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Inositol polyphosphate multikinase regulates inositol 1,4,5,6-tetrakisphosphate ☆

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Abstract

The human inositol phosphate multikinase (IPMK, 5-kinase) has a preferred 5-kinase activity over 3-kinase and 6-kinase activities and a substrate preference for inositol 1,3,4,6-tetrakisphosphate (Ins(1,3,4,6)P_4) over inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4). We now report that the recombinant human protein can catalyze the conversion of inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P_4) to Ins(1,3,4,5,6)P_5 in vitro; the reaction product was identified by HPLC to be Ins(1,3,4,5,6)P_5. The apparent V_{max} was 42 nmol of Ins(1,3,4,5,6)P_5 formed/min/mg protein, and the apparent K_m was 222 nM using Ins(1,3,4,6)P_4 as a substrate; the catalytic efficiency was similar to that for Ins(1,4,5)P_3. Stable over-expression of the human protein in HEK-293 cells abrogates the in vivo elevation of Ins(1,4,5,6)P_4 from the *Salmonella dublin* SopB protein. Hence, the human 5-kinase may also regulate the level of Ins(1,4,5,6)P_4 and have an effect on chloride channel regulation.

Keywords: Inositol phosphates; Kinase; Enzymology; Tissue culture cells; *Salmonella dublin*

In human cells, inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P_5) and inositol hexakisphosphate (InsP_6) exist at micromolar concentrations and are the major inositol phosphates in most mammalian cells [1,2]. The biological significance of these inositol phosphates and their biosynthesis is an area of active investigation. While the functional role of Ins(1,3,4,5,6)P_5 is unclear in human cells, InsP_6 has been implicated in diverse cellular processes including nuclear mRNA export [3,4], apoptosis [5], non-homologous DNA end joining [6,7], endocytosis [8], binding to the clathrin assembly proteins AP2 and AP180 [9–11], and in vitro inhibition of clathrin cage assembly [12,13].

Recently, the biochemical pathway for InsP_6 synthesis in human cells was described [14]. As part of this pathway, the human inositol phosphate multikinase (IPMK) is postulated to catalyze the bulk synthesis of Ins(1,3,4,5,6)P_5 in human cells. We have demonstrated that the human IPMK is a 5-kinase catalyzing the phosphorylation of Ins(1,3,4,6)P_4 yielding Ins(1,3,4,5,6)P_5 [15]. Kinetically, Ins(1,3,4,6)P_4 is the preferred substrate when compared to Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. These findings are consistent with the postulated biosynthetic pathway for human cells [16].

The yeast protein IPK2 is an orthologue of the human IPMK and phosphorylates Ins(1,4,5)P_3 to Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 in two sequential reactions. Previously, a G344R mutant (*ipk2-3*) of the yeast protein was described that retained the 6-kinase activity of the wild-type IPK2 protein but not the 3-kinase activity; this results in the synthesis of Ins(1,4,5,6)P_4 but not Ins(1,3,4,5,6)P_5 [4,17]. From a previous work, the human protein was not able to fully complement the yeast orthologue IPK2 protein but can complement the mutant IPK2-3 protein [15]. When expressed in the *ipk2-3* mutant yeast, the human IPMK was able to utilize the Ins(1,4,5,6)P_4 as a substrate and

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synthesize Ins(1,3,4,5,6)P_5; hence, Ins(1,4,5,6)P_4 is likely a substrate of the human IPMK.

The synthesis of Ins(1,4,5,6)P_4 can result from either the phosphorylation of an InsP_3 isomer or from the dephosphorylation of InsP_5. Currently, the only characterized synthetic reaction from Ins(1,4,5)P_3 to Ins(1,4,5,6)P_4 is from yeast [4,17]; we do not believe this occurs for the bulk biosynthesis in human cells [14]. The dephosphorylation of Ins(1,3,4,5,6)P_5 to Ins(1,4,5,6)P_4 has been previously demonstrated in rat AR4-2J pancreatoma cell homogenates [16], and the rat multiple inositol polyphosphate phosphatase (MIPP) was able to catalyze this reaction in an in vitro assay system [18]. It is plausible that MIPP is responsible for the synthesis of Ins(1,4,5,6)P_4 from Ins(1,3,4,5,6)P_5.

Likewise, the degradation of Ins(1,4,5,6)P_4 can result from either phosphorylation or dephosphorylation. The phosphorylation of Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5 was also demonstrated in rat AR4-2J cell homogenates [16]; however, the protein responsible for this reaction has not been identified. The dephosphorylation of Ins(1,4,5,6)P_4 to Ins(1,4,5)P_3 was observed for the rat MIPP [18]. However, in in vitro assays of whole-cell homogenates, labeled Ins(1,4,5,6)P_4 was phosphorylated to Ins(1,3,4,5,6)P_5, not dephosphorylated to an InsP_3 isomer [16]; this suggests phosphorylation as the predominant mechanism for metabolism of Ins(1,4,5,6)P_4.

