reduction in PtdIns 4-kinase activity in A431 cell membranes treated with a protein phosphotyrosine phosphatase (PTPase) [15], while relatively recent studies have shown an increase in PtdIns 4-kinase activity upon dephosphorylation at tyrosine residues [14]. Dephosphorylation at serine residues is associated with reduction in PtdIns 4-kinase activity. In contrast to A431 cells, a type II PtdIns 4-kinase was suggested to be activated by tyrosine phosphorylation in Con A-stimulated splenic lymphocytes [11]. The present paper addresses this discrepancy by providing a direct evidence for in vitro tyrosine phosphorylation and activation of a splenic PtdIns 4-kinase by protein tyrosine kinase(s) (PTK) from Con A-stimulated splenic lymphocytes.

2. Materials and methods

2.1. Materials

Phosphatidylinositol, Triton X-100, phenylmethylsulfonylfluoride (PMSF), RPMI 1640 medium were from Sigma, St. Louis, MO, USA. Silica coated aluminium *tlc* plates were from Merck, Germany. Nitrocellulose sheets were from Amersham International. Protein A agarose beads, anti-rabbit IgG conjugated to alkaline phosphatase, concanavalin A (Con A) and monoclonal anti-phosphotyrosine antibodies (clone 3-365-10) were from Boehringer Mannheim, Germany. [γ-^{32}P]ATP (3000 Ci/mmol) was from Board of Radiation and Isotope Technology, Bombay, India. Wistar rats were from Haffkine Biopharmaceuticals, Bombay, India. All other chemicals were of analytical grade. Rat splenic protein tyrosine phosphatase was a generous gift from G. Swarup, Centre for Cellular and Molecular Biology, Hyderabad, India.

2.2. Purification of PtdIns 4-kinase from spleen particulate fraction

Particulate fractions from adult rat tissues were obtained as described [16]. Briefly, tissues were homogenized in buffer containing 50 mM Tris (pH 7.6), 1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF, 0.1 mM Pefabloc and 1 μg/ml soyabean trypsin inhibitor. The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was centrifuged at $35000 \times g$ for 40 min to obtain the particulate fraction. PtdIns 4-kinase was purified to homogeneity from rat spleen particulate fraction sequentially on phosphocellulose, DEAE-Sephacel, hep-

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Abbreviations: PtdIns(4,5)P_2, D-*myo*-phosphatidylinositol 4,5-bisphosphate; PtdIns4*P*, D-*myo*-phosphatidylinositol 4-phosphate; PtdIns, D-*myo*-phosphatidylinositol; PtdIns 4-kinase, phosphatidylinositol 4-kinase; EGF, epidermal growth factor; PMSF, phenylmethylsulfonyl fluoride; Pefabloc SC, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; Con A, concanavalin A; pNPP, *para*-nitrophenyl phosphate; PTK, protein tyrosine kinase; PTPase, protein phosphotyrosine phosphatase; PP2A, protein phosphatase 2A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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arin acrylamide and on hydroxyapatite column chromatography as described (Verghese, M. et al., manuscript submitted). The enzyme was assayed in 0.3% Triton X-100 as described earlier [11].

2.3. Immunoprecipitation of phosphotyrosyl proteins from Con A stimulated rat splenic lymphocytes

Isolation and stimulation of rat splenic lymphocytes with Con A were described earlier [11]. Briefly, about 5×10^6 splenic lymphocytes in 0.2 ml RPMI 1640 medium were treated with Con A (5 μg/ml) at 37°C for 2 min. The incubation was terminated with the addition of 1 ml of ice cold RPMI medium. Cells were lysed in 0.2 ml of lysis buffer containing 1% Triton X-100 (v/v), 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 0.25 mM sodium orthovanadate, 1 mM PMSF and 10 μg/ml benzamidine. Phosphotyrosyl proteins were immunoprecipitated from the lysates with anti-phosphotyrosine antibodies (1 μg/ml) at 4°C for 4 h. The immunoprecipitates were collected with protein A agarose beads precoated with bovine serum albumin at 4°C for 1 h. The protein A agarose beads were washed thrice with lysis buffer. Phosphotyrosyl proteins were eluted with 0.2 ml of 0.1 M *p*-nitrophenyl phosphate (pNPP). The eluates were assayed for protein tyrosine kinase activity with poly(glu,tyr) (4:1) as substrate in 25 mM (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), pH 7.4, 20 mM $MgCl_2$ and 0.1 mM vanadate [17]. One unit of protein tyrosine kinase activity is defined as the amount of enzyme required to produce 1 nmol of phosphorylated poly(glu,tyr) per minute at 25°C.

