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On the contribution of stereochemistry to human ITPK1 specificity: Ins(1,4,5,6)P_4 is not a physiologic substrate

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Abstract **Ins(1,4,5,6)P_4, a biologically active cell constituent, was recently advocated as a substrate of human Ins(3,4,5,6)P_4 1-kinase (hITPK1), because stereochemical factors were believed relatively unimportant to specificity [Miller, G.J., Wilson, M.P., Majerus, P.W. and Hurley, J.H. (2005) Specificity determinants in inositol polyphosphate synthesis: crystal structure of inositol 1,3,4-triphosphate 5/6-kinase. Mol. Cell. 18, 201–212]. Contrarily, we provide three examples of hITPK1 stereospecificity. hITPK1 phosphorylates only the 1-hydroxyl of both Ins(3,5,6)P_3 and the *meso*-compound, Ins(4,5,6)P_3. Moreover, hITPK1 has >13,000-fold preference for Ins(3,4,5,6)P_4 over its enantiomer, Ins(1,4,5,6)P_4. The biological significance of hITPK1 being stereospecific, and not physiologically phosphorylating Ins(1,4,5,6)P_4, is reinforced by our demonstrating that Ins(1,4,5,6)P_4 is phosphorylated (K_m = 0.18 μM) by inositol-phosphate-multikinase.**
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1. Introduction

Inositol phosphates comprise a large family of intracellular signals which play many important roles in cell biology [1–3]. These biological actions can be quite specific to one particular inositol phosphate. For example, Ins(3,4,5,6)P_4 is the only naturally occurring inositol phosphate that can inhibit the conductance of CaMKII-activated Cl^- channels [4].

In contrast, some of the enzymes that metabolize inositol phosphates can be relatively promiscuous [5]. For example, mammalian ITPK1 phosphorylates Ins(1,3,4)P_3 at both the 5- and 6-positions [6,7] while also acting as a physiologically reversible Ins(3,4,5,6)P_4 1-kinase and Ins(1,3,4,5,6)P_5 1-phosphatase [8,9]. This enzyme, by determining cellular Ins(3,4,5,6)P_4 levels, regulates cellular Cl^- channel activities [4]. One explanation for the catalytic versatility of ITPK1 [8] is based on a long-standing hypothesis [10] that recognizes that some inositol phosphates may interact with the binding sites of receptors and enzymes in more than one orientation (i.e., "mode"), enabling one inositol phosphate to mimic another by presenting to the docking site some key recognition features. These determinants of ligand binding are typically considered not to be restricted to the two-dimensional arrangement of phosphates and hydroxyls around the inositol ring, but also to include the three-dimensional stereochemistry at each position of the ring. Thus, our model for ligand binding to ITPK1 is stereochemically based, and it proposes that the enzyme uses three different binding modes [8] (Fig. 1): Mode 1 binding (permitting 1-kinase activity) was designated for Ins(3,4,5,6)P_4. We further proposed that Ins(1,3,4)P_3 could bind to the active site in two orientations, designated mode 2 (permitting 6-kinase activity) and mode 3 (permitting 5-kinase activity). Mode 3 also accounts for the 5-hydroxyl phosphorylation of the non-physiological substrate, Ins(1,2,4)P_3 [11]. Three substituents on the inositol ring (groups coloured red in Fig. 1), are common to each binding mode, but by themselves, these are insufficient to fully define ligand specificity, since all three groups are also present on Ins(1,4)P_2, which is not a substrate [8,12]. We therefore proposed that ligand recognition is combinatorial in nature, with some groups making additional contributions, but only in specific binding modes [8]. This model for ligand binding suggests that ITPK1 might be one of those proteins [13] that utilizes both rigid and flexible regions in its active site.

Efforts to test our predictions by determining the crystal structure of the human ITPK1 (hITPK1) have so far not been successful (G. Miller, J. Hurley and S. Shears, unpublished data). It was therefore a substantial advance when Miller et al. [14] recently solved the structure of the ITPK1 homologue which is present in *Entamoeba histolytica*. This enabled the modeling of the structural determinants of ligand specificity for the amoeboid enzyme. Miller et al. [14] also comprehensively confirmed the nature of the active site of the human enzyme by site-directed mutagenesis, so they applied their ligand-binding model to hITPK1. This model [14] has the same three binding modes that we [8] originally put forward for Ins(3,4,5,6)P_4, Ins(1,3,4)P_3 and Ins(1,2,4)P_3, but their hypothesis [14] still differs from ours in two key aspects. First, in order to rationalize the phosphorylation of Ins(1,3,4)P_3 at both the 5- and 6-hydroxyl, both of these