The above observations suggest a model where the interconversion of Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 results in the regulation of Ins(1,4,5,6)P_4 levels [16]. Given that the human IPMK can use Ins(1,4,5,6)P_4 as a substrate, we feel that the human IPMK may be responsible for the conversion of Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5 in vivo and participate in the regulation of Ins(1,4,5,6)P_4 levels.

Ins(1,4,5,6)P_4 has been shown to regulate chloride channel activity in *Salmonella enteritis*. As part of the machinery of *Salmonella*-induced diarrhea, the virulence factor SopB is introduced into the gastrointestinal cell via the Type III secretion system [19,20], and deletion of the SopB gene abrogates diarrhea and inflammation [21]. The SopB protein is an inositol phosphatase and catalyzes the dephosphorylation of Ins(1,3,4,5,6)P_5 to Ins(1,4,5,6)P_4; the net result is a significant increase in Ins(1,4,5,6)P_4, and elevation of Ins(1,4,5,6)P_4 results in increased chloride channel activity by antagonizing the inhibitory effect of phosphatidylinositol 3,4,5-trisphosphate [3,22].

We have formally defined the enzymatic activity of the human IPMK toward Ins(1,4,5,6)P_4 and established a role for the human IPMK activity in regulating Ins(1,4,5,6)P_4 levels.

Experimental procedures

Materials. All chemicals were of reagent grade or better. Restriction endonucleases, DNA modifying enzymes, and general reagents were from Amersham, Boehringer–Mannheim, Fisher, Invitrogen, New England Biolabs, Promega, Roche, and Stratagene unless stated otherwise. Acrylamide solution, Bio-Safe Coomassie Stain, Bradford Protein Assay Kit, Mini-Protean II Cell, and Mini Trans-Blot Cell used for protein work were purchased from Bio-Rad. SuperSignal West Pico Kit used for detection of Western transfer blots was from Pierce. Radiolabeled inositol phosphates [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4,5)P_4, and [^{3}H]InsP_6 were purchased from NEN and Amersham. General reagents and chemicals were purchased from Sigma and Fisher unless stated otherwise.

Strains, plasmids, and growth conditions. Methods for bacterial growth and selection were described previously [23,24]. *Escherichia coli* strain XL-1Blue (Stratagene) was used as the bacterial host for all plasmids unless stated otherwise. Bacterial strains were cultured in LB (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.4) medium supplemented with ampicillin (100 μg/ml) where appropriate and transformed by standard methods [23,24]. All bacterial strains were grown at 37 °C. Agar plates were made by the addition of agar to 1.5% final concentration. Methods for *Saccharomyces cerevisiae* growth and selection were described previously [25–27]. The *S. cerevisiae* strain used in this study is SWY1852 (*MATα*; *ipk2-3*; *gle1-2*; *ade2*; *ade3*; *ura3*; *his3*; *leu2*; *trp1*; *LYS2*) [17]. This strain of yeast was grown at 23 °C in synthetic minimal medium plus appropriate amino acids supplemented with 2% glucose. Methods for generating the T-REx-293 human embryonic cells (Invitrogen) stably transfected with the Myc-tag human IPMK fusion protein cDNA under the regulation of a tetracycline-inducible promoter were previously described [14]; stably transfected T-REx-293 human embryonic cells were grown in medium containing DMEM with 10% FBS (Tet system approved, Clontech), 2 mM glutamine, 5 μg/ml blasticin, and 0.3 mg/ml zeocin. For induction of human IPMK expression, 0.2 μg/ml of tetracycline was added.

Synthesis of radiolabeled inositol phosphates. The synthesis of the substrate [^{3}H]Ins(1,3,4,6)P_4 was described previously [15]. The substrate [^{3}H]Ins(1,4,5,6)P_4 was purified from the yeast strain SWY1852 (kindly provided by Susan R. Wente). Yeast cells were grown in 100 ml of synthetic minimal medium minus leucine with 50 μCi/ml [^{3}H]inositol to a density of 3×10^7 cells/ml at 23 °C. Cells were pelleted and washed twice with ice-cold water. Soluble inositol phosphates were extracted as previously described [28], lyophilized, and resuspended in 2 ml of deionized water. A diagnostic sample of 100 μl was analyzed by anion-exchange HPLC (Adsorbosphere SAX HPLC, Alltech) column with a linear gradient of 0–100% 1 M ammonium phosphate, pH 3.5, at 1 ml/min over 120 min; identification of Ins(1,4,5,6)P_4 was made by its elution time relative to [^{32}P]Ins(1,3,4,5)P_4 and commercially purchased [^{3}H]Ins(1,3,4,5)P_4 standards [29,30]. A preparative sample of 900 μl was separated by anion-exchange HPLC as noted above; fractions corresponding to [^{3}H]Ins(1,4,5,6)P_4 were collected based on the diagnostic run, pooled, and desalted using the Bio-Rad AG 1-X8 (formate form; 200–400 mesh) anion-exchange column followed by Bio-Rad AG 50 W-X8 (H+ form; 25–50 mesh) cation-exchange column [31]. The [^{3}H]Ins(1,4,5,6)P_4 substrate was analyzed for purity and identity by its elution time relative to [^{32}P]Ins(1,3,4,5)P_4 and commercially purchased [^{3}H]Ins(1,3,4,5)P_4 on an Adsorbosphere SAX HPLC column as described above [29].