2.4. In vitro phosphorylation of PtdIns 4-kinase with phosphotyrosyl proteins

Rat splenic PtdIns 4-kinase was incubated with PTK activity (8 mU) from *p*NPP eluates from Con A stimulated splenic lymphocytes in 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 0.25 mM EGTA, 0.1 mM vanadate, 100 μM [γ-^{32}P]ATP (200–300 cpm/pmol), 0.3% Triton X-100, 1 mM PMSF, 10 μg/ml benzamidine in the presence or absence of 150 μM genistein. Phosphorylation was initiated with the addition of labeled ATP and incubated at room temperature (~25°C) for 30 min. The reaction was terminated with the addition of SDS-PAGE sample buffer and electrophoresed on 12% SDS-PAGE. The gel was stained with Coomassie blue, dried and subjected to autoradiography. Phosphoamino acid analysis of the labeled PtdIns 4-kinase was performed as described [18,19]. The phosphoamino acids were visualized with ninhydrin followed by autoradiography.

For determination of stoichiometry and effect of phosphorylation on PtdIns 4-kinase, the enzyme was preincubated with *p*NPP eluates as described above for different time points. At the end of each incubation period, aliquots were assayed for PtdIns 4-kinase activity and analyzed on SDS-PAGE. PtdIns 4-kinase activity was assayed by addition of PtdIns (100 μg/ml) to the preincubated samples at room temperature for 3 min. The phospholipids were extracted and analyzed as described [11,20]. The PtdIns 4-kinase band was excised from SDS-PAGE and the radioactivity in the protein was quantified by liquid scintillation counting.

2.5. Dephosphorylation of PtdIns 4-kinase with PTPase and PP2A

PtdIns 4-kinase was phosphorylated with PTKs as described above. The phosphorylated PtdIns 4-kinase was further incubated for 10 min in the presence of protein phosphatases (PTPase or PP2A) and assayed for PtdIns 4-kinase activity. PTPase did not dephosphorylate PtdIns4*P* or ATP under the experimental conditions.

2.6. Other techniques

Gel electrophoresis was performed on 7–15% gradient or 12% SDS-PAGE as described by Laemmli [21]. Immunoblots were performed as described [22]. Protein concentrations were estimated by dye binding method [23].

3. Results

3.1. Con A stimulates protein tyrosine kinase(s) activity in splenic lymphocytes

Earlier studies from our lab had shown that Con A modulates tyrosine phosphorylation and activation of a PtdIns 4-kinase in rat splenic lymphocytes within two minutes [11]. These studies suggest that PTKs responsible for phosphorylation of PtdIns 4-kinase in splenic lymphocytes may also be activated within this period. To address the effect of Con A on PTKs, rat splenic lymphocytes were incubated with Con A and phosphotyrosyl proteins were immunoprecipitated with anti-phosphotyrosine antibodies. Proteins were affinity eluted with *p*NPP from the immunoprecipitates and assayed for PTK activity. The enzyme activity was approximately 3-fold higher

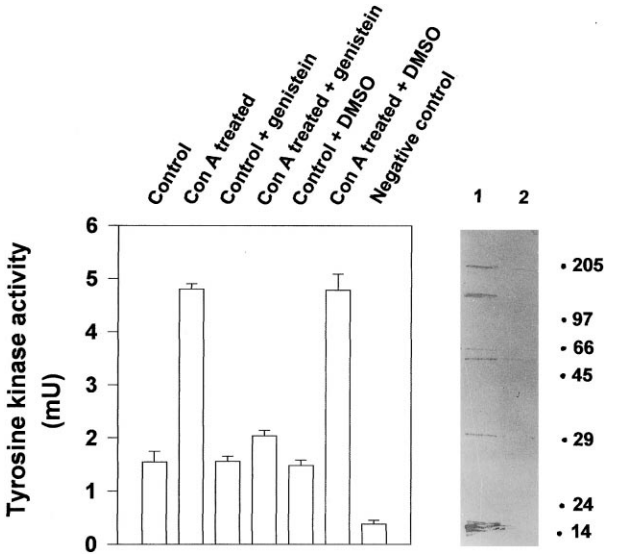

Fig. 1. Protein tyrosine kinase activity in anti-phosphotyrosine immunoprecipitates. Phosphotyrosyl proteins from Con A-stimulated and control splenic lymphocytes were immunoprecipitated with anti-phosphotyrosine antibodies and were affinity eluted with *p*NPP as described in Section 2. These eluates were assayed for tyrosine kinase activity with poly(glu,tyr) (4:1) as substrate in the presence or absence of genistein. The right panel shows the immunoblot analysis of the phosphotyrosyl proteins from Con A-stimulated (lane 1) and control (lane 2) splenic lymphocytes. The *p*NPP eluates from the anti-phosphotyrosine immunoprecipitates were electrophoresed on 7.5–15% gradient SDS-PAGE and transferred onto nitrocellulose sheet. The blot was probed with anti-phosphotyrosine antibodies. The molecular weight markers in kDa are indicated to the right of the figure.