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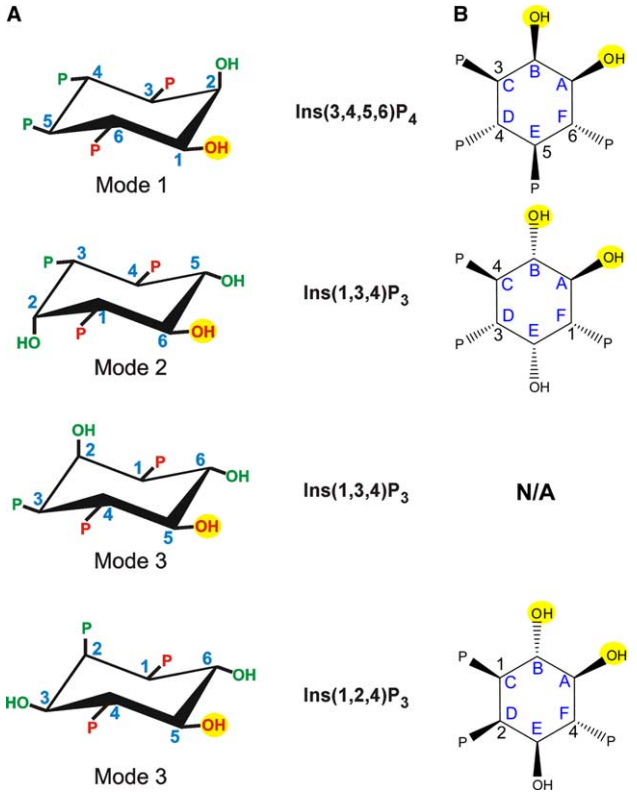

Fig. 1. Two models of the structural determinants of ligand specificity for hITPK1. The figure depicts in column 'a' our earlier proposal that there are three modes of binding of inositol phosphates to hITPK1 [8]. It can be illuminating to consider these different binding modes (i.e., "1", "2" and "3") as permitting 1-kinase, 6-kinase and 5-kinase activities, respectively. These phosphorylation sites are marked with a yellow circle. Three groups in Ins(3,4,5,6)P_4, Ins(1,3,4)P_3 and Ins(1,2,4)P_3 (coloured red) are conserved in all three of these proposed binding modes. We have previously noted that these groups by themselves are insufficient to designate substrate specificity, so we have proposed a combinatorial recognition model in which some of the additional groups (coloured green) contribute to ligand recognition in a mode-specific manner [8]. Miller et al. [14] have put forward a simpler, but more promiscuous version of this model (column 'b') in which stereochemical factors are not significant determinants of ligand binding. Although this model developed primarily from experiments with ITPK1 from *Entamoeba histolytica*, it was proposed to be also applicable to hITPK1. They proposed that, irrespective of stereochemistry, substrate specificity was determined by the presence of phosphates at sites 'C', 'D' and 'F' and not at sites 'A' and 'B'; in this model, either (but not both) of the hydroxyl groups can be phosphorylated. Position 'E' was considered unimportant. N/A, not applicable (i.e., model 'b' does not accept that Ins(1,3,4)P_3 can bind in this orientation). See text for further details.

acceptor groups were proposed to be sufficiently close to the γ-phosphate of ATP that either (but not both) could be phosphorylated by direct inline transfer [14]. In that context, only one binding mode is necessary in order to explain the two products of Ins(1,3,4)P_3 phosphorylation (equivalent to our mode 2; Fig. 1).

A second, and unique aspect of the model of Miller et al. [14] is the proposal that, for the amoeboid ITPK1, ligand specificity is not significantly affected by either the hydroxyl groups, nor by their orientation (i.e., axial vs. equatorial), nor by the stereochemistry at any of the six stereogenic centres of the inositol ring (Fig. 1). In the current study, we have investigated to what extent these observations might also apply to hITPK1. We were also intrigued by the range of additional inositol phosphates that Miller et al. [14] proposed were metabolized by both the amoeboid and human ITPK1. Most of these additional inositol phosphates are not constituents of mammalian cells, and as such, the veracity of this proposal does not impact upon our understanding of hITPK1 function in vivo. However, Ins(1,4,5,6)P_4, which is an intracellular constituent in mammalian cells [15], is one of the proposed amoeboid ITPK1 substrates that was also suggested as a substrate for hITPK1. Ins(1,4,5,6)P_4 is also biologically active; it has been shown to regulate Cl^- transport [16], to be a PtdIns(3,4,5)P_3 antagonist [17], and to bind tightly to some pleckstrin homology domains [18], and finally, it may contribute to transcriptional regulation [19]. Therefore, it is important to understand how the cellular levels of this inositol phosphate are regulated. If hITPK1 were to participate in maintaining steady-state levels of Ins(1,4,5,6)P_4, then we would need to determine the biological impact of competition from other hITPK1 substrates.