^{32}P-labeled standards for HPLC analysis were synthesized based on previously published methods for [^{32}P]Ins(1,4,5)P_3 [32]. [^{32}P]Ins(1,2,3,4,5,6)P_6 was synthesized by a modification of a previously published protocol [33]. Mung beans were germinated in water with [^{32}P]P_i in the dark for 3 days, harvested, and homogenized in water. The homogenized plant extract was loaded onto a Dowex-HCl anion-exchange column and washed with 0.25 N HCl; [^{32}P]Ins(1,2,3,4,5,6)P_6 was eluted with 1.5 N HCl. The standards [^{32}P]Ins(1,3,4,5)P_4 and [^{32}P]Ins(1,3,4,5,6)P_5 were synthesized by incubating [^{32}P]Ins(1,4,5)P_3 with 5 μg of the human IPMK for 1 and 3 h, respectively, in reaction conditions previously described [15]. All standards were confirmed by their elution time relative to commercially purchased ^{3}H-labeled inositol phosphates on Adsorbosphere SAX HPLC column as described above.

Enzyme activity assay. Enzymatic activity was determined as described previously and summarized below [15]. A known amount of enzyme was added to 50 mM Hepes, pH 7.2, 100 mM KCl, 100 μg/ml BSA, 8 mM $MgCl_2$, 5 mM ATP, and 1000–2000 cpm of ^{3}H-labeled substrate to a total reaction volume of 50 μl at 37 °C for the desired times. The reaction was stopped by the addition of 1 ml H_2O, and the sample was loaded onto a 500 μl Dowex-formate column (AG 1-X8, formate, mesh 200–400, Bio-Rad) equilibrated in water. The column was washed with eight 1-ml

aliquots of 1 M ammonium formate/0.1 M formic acid to elute the substrate. The product was eluted with two 1-ml aliquots of 2 M ammonium formate/0.1 M formic acid and counted in a liquid scintillation counter.

Analysis of soluble inositol phosphates. Cells were grown in complete media containing 10 μCi/ml [^{3}H]inositol for at least 72 h with or without tetracycline induction. Cells were trypsinized, washed with sterile PBS × 2, lysed in 0.5 ml methanol/0.5 N HCl (2:1), and extracted with 0.5 ml chloroform. The aqueous phase was separated, dried, and resuspended in 0.5 ml distilled water. ^{32}P-labeled standards were added to each sample, and the samples were applied to an Adsorbosphere SAX HPLC column as described above. Radioactivity was measured using the inline detector β-RAM (IN/US System), and the identities of the individual inositol polyphosphates were assigned on the basis of co-elution with known standards.

Results

Phosphorylation of Ins(1,4,5,6)P$_4$ to Ins(1,3,4,5,6)P$_5$ by human IPMK

Based on our previous work [15], human IPMK is able to phosphorylate Ins(1,4,5,6)P_4 when expressed in the *ipk2-3* mutant yeast strain. To determine the enzymatic properties of human IPMK with respect to the substrate Ins(1,4,5,6)P_4, in vitro enzyme analysis was performed.

The ^{3}H-labeled substrate was synthesized in vivo by metabolic labeling of the *ipk2-3* mutant yeast strain SWY1852 with [^{3}H]inositol followed by purification using anion-exchange HPLC and desalting ion-exchange chromatography as described in Experimental procedures. Analysis of the product using Adsorbosphere SAX HPLC showed a single peak that eluted 3–4 min after the [^{32}P]Ins(1,3,4,5)P_4 standard (data not shown); this demonstrates purity and identity of the correct isomer of $InsP_4$ [29,30].

Recombinant human IPMK was isolated as previously described [15] and incubated with 100 nM [^{3}H]Ins(1,4,5,6)P_4 for 0 min (Fig. 1A) or 60 min (Fig. 1B) at 37 °C. The enzyme reaction was terminated, mixed with ^{32}P-labeled standards, and separated on an Adsorbosphere SAX HPLC column. The product of the reaction was formally identified as Ins(1,3,4,5,6)P_5 based on co-elution with known ^{32}P-labeled standard; this indicates that Ins(1,4,5,6)P_4 is a substrate for the human IPMK resulting in phosphorylation at the D-3 position and generating Ins(1,3,4,5,6)P_5 as the product.