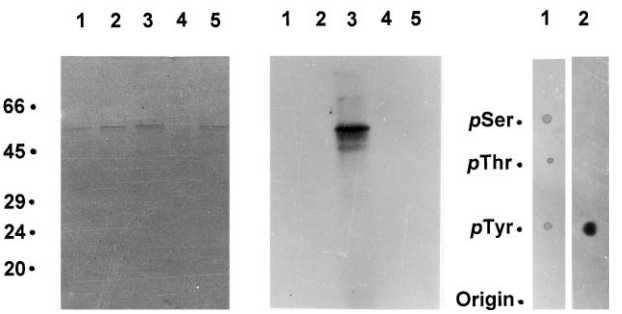

Fig. 2. In vitro phosphorylation of PtdIns 4-kinase by activated protein tyrosine kinases. PtdIns 4-kinase was incubated with or without PTKs from Con A-stimulated splenic lymphocytes under phosphorylating conditions in the presence of labeled ATP. The left panel shows the Coomassie blue-stained gel and the center panel shows the autoradiograph of the same gel. Lane 1: PtdIns 4-kinase; lane 2: PtdIns 4-kinase with 0.1 M pNPP; lane 3: PtdIns 4-kinase in the presence of stimulated PTKs; lane 4: stimulated PTKs; lane 5: PtdIns 4-kinase in the presence of stimulated PTKs and genistein. The molecular weight markers in kDa are indicated on the extreme left. The right panel shows the phosphoamino acid analysis of phosphorylated PtdIns 4-kinase. Lane 1 shows the phosphoamino acid standards stained with ninhydrin and lane 2 the autoradiograph.

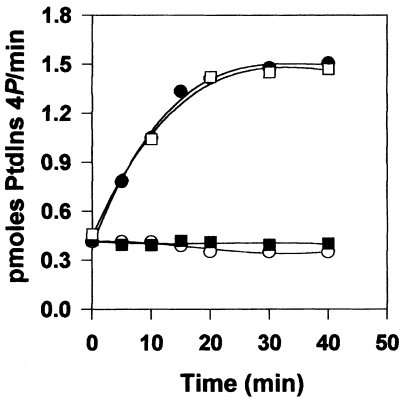

Fig. 3. PTKs activate PtdIns 4-kinase in vitro. PtdIns 4-kinase was incubated for specified time periods under phosphorylating conditions in the presence of PTKs from control cells (○ – ○) or Con A-treated cells (● – ●) with genistein (■ – ■) or DMSO (□ – □). At the end of the incubation, aliquots were assayed for PtdIns 4-kinase activity.

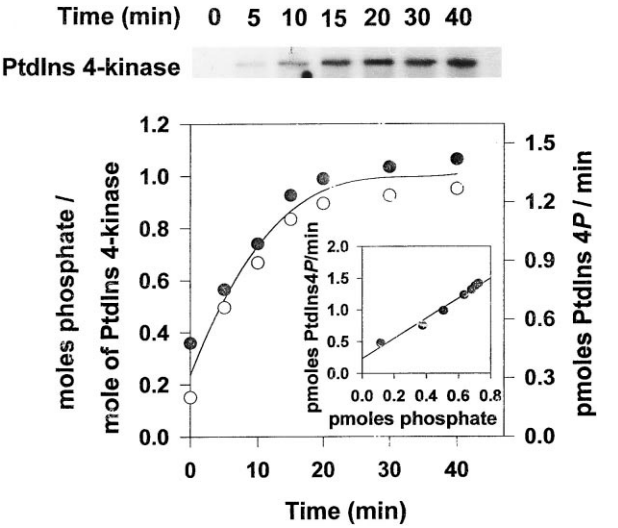

Fig. 4. Stoichiometric analysis of phosphorylation of PtdIns 4-kinase. PtdIns 4-kinase was incubated with PTKs from Con A-stimulated cells. Aliquots were taken at the indicated time points and were assayed for PtdIns 4-kinase and were electrophoresed on SDS-PAGE. The radioactivity in PtdIns 4-kinase was counted. (● – ●) PtdIns 4-kinase activity; (○ – ○) moles of Pi/mole of PtdIns 4-kinase. The inset shows the correlation of phosphorylation and activation of PtdIns 4-kinase. The upper panel shows the autoradiogram of the labeling of PtdIns 4-kinase as a function of time.