In the current study we show that stereochemical factors are important determinants of ligand specificity for hITPK1. In so doing, we show that hITPK1 is not a physiologically relevant Ins(1,4,5,6)P_4 kinase; instead, we show that function is performed by another enzyme, the inositol polyphosphate multikinase.

2. Methods

2.1. Materials

Recombinant human ITPK1 (hITPK1) was prepared as previously described [9,20]. Recombinant human IPMK was prepared as follows. The cDNA clone IMAGE:4510867 (GenBank2 accession no. BG258567) was obtained from ATCC. The superfluous nucleotide at position 451 was removed by site-directed mutagenesis, as described in Ref. [21]. The open reading frame was completely resequenced. *Bam*HI and *Kpn*I restriction sites were introduced at the 5′ and 3′ ends, respectively. Then the cDNA was subcloned into the pTrcHisA vector and the protein was expressed in *Escherichia coli*. Recombinant poly(His)-tagged protein was purified using Ni-agarose (Clontech), which was eluted with imidazole according to the manufacturer's instructions.

[^{3}H]Ins(3,4,5,6)P_4 and [^{3}H]Ins(1,4,5,6)P_4 were prepared as previously described (Refs. [20,22], respectively). Non-radiolabeled Ins(3,4,5,6)P_4, Ins(1,4,5,6)P_4 and Ins(1,3,4)P_3 were purchased from CellSignals Inc (Lexington, KY). Ins(3,5,6)P_3 and Ins(4,5,6)P_3 were synthesized as previously described [23,24] and their identities were confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy [23–25]. Previously unpublished ^{1}H, and ^{13}C NMR data for Ins(4,5,6)P_3 are as follows: ^{1}H NMR (D_2O, pH 10) δ 3.92 (dd, J = 2.5, 9.3 Hz, 2H, H-1 and H-3), 4.19 (q, J = 9.3 Hz, 1H, H-5), 4.25 (t, J = 2.5 Hz, 1H, H-2), 4.39 (q, J = 9.3 Hz, 2H, H-4 and H-6); ^{13}C NMR (D_2O, pH 10) δ 73.87, 74.60 (2C), 78.32 (2C), 79.10.

The purity of Ins(3,5,6)P_3 and Ins(4,5,6)P_3 was determined by HPLC separation followed by metal dye detection [26] using two complementary HPLC procedures that employ either an acidic or an alkaline gradient [27]. In the acidic gradient, Ins(4,5,6)P_3 has a unique elution position that is well-resolved from all other InsP_3 isomers [8,27,28]. Ins(3,5,6)P_3 was resolved from other possible InsP_3 isomers by using both gradients [8,11,27,28]. Thus, we were able to show that Ins(4,5,6)P_3 was >97% pure (the approx. 3% contaminant is Ins(3,4,5)P_3; Fig. 2 and see Section 3). Ins(3,5,6)P_3 had no detectable contaminants (Fig. 2 and see Section 3). Finally, this batch of Ins(3,5,6)P_3 has also been shown to be enantiomerically pure (i.e., it does not contain any Ins(1,4,5)P_3) because no inorganic phosphate was released when Ins(3,5,6)P_3 was incubated with *Schizosaccharomyces pombe* synaptojanin, an active Ins(1,4,5)P_3 5-phosphatase [29]. Enantiomeric contamination is not an issue for Ins(4,5,6)P_3, which is a *meso*-compound.