Given prior report of 6-kinase activity of the human IPMK [34], we tested whether Ins(1,4,5)P_3 can be phosphorylated at the D-6 position. Radiolabeled Ins(1,4,5)P_3 was incubated with 50 ng of human IPMK for 60 min. The product was identified using an Adsorbosphere SAX HPLC column. Greater than 95% of the Ins(1,4,5)P_3 substrate was converted to Ins(1,3,4,5)P_4; no production of the Ins(1,4,5,6)P_4 isomer was observed (data not shown). Using 5 μg of human IPMK incubated with the Ins(1,4,5)P_3 substrate, again the only $InsP_4$ isomer observed was Ins(1,3,4,5)P_4; however, at the higher enzyme level, we did see the production of Ins(1,3,4,5,6)P_5 but only after almost all of the Ins(1,4,5)P_3 substrate was converted to Ins(1,3,4,5)P_4 (data not shown). The human IPMK is a poor 6-kinase and will phosphorylate at either the D-5 position or D-3 position before the D-6 position. Therefore, human IPMK does not catalyze the phosphorylation of Ins(1,4,5)P_3 to Ins(1,4,5,6)P_4.

Enzyme kinetics of human IPMK

To further define Ins(1,4,5,6)P_4 as a substrate of the human IPMK, Michaelis–Menten kinetics were performed. The conversion of substrate to product was determined at a fixed amount of enzyme and a given substrate concentration at time 0, 10, 20, and 30 min; the resulting product

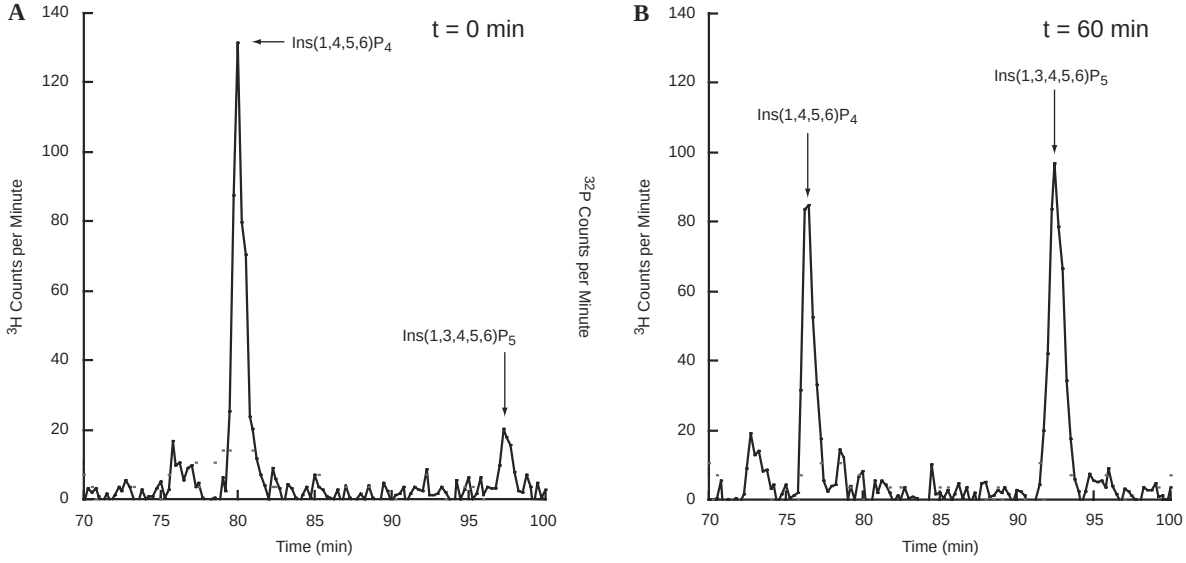

Fig. 1. Phosphorylation of Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5 by human IPMK. [^{3}H]Ins(1,4,5,6)P_4 was incubated with 50 ng of recombinant human IPMK for 0 min (A) or 60 min (B) at 37 °C. The enzyme reaction was terminated, mixed with ^{32}P-labeled standards, and separated on an Adsorbosphere SAX HPLC column as described under Experimental procedures. The HPLC chromatograms for isomers of $InsP_4$ and $InsP_5$ are shown. Identification of the isomers of inositol phosphates was by comparison with known ^{32}P-labeled internal standards.

vs. time plot generated a line using the least mean square method whose slope represented the velocity of the reaction at the given substrate concentration (data not shown). The conversion of substrate to product was linear with respect to time within our experimental system, indicating that product inhibition is unlikely under our experimental conditions. Next the 1/velocity was plotted against 1/[substrate], and the kinetic parameters K_m and V_{max} were determined from linear curve fit of the Lineweaver–Burk plot from the average values of three independent experiments (Fig. 2).