in Con A-stimulated cells. Immunoblots probed with anti-phosphophotyrosine antibodies showed a wide range of proteins from 12.5 kDa to 209 kDa in *p*NPP eluates from Con A-treated cells (Fig. 1). The time scale of activation of PTKs indicates that it may be due to posttranslational modification rather than de novo synthesis of enzymes. The increased PTK activity in pNPP eluates was inhibited by genistein. These results suggest that Con A activates PTKs in splenic lymphocytes. PTK activity in *p*NPP eluates was used as source for in vitro phosphorylation of a type II PtdIns 4-kinase from rat spleen.

3.2. *A type II PtdIns 4-kinase was phosphorylated by activated protein tyrosine kinase(s) from splenic lymphocytes in vitro*

A type II PtdIns 4-kinase from rat spleen particulate fraction was purified to homogeneity on conventional column chromatography (Verghese, M. et al., manuscript submitted). The enzyme showed a single band of 55 kDa on SDS-PAGE stained with Coomassie blue. The enzyme was incubated in the presence of PTK activity from Con A-stimulated splenic lymphocytes under phosphorylating conditions. PtdIns 4-kinase did not show any phosphorylation in the absence of PTKs. However, the enzyme was phosphorylated in the presence of PTKs (Fig. 2). Addition of genistein inhibited phosphorylation of PtdIns 4-kinase. However, absence of radiolabelling of protein in *p*NPP eluates was intriguing. It suggests that PTKs were already cold phosphorylated in vivo (Fig. 1). Phosphoamino acid analysis of labeled PtdIns 4-kinase showed only phosphotyrosine (Fig. 2). These results strongly support that PtdIns 4-kinase is phosphorylated in vitro by PTK activity from Con A-stimulated splenic lymphocytes.

3.3. *Tyrosine phosphorylation activates type II PtdIns 4-kinase in vitro*

PtdIns 4-kinase showed nearly 3-fold activation in the presence of PTKs from Con A-stimulated but not from control cells under phosphorylating conditions (Fig. 3). Stoichiometric analysis showed that one mole of phosphate was incorporated per mole of PtdIns 4-kinase (Fig. 4). A strong correlation was observed between phosphate incorporation and activation of splenic PtdIns 4-kinase (Fig. 4). Kinetic analysis showed that tyrosyl phosphorylation of PtdIns 4-kinase reduced the K_m for PtdIns from 22 μM to 7.3 μM and increased V_{max} by 2-fold. These results suggest that tyrosyl phosphorylation activates PtdIns 4-kinase.

Incubation of phosphorylated PtdIns 4-kinase with PTPases abolished the stimulated activity, while incubation with PP2A had no effect. Addition of vanadate restored the stimulated activity (Table 1). These results suggest that protein phosphotyrosyl dephosphorylation down-regulates splenic type II PtdIns 4-kinase.

Table 1
Effect of protein phosphatases on stimulated PtdIns 4-kinase activity

S. no.	Treatment	% PtdIns 4-kinase activity
1	PtdIns 4-kinase	100
2	PtdIns 4-kinase+pNPP eluates from control cells	104
3	PtdIns 4-kinase+pNPP eluates from Con A-stimulated cells	277
4	PtdIns 4-kinase+pNPP eluates from Con A-stimulated cells+PTPase	107
5	PtdIns 4-kinase+pNPP eluates from Con A-stimulated cells+PTPase+vanadate	273
6	PtdIns 4-kinase+pNPP eluates from Con A-stimulated cells+PP2A	265

Phosphorylated PtdIns 4-kinase was incubated with PTPase/PP2A for 10 min and assayed for PtdIns 4-kinase activity as described in Section 2.