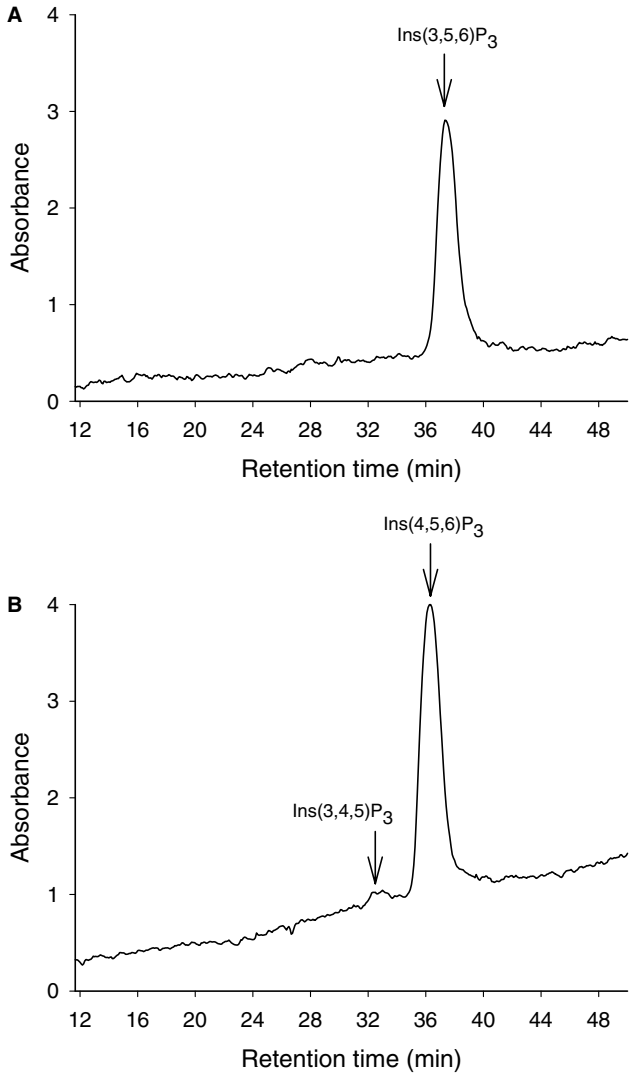

Fig. 2. HPLC analysis of the purity of Ins(3,5,6)P_3 and Ins(4,5,6)P_3. 6 nmol of either Ins(3,5,6)P_3 (panel A) or Ins(4,5,6)P_3 (panel B) were analyzed by HPLC using an alkaline gradient (see Section 2 and Ref. [27]). Isomeric assignments are based on the complementary nature of the alkaline gradient when used in parallel with an acidic gradient (see Section 3 and Ref. [27]), which together resolve Ins(3,5,6)P_3 and Ins(4,5,6)P_3 from all other InsP_3 isomers.

2.2. Enzyme assays

The hITPK1 activity was assayed at 37 °C in 100–200 μl buffer containing 100 mM KCl, 20 mM HEPES pH 7.2, 5 mM ATP, 10 mM phosphocreatine, 6 mM $MgSO_4$, 0.3 mg/ml bovine serum albumin and 3.6 U/ml phosphocreatine kinase (Calbiochem). Other assay details are given in the figure legends. Assays with radiolabeled inositol phosphates were quenched with PCA and neutralized with K_2CO_3 as previously described [30]. Some of these assays were analyzed using gravity-fed ion-exchange columns [30]. Other assays were analyzed by HPLC using a 12.5 × 4.6 mm Partisphere SAX column (Krackler Scientific, NC) as previously described [31]. Kinetic parameters for IPMK-dependent phosphorylation of [^{3}H]Ins(1,4,5,6)P_4 were determined with assays that contained a range of Ins(1,4,5,6)P_4 concentrations from 0.05 to 1.8 μM. K_m and V_{max} were determined as previously described [32], by non-linear curve fitting to the Michaelis–Menten equation, using SigmaPlot. Other assays that consisted entirely of non-radiolabeled inositol phosphates were quenched by boiling for 3 min, and then processed for analysis by HPLC separation followed by metal dye detection [26] using an acidic gradient as described by Adelt et al. [27]. Data were exported as ASCII files into SigmaPlot.

3. Results and discussion

3.1. Is Ins(3,5,6)P_3 a substrate of hITPK1?

Ins(3,5,6)P_3 is not a physiologically relevant inositol phosphate, but it has been utilized in the current study to provide useful information on the regioselectivity of human ITPK1 (hITPK1). Our model predicts that Ins(3,5,6)P_3 is a candidate mode 1 substrate, and, therefore, it would only be phosphorylated at the 1-position (column 'a', Fig. 3). An alternative model of the activities of mammalian ITPK1 predicts that it will phosphorylate either (although not both) of the 1- and 2-hydroxyls of Ins(3,5,6)P_3 (column 'b' in Fig. 3); the latter prediction is based on a structural characterization of an ITPK1 homologue from *E. histolytica* [14].

We tested these different ideas concerning positional specificity of hITPK1 using non-radiolabeled Ins(3,5,6)P_3, the metabolism of which was recorded using an on-line mass-detection HPLC technique [26,27]. As a control, we also recorded the phosphorylation of non-radiolabeled Ins(1,3,4)P_3 (Fig. 4). It should be noted that the sensitivity of this method increases when there are a larger number of phosphate groups around the inositol ring [26]. For example, an InsP_4 will yield a larger signal than an equivalent concentration of InsP_3 [26]. This technical point is apparent following an analysis of Ins(1,3,4)P_3 phosphorylation by ITPK1; the sizes of the peaks of the two products (Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4) are larger than the peak of the original substrate (Fig. 4A).