The apparent V_{max} was 42 nmol of Ins(1,3,4,5,6)P_5 formed/min/mg protein, and the apparent K_m was 222 nM of Ins(1,3,4,6)P_4. The above kinetic values translate to catalytic processivities (k_{cat}), derived from V_{max}/K_m, of 0.19 L/min/mg for Ins(1,4,5,6)P_4. Table 1 shows the kinetic parameters of Ins(1,4,5,6)P_4 in addition to those of the previously characterized substrates Ins(1,3,4,6)P_4, Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4 [15]. Comparing the kinetic parameters, Ins(1,3,4,6)P_4 is the preferred substrate and is 43 times better as a substrate compared to Ins(1,3,4,5)P_4, 2 times better as a substrate compared to Ins(1,4,5,6)P_4, and 1.6 times better as a substrate compared to Ins(1,4,5)P_3. Hence, the human IPMK's specificity for the different substrates would be in the following order: Ins(1,3,4,6)P_4 > Ins(1,4,5,6)P_4 ≅ Ins(1,4,5)P_3 ≫ Ins(1,3,4,5)P_4. This result is in line with the role of human IPMK in the synthesis of InsP_6 [15]. Nevertheless, Ins(1,4,5,6)P_4 is a good substrate; hence, the human IPMK may play a role in the regulation of Ins(1,4,5,6)P_4 levels in human cells.

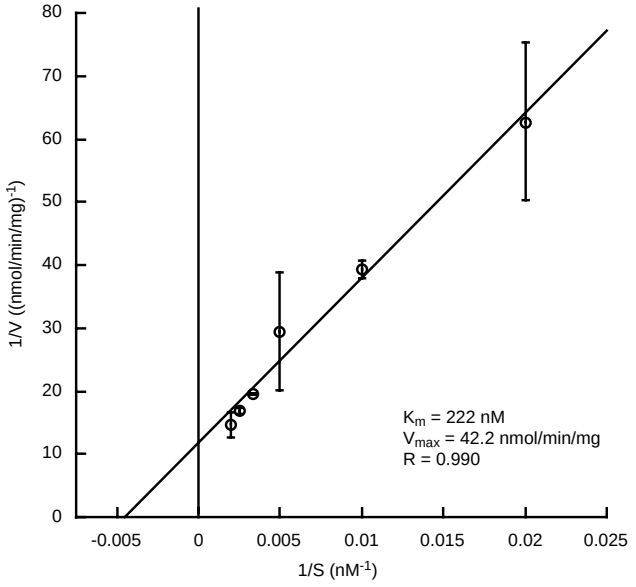

Fig. 2. Enzyme kinetics of human IPMK with the substrate Ins(1,4,5,6)P_4. Recombinant human IPMK was assayed for enzymatic activity as described under Experimental procedures. The ATP concentration was fixed at 5 mM while varying the Ins(1,4,5,6)P_4 substrate concentration. Kinetic parameters K_m and V_{max} were determined from linear curve fit of the Lineweaver–Burk plot from the average values of three independent experiments. The error bars represent the standard deviation of each data set.

Table 1
Kinetic parameters for the substrates of human IPMK[a]

Substrate	Product	V_{max} (nmol/min/mg)	K_m (nM)	V_{max}/K_m
Ins(1,3,4,6)P_4	Ins(1,3,4,5,6)P_5	114	295	0.39
Ins(1,4,5)P_3	Ins(1,3,4,5)P_4	27	112	0.24
Ins(1,4,5,6)P_4	Ins(1,3,4,5,6)P_5	42	222	0.19
Ins(1,3,4,5)P_4	Ins(1,3,4,5,6)P_5	1.1	129	0.01

[a] Kinetics values for Ins(1,3,4,6)P_4, Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4 were previously reported [15].

In vivo phosphorylation of Ins(1,4,5,6)P_4 by human IPMK

After the in vitro characterization of the kinetics of Ins(1,4,5,6)P_4 phosphorylation by human IPMK, an in vivo approach was sought. Little is known of the regulation of Ins(1,4,5,6)P_4 within the cell, and Ins(1,4,5,6)P_4 has been shown to be produced by two enzymes, the endogenous MIPP and the *Salmonella dublin* SopB protein, both by dephosphorylation of Ins(1,3,4,5,6)P_5 [3,18,22,30,35]. The regulation of the former is not known, and the introduction of the latter is the result of *S. dublin* infection and significantly increases the Ins(1,4,5,6)P_4 level within the cell [3,22,30].

Because the level of Ins(1,4,5,6)P_4 in human cells is very low, it is difficult to definitively measure changes in Ins(1,4,5,6)P_4 levels, we chose to increase the level of Ins(1,4,5,6)P_4 by infecting tissue culture cells with *S. dublin*. To test if human IPMK can phosphorylate Ins(1,4,5,6)P_4 in vivo and regulate the level of Ins(1,4,5,6)P_4, we over-expressed the human IPMK in HEK-293 cells infected with *S. dublin* bacteria. We predict a decrease in the production of Ins(1,4,5,6)P_4, associated with SopB activity of *S. dublin*.

HEK-293 cells were stably transfected with the human IPMK cDNA construct under the regulation of a tetracycline-inducible expression system [14]. Cells were grown to 90% confluency in media containing 10 μCi/ml [^{3}H]inositol in the presence (induced condition) or absence (uninduced condition) of tetracycline and were infected with wild-type *S. dublin* (strain 2229) for 30 min at 37 °C. Soluble inositol phosphates were extracted, mixed with ^{32}P-labeled standards, and separated by HPLC. The HPLC chromatographs of isomers of InsP_4, InsP_5, and InsP_6 are shown in Fig. 3; other parts of the chromatographs were essentially identical.