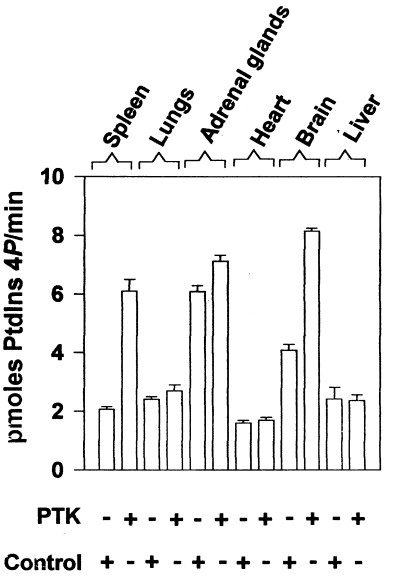

Fig. 5. Differential regulation of PtdIns 4-kinase by PTKs in adult rat tissue. Particulate membrane fractions from different tissues were incubated with or without PTKs under phosphorylating conditions for 30 min at room temperature. At the end of the incubation, the membrane fractions were assayed for PtdIns 4-kinase activity with exogenously added PtdIns.

3.4. Tissue-specific regulation of PtdIns 4-kinase

The effect of tyrosyl phosphorylation on splenic type II PtdIns 4-kinase is in contrast to the results obtained with studies on PtdIns 4-kinase regulation in A431 cell membranes [14]. This discrepancy may be due to differences in cell types used for study and suggests tissue-specific regulation of PtdIns 4-kinase. To address this hypothesis, particulate (membrane) fractions from adult rat tissue were incubated with activated PTKs under phosphorylating conditions and assayed for PtdIns 4-kinase activity. Spleen and brain particulate fractions showed activation of PtdIns 4-kinase in the presence of PTKs while particulate fractions from lung, adrenal glands, heart and liver are not affected by the presence of PTKs (Fig. 5). These results support that PtdIns 4-kinases are regulated in tissue-specific fashion. To the best of our knowledge, this is the first report on tissue-specific regulation of PtdIns 4-kinase.

4. Discussion

Earlier studies have shown that type II PtdIns 4-kinases are phosphorylated in vivo and are associated with both protein tyrosine kinases and serine kinases in response to external stimuli [14,24,25]. However, these studies fail to demonstrate direct phosphorylation of the enzyme in in vitro assays. In the absence of direct evidence for phosphorylation by protein kinases and the stoichiometry, the effect of phosphorylation on PtdIns 4-kinase remains debatable.

Activation of rat splenic lymphocytes by Con A has shown 3-fold increase in PTK activity. The increase in PTK activity may be due to specific activation of a protein tyrosine kinase or a cumulative effect of different PTK activities. Activation of lymphocytes by antigens, anti-TCR-CD3 monoclonal antibodies are known to activate protein tyrosine kinases belonging to *src, syk, zap* and *csk* families and phosphorylation of a wide array of proteins, including a type II PtdIns 4-kinase [11,26]. The effect of Con A on these PTKs is not well defined. The majority of these PTKs are known to autophosphorylate at tyrosine in vivo. Anti-phosphotyrosine immunoblots from Con A-stimulated cells showed proteins in the range of ~209–~12.5 kDa. While the low molecular weight protein (~12.5 kDa) may be the subunit of the TCR-CD3 complex, the identity of the high molecular proteins is not clear. The molecular weights of some of these proteins (~57 kDa and ~66 kDa) are similar to reported PTKs from lymphocytes suggesting that Con A may be activating more than one kinase. The protein tyrosine kinase activity(ies) from Con A-stimulated lymphocytes was used as a source to study in vitro phosphorylation of PtdIns 4-kinase.

PtdIns 4-kinase from rat spleen does not show any associated protein kinase activity in vitro (Fig. 2). In vitro tyrosyl phosphorylation and activation of the enzyme by PTKs from Con A-stimulated cells suggest that regulation of PtdIns 4-kinase is coupled to activation of protein tyrosine kinases. Stoichiometric analysis suggests a single tyrosine phosphorylation site on the enzyme. Down-regulation of PtdIns 4-kinase by protein phosphotyrosine dephosphorylation suggests that the enzyme is stringently regulated by the balanced activities of PTKs and PTPases. The absence of serine/threonine phosphorylation and no effect of PP2A on PtdIns 4-kinases in vitro suggest that serine/threonine kinases may not be regulating the enzyme directly. The observed reduction in K_m for PtdIns may be a mechanism for PtdIns 4-kinase to overcome the limiting substrate concentrations during early signal transduction.

The regulatory mechanisms for PtdIns 4-kinases appear to be tissue-specific. While PTKs from Con A-stimulated splenic lymphocytes activate the enzyme from spleen and brain, they have no effect on PtdIns 4-kinases in lung, adrenal gland, heart and liver tissue. These results suggest that PtdIns 4-kinase(s) are a family of related enzymes, regulated in a tissue-specific manner and the controversial effects of tyrosyl phosphorylation on PtdIns 4-kinase may be due to differences in cell type/tissue used for study.

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