There have been efforts to rationalize why two InsP_4 isomers are formed upon phosphorylation of Ins(1,3,4)P_3 by ITPK1. At least for the ITPK1 homologue in *E. histolytica*, Miller et al. [14] have proposed that the phosphorylation of Ins(1,3,4)P_3 at both the 5- and 6-hydroxyl is possible in one substrate-binding mode. Both of these hydroxyl groups are

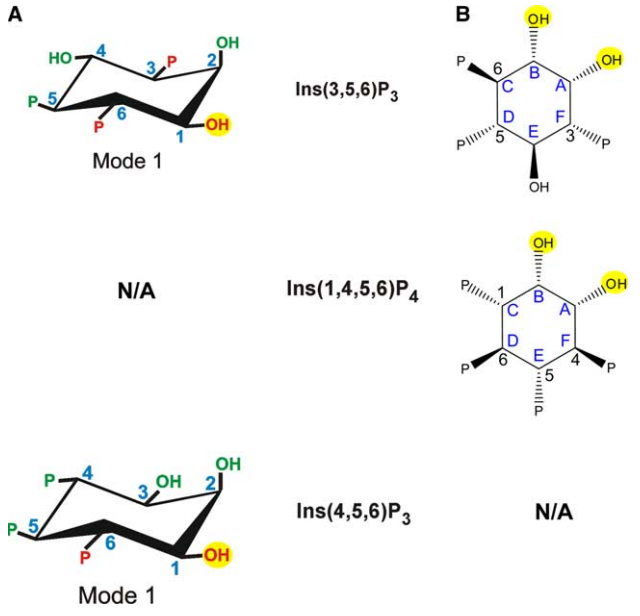

Fig. 3. Two models of the predicted binding and phosphorylation of Ins(3,5,6)P_3, Ins(4,5,6)P_3 and Ins(1,4,5,6)P_4 by hITPK1. The binding of inositol phosphates to hITPK1, and the site of phosphorylation (yellow circle) is predicted according to the model proposed either by Ho et al. (column 'a'; Ref. [8]) or Miller et al. (column 'b' and Ref. [14]). N/A, not applicable to this model. See Fig. 1 for the significance of other colour-coding.

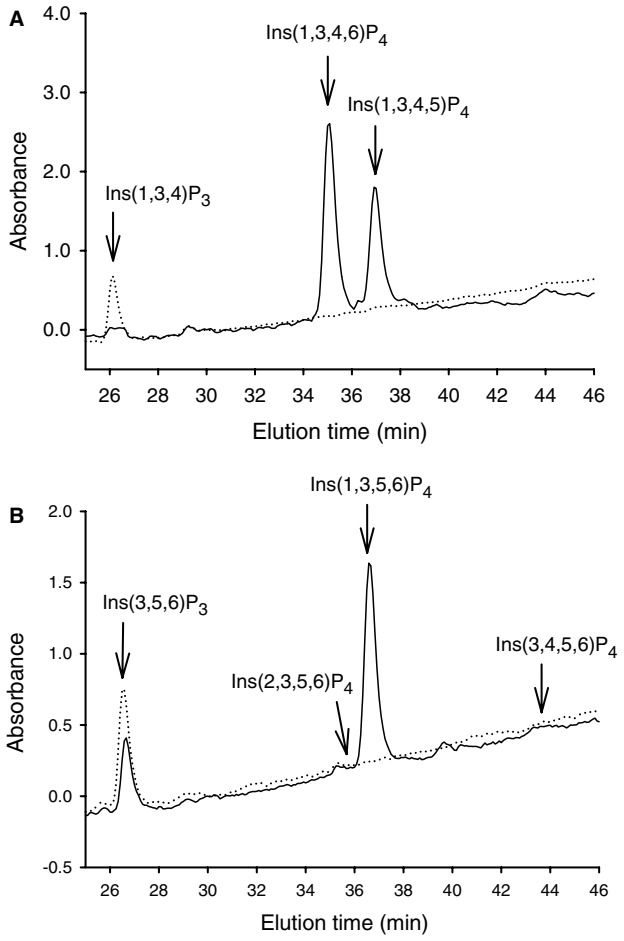

Fig. 4. HPLC Analysis of the phosphorylation of Ins(1,3,4)P_3 and Ins(3,5,6)P_3 by hITPK1. Recombinant hITPK1 (3400 ng) was incubated in 200 μl assay buffer for 0 (dotted trace) or 4 h (solid trace) with 50 μM of either Ins(1,3,4)P_3 (panel A) or Ins(3,5,6)P_3 (panel B) as described in Section 2. Samples were quenched by boiling and analyzed by an on-line mass detection HPLC technique that uses an acidic gradient (see Section 2). The absorbance at the 30 min elution time was arbitrarily set to zero. The elution positions of the named InsP_4 isomers are arrowed; these were determined using genuine standards [11]. Note that sensitivity of this technique depends upon the number of phosphate groups; thus, an InsP_4 yields a larger signal than an equivalent concentration of InsP_3 [26,27].