In HEK-293 cells stably transfected with human 5-kinase without induction, endogenous level of Ins(1,4,5,6)P_4 is small compared to InsP_5 and InsP_6, and are indistinguishable from the Ins(3,4,5,6)P_4 isomer by our HPLC system (see Fig. 3A). The Ins(1,4,5,6)P_4 level is similar to that seen for HEK-293 cells stably transfected with vector plasmid (data not shown). The total count in the Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 peak was 291 cpm. When the HEK-293 cells

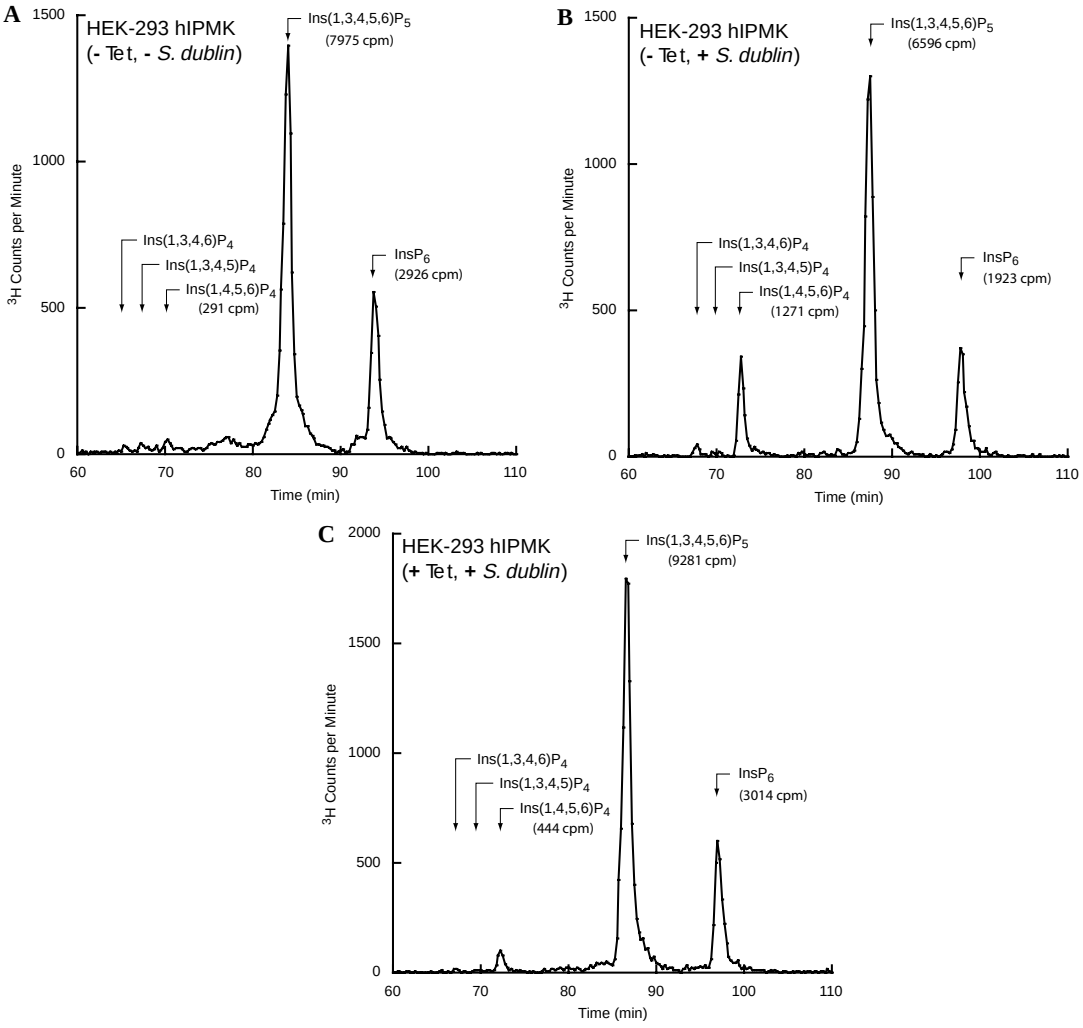

Fig. 3. In vivo phosphorylation of Ins(1,4,5,6)P_4 by human IPMK. HEK-293 cells stably transfected with the human IPMK cDNA construct under the regulation of a tetracycline-inducible expression system were grown in media with or without tetracycline with 10 μCi/ml [^{3}H]inositol to 90% confluency and were infected with wild-type *S. dublin* (strain 2229) for 30 min. Soluble inositol phosphates were extracted, mixed with ^{32}P-labeled standards, and separated on an Adsorbosphere SAX HPLC column as described in Experimental procedures. The HPLC chromatographs of isomers of $InsP_4$, $InsP_5$, and $InsP_6$ are shown for cells without induction of human IPMK and without *S. dublin* infection (−Tet, −*S. dublin*) (A), cells without induction of the human IPMK and with *S. dublin* infection (−Tet, +*S. dublin*) (B), and cells with induction of human IPMK and with *S. dublin* infection (+Tet, +*S. dublin*) (C). The elution times for the ^{32}P-labeled standards are shown by the arrows. The radioactivity (cpm) of each sample was normalized to the total cell number, and the cpm for Ins(1,4,5,6)P_4, Ins(1,3,4,5,6)P_5, and $InsP_6$ peaks is as noted in each of the chromatograms.

are incubated with *S. dublin*, the SopB protein, a virulence factor, is introduced into the cytosol of the HEK-293 cell and catalyzes the dephosphorylation of $InsP_5$ (Fig. 3B); the resulting Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 peak increased to 437% (1271 cpm) of that of uninfected cells. The isomer of $InsP_4$ that accounts for the increase is Ins(1,4,5,6)P_4 as previously published [3,22,30]. A comparable increase in Ins(1,4,5,6)P_4 was observed in naive HEK-293 cells and HEK-293 cells stably transfected with vector grown in media with or without tetracycline (data not shown); hence, induction with tetracycline did not affect the ability of *S. dublin* strain 2229 to introduce SopB into HEK-293 cells.

Over-expression of human IPMK in HEK-293 cells significantly attenuated the increase in Ins(1,4,5,6)P_4 seen with *S. dublin* infection (Fig. 3C). In the presence of tetracycline (induction condition), a tenfold increase in human IPMK activity was observed in HEK-293 cells stably transfected with the human IPMK (data not shown). This activity resulted in a significant reduction of Ins(1,4,5,6)P_4 levels of cells infected with *S. dublin* compared to uninduced cells; the Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 peak is decreased to 153% (444 cpm) of uninfected cells. No significant change in $InsP_5$ and $InsP_6$ was noted in our experimental conditions. Hence, human IPMK can catalyze the phosphorylation of Ins(1,4,5,6)P_4 in vivo and, when over-expressed, attenuate the increase of Ins(1,4,5,6)P_4 resulting from *S. dublin* infection. In addition, over-expression of human IPMK decreased the chloride channel activity seen in *S. dublin* infection (data not shown); hence, the human IPMK may regulate the level of Ins(1,4,5,6)P_4 in cells.

Discussion

We have demonstrated that Ins(1,4,5,6)P_4 is a substrate of the human IPMK in vitro and in vivo. Using an in vitro assay system, we defined the product of the reaction as Ins(1,3,4,5,6)P_5, and therefore, human IPMK possesses 3-kinase activity toward Ins(1,4,5,6)P_4. Kinetic analysis revealed that Ins(1,4,5,6)P_4 is a good substrate. In vivo analysis showed that human IPMK is able to attenuate the increase of Ins(1,4,5,6)P_4 observed in *S. dublin* infection in human tissue culture cells.

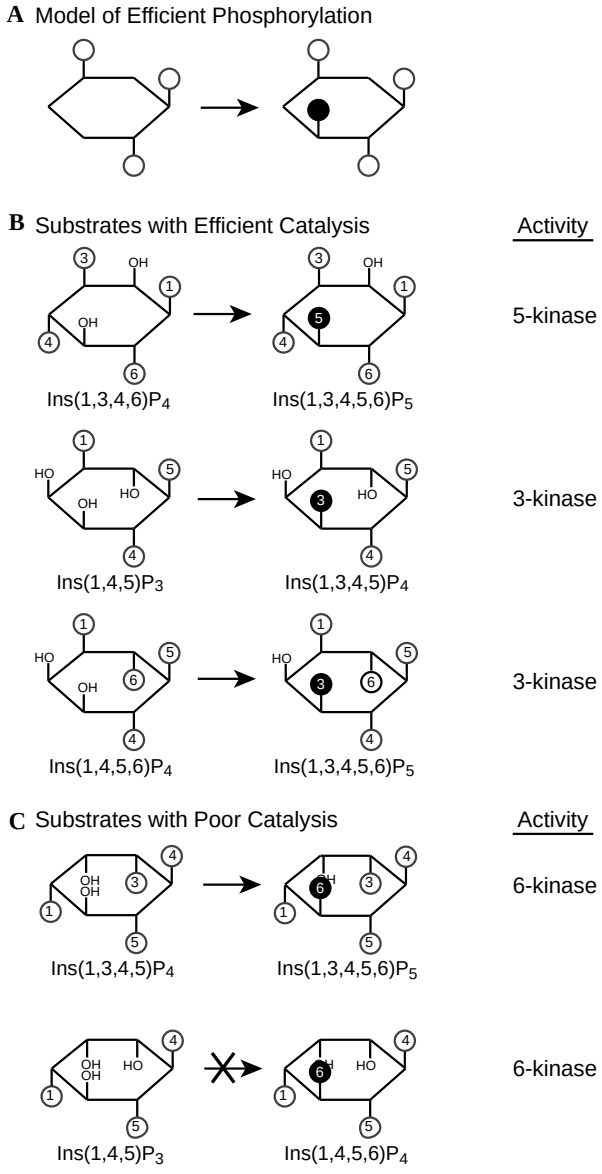

Fig. 4. Proposed model for efficient substrate binding by human IPMK. A model for efficient substrate binding by human IPMK is outlined in (A). The inositol ring is presented in planar conformation for easier visualization. Three phosphates (open circles) in the specified orientation are required for efficient addition of the solid black phosphate to the inositol ring. So far, all substrates with good catalytic efficiency (B) obey this model, and those that do not obey this model have poor catalytic efficiency (C). See text for details.