sufficiently close to the γ-phosphate of ATP that either (but not both) can, in principle, be phosphorylated by direct inline transfer. The latter proposal is distinct from our earlier hypothesis that mammalian ITPK1 has two binding modes for Ins(1,3,4)P_3, which separately determine whether it is the 5-hydroxyl or the 6-hydroxyl that is phosphorylated [8]. None of the new data that are presented in the current study are able to distinguish between these two alternate models.

In agreement with the prediction of Miller et al. [14], we found that Ins(3,5,6)P_3 was phosphorylated by hITPK1 (Fig. 4B). A single InsP_4 product was identified which co-eluted with standards of Ins(1,3,5,6)P_4. No InsP_5 was formed from Ins(1,3,5,6)P_4 (data not shown). More importantly, no product accumulated with the retention times of Ins(2,3,5,6)P_4 or Ins(3,4,5,6)P_4 (Fig. 4B). These data are in agreement with our stereochemically based model for ligand binding, which does not permit phosphorylation of either the 2- or 4-hydroxyls of Ins(3,5,6)P_3 (Fig. 4). Clearly, the stereochemistry of the inositol ring at C-2 prevents phosphorylation of the 2-OH group by hITPK1 in both Ins(3,4,5,6)P_4 and Ins(3,5,6)P_3, either by dictating possible binding modes, and/or because an axial hydroxyl group cannot be presented to the active site in the correct orientation for phosphoryl transfer.

According to our model of ligand binding (Fig. 3, column 'a'), regioselective phosphorylation of Ins(3,5,6)P_3 at the 1-position characterizes it as a mode 1 substrate. Since Ins(3,4,5,6)P_4 is also a mode 1 substrate (Fig. 1), we determined the affinity of hITPK1 for Ins(3,5,6)P_3 to quantify the contribution of the 4-phosphate to ligand binding. We considered this to be an important experiment because the 4-phosphate of Ins(3,4,5,6)P_4 is not one of those groups that has an equivalent in all three of our proposed ligand-binding modes (Figs. 1 and 3). Therefore, this situation gave us an opportunity to test our combinatorial hypothesis that the 4-phosphate might be one of those determinants of ligand binding that are mode-specific.

The value of the IC_{50} with which Ins(3,5,6)P_3 inhibited Ins(3,4,5,6)P_4 phosphorylation was used to calculate the affinity of the inhibitor (Fig. 5). As a control, we also checked the affinity for Ins(1,3,4)P_3 using the same method (Fig. 5). As expected, Ins(1,3,4)P_3 potently inhibited hITPK1 activity towards Ins(3,4,5,6)P_4 (IC_{50} = 7.6 μM; K_i = 0.15 μM, Fig. 5A). This method for measuring substrate affinity is validated by the estimated affinity of Ins(1,3,4)P_3 being very close to the previously determined K_m value (0.3 μM; Ref. [9]). The affinity of Ins(3,5,6)P_3 for hITPK1 (IC_{50} = 100 μM; K_i = 2 μM; Fig. 5B) is around 13-fold lower than that for Ins(3,4,5,6)P_4 (0.1 μM, Ref. [9]). In other words, removal of the 4-phosphate from Ins(3,4,5,6)P_4 can be concluded to reduce ligand affinity 13-fold. The 4-phosphate has this function in mode 1, and there is a surrogate in mode 2 (Fig. 1). There is not an equivalent group in mode 3 (Fig. 1). Thus, the 4-phosphate in Ins(3,4,5,6)P_4 may be one of those groups that we have proposed makes a contribution to ligand binding in a mode-specific manner.

3.2. Is Ins(1,4,5,6)P_4 a substrate of hITPK1?

Ins(1,4,5,6)P_4 is another inositol phosphate that can distinguish between the two different models of ligand binding described in Figs. 1 and 3. In our model, this inositol phosphate is not predicted to be a substrate, as it cannot present the key recognition features to the active site in any of our three binding modes. In contrast, Miller et al. [14] consider Ins(1,4,5,6)P_4 to be a potential substrate of hITPK1, based on their model of the amoeboid homologue, because Ins(1,4,5,6)P_4 contains three appropriately positioned phosphate groups, but this would only be valid if stereochemical factors are not significant determinants of ligand specificity (Figs. 1 and 3). The resolution of this issue has physiological relevance because Ins(1,4,5,6)P_4 is proposed to be a biologically active constituent of mammalian cells (see Section 1). It is therefore an important goal to understand the nature of the enzymes that metabolize Ins(1,4,5,6)P_4 in vivo.