From our current and previous studies, we have characterized the enzyme kinetics of the human IPMK using four different substrates (Table 1). The preferred substrate continues to be Ins(1,3,4,6)P_4 having the best V_{max} and catalytic processivity (V_{max}/K_m). Ins(1,4,5,6)P_4 is a good substrate having kinetic parameters comparable to those of Ins(1,4,5)P_3. The above inositol phosphates are significantly better substrates than Ins(1,3,4,5)P_4. These data further support the idea that the human IPMK is predominantly a 5-kinase converting Ins(1,3,4,6)P_4 to Ins(1,3,4,5,6)P_5, which is consistent with the proposed biosynthetic pathway for InsP_6 synthesis in human cells [14].

From our kinetic data, we noted common features among the good substrates, and we propose a modification of the previously published model for efficient catalysis [36,37]. The key recognition features that are common in substrates with high catalytic efficiency are summarized in Fig. 4. This model proposes that the human IPMK can bind to different substrates in recognition of the three phosphate groups (open circles) in a specific relative orientation to the phosphorylation site (filled circle) as depicted in Fig. 4A; essentially, one needs three phosphates at the specified orientation for efficient addition of the solid black phosphate to the inositol ring. Absence of one of these three phosphates or a phosphate group in a different orientation results in poor catalysis. So far, this model accurately predicts which inositol phosphates are substrates of human IPMK (Figs. 4B and C).

Therefore, human IPMK is predominantly a 5-kinase phosphorylating Ins(1,3,4,6)P_4 to Ins(1,3,4,5,6)P_5. The 3-kinase activity is second best. The phosphorylation of Ins(1,4,5)P_3 at the D-3 position is unlikely to be physiologically significant, and the phosphorylation of Ins(1,4,5,6)P_4 at the D-3 position will be discussed below. The 6-kinase activity is the least catalytically efficient (see Chang et al. [15] for detailed discussion on the substrate Ins(1,3,4,5)P_4). The phosphorylation of Ins(1,4,5)P_3 to Ins(1,4,5,6)P_4 by human IPMK has not been observed [15,34]; in our experimental system, we only observed the phosphorylation at the D-3 position. Formally, phosphorylation at the D-6 position of Ins(1,4,5)P_3 is possible, as seen in the yeast IPK2 orthologue [17], but the human IPMK will preferentially phosphorylate Ins(1,4,5)P_3 at the D-3 position first and only then will phosphorylate Ins(1,3,4,5)P_4 at the D-6 position, with a significantly lower catalytic efficiency. Therefore, Ins(1,4,5,6)P_4 is synthesized from Ins(1,3,4,5,6)P_5 in human cells.

The ability of human IPMK to convert Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5 presents the possibility that the human protein is involved in the interconversion of Ins(1,3,4,5,6)P_5 and Ins(1,4,5,6)P_4. An Ins(1,4,5,6)P_4–Ins(1,3,4,5,6)P_5 cycle was initially proposed by following labeled intermediates in vitro using whole-cell homogenates [16]. In human cells, Ins(1,4,5,6)P_4 is likely to be synthesized by a 3-phosphatase reaction from Ins(1,3,4,5,6)P_5; an enzyme shown to catalyze this reaction is MIPP [18]. The 3-kinase activity converting Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5 was initially noted in

whole-cell homogenates of rat AR4-2J pancreatoma cells [16]. We have now characterized in detail that the human IPMK can catalyze this reaction. The next logical question is whether human IPMK can participate in the conversion of Ins(1,4,5,6)P_4 back to Ins(1,3,4,5,6)P_5 in vivo.

Using a stably transfected cell line, we demonstrated that over-expressing the human IPMK significantly attenuated the increased Ins(1,4,5,6)P_4 observed in *S. dublin* infection. This supports the hypothesis that Ins(1,4,5,6)P_4 is a substrate in vivo and that human IPMK is able to regulate the level of Ins(1,4,5,6)P_4 by conversion back to Ins(1,3,4,5,6)P_5. Therefore, the human IPMK plays a role in the cycling between Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5. The significance of the cycling between Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 may be the regulation of chloride channel activity. Previous studies have demonstrated that Ins(1,4,5,6)P_4 indirectly increased chloride channel activity [22,30]. Although, the exact mechanism is unclear.

In conclusion, we have presented enzymology establishing that human IPMK is able to catalyze the phosphorylation of Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5 in vitro and in vivo. This activity is able to regulate the levels of Ins(1,4,5,6)P_4 in the cell and is the activity previously described in the cycling of Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5.

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