In order to maximize the sensitivity of these kinase assays, we incubated hITPK1 with [^{3}H]Ins(1,4,5,6)P_4, and we measured its ability to be metabolized under first-order conditions. There was no phosphorylation (Fig. 6A). In contrast, 42% of added [^{3}H]Ins(3,4,5,6)P_4 was phosphorylated by a concentration of hITPK1 (Fig. 6B) that was approx. 1600-fold lower than that

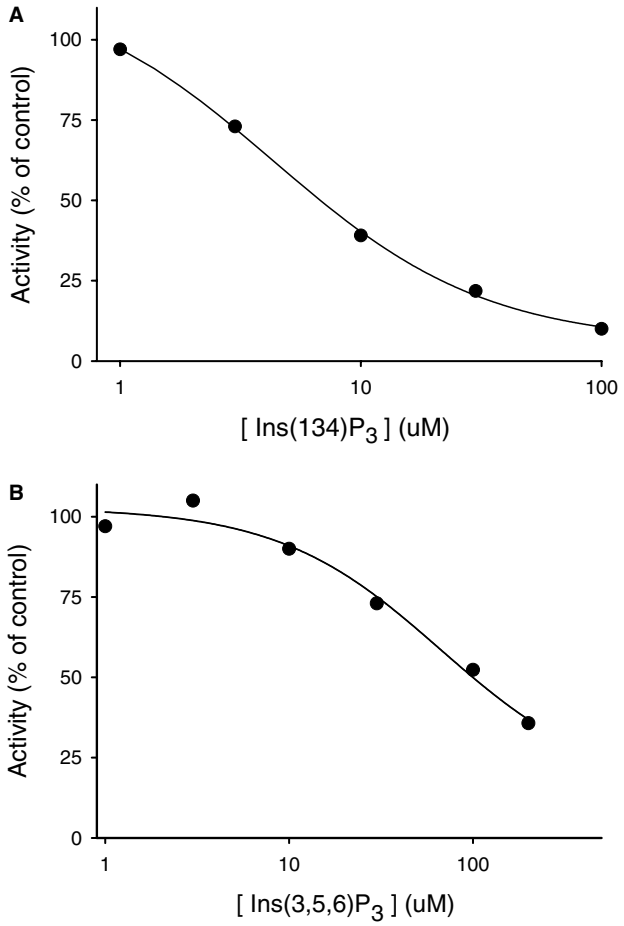

Fig. 5. Determination of the affinity of $Ins(1,3,4)P_3$ and $Ins(3,5,6)P_3$ for hITPK1. Recombinant hITPK1 (24 ng) was incubated in 100 μl assay buffer for 30 min with 5 μM [^{3}H]$Ins(3,4,5,6)P_4$ as described in Section 2, plus the indicated concentrations of either $Ins(1,3,4)P_3$ (panel A) or $Ins(3,5,6)P_3$ (panel B). Samples were acid quenched, neutralized, and analyzed by gravity-fed columns as described in Section 2. Data shown are from a representative experiment performed in duplicate (typical of three). K_i values were estimated from the empirically determined IC_{50} value using the following equation: $K_i = (IC_{50} \times K_m)/([S] + K_m)$; $[S]$, substrate concentration and the value of the K_m for $Ins(3,4,5,6)P_4$ is 0.1 μM [9].

used in the assays with [^{3}H]$Ins(1,4,5,6)P_4$ (Fig. 6A). By taking 5% phosphorylation as a conservative estimate of the minimal level of detection, we can calculate that we would have been able to measure any $Ins(1,4,5,6)P_4$ phosphorylation that occurred at a rate that was up to 13,000-fold slower than that of $Ins(3,4,5,6)P_4$. We therefore conclude that $Ins(1,4,5,6)P_4$ is not a physiologically relevant hITPK1 substrate. This difference in the reactivity of hITPK1 towards $Ins(3,4,5,6)P_4$ vs. $Ins(1,4,5,6)P_4$ is particularly pertinent to the current study because these two compounds are enantiomers; our data (Fig. 6) therefore again verify that stereochemistry is an important determinant of the substrate specificity of hITPK1.

While hITPK1 cannot phosphorylate $Ins(1,4,5,6)P_4$, there is an enzyme that was previously observed in mammalian cell lysates that does show $Ins(1,4,5,6)P_4$ 3-kinase activity [15,33,34]. Majerus and colleagues [32] have proposed that this mammalian 3-kinase activity is performed by IPMK (also known as IPK2). In support of this idea, the latter group heterologously overexpressed rat IPMK in a strain of *S. cerevisiae* that lacks

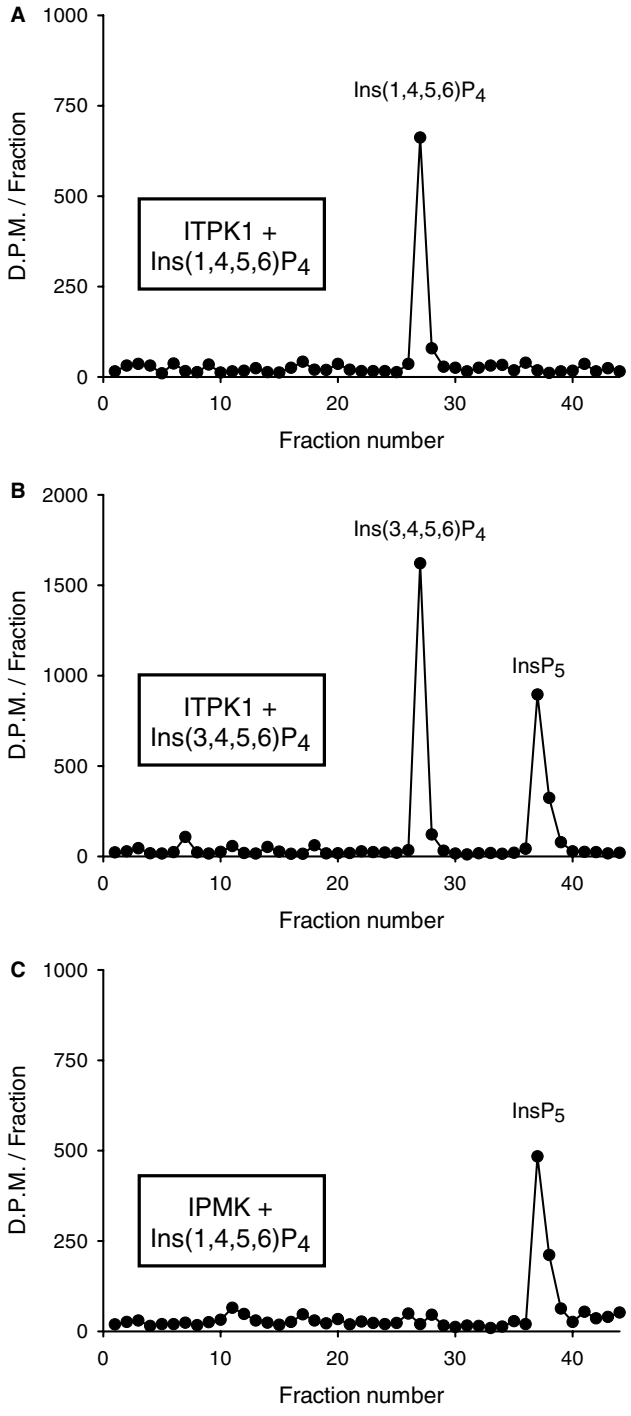

Fig. 6. $Ins(1,4,5,6)P_4$ is phosphorylated by hIPMK, but not by hITPK1. Assays were performed for 10 min in 100 μl assay buffer as described in Section 2 with either (panel A) 800 D.P.M. [^{3}H]$Ins(1,4,5,6)P_4$ plus 7500 ng hITPK1, or (panel B) 2500 D.P.M. [^{3}H]$Ins(3,4,5,6)P_4$ plus 4.8 ng hITPK1, or (panel C) 800 D.P.M. [^{3}H]$Ins(1,4,5,6)P_4$ plus 53 ng hIPMK. Assays were acid quenched, neutralized and analyzed by HPLC as described in Section 2.

the inherent ability to phosphorylate $Ins(1,4,5,6)P_4$ to $InsP_5$; in these yeast cells, rat IPMK rescued $InsP_5$ synthesis [32]. We have provided support to the latter observations by demonstrating that $Ins(1,4,5,6)P_4$ is phosphorylated by purified recombinant human IPMK (Fig. 6C). Moreover, the kinetic

parameters that we determined for this Ins(1,4,5,6)P_4 3-kinase activity (K_m = 0.18 μM; V_{max} = 139 nmol/mg protein/min) are similar to those for its other substrates [21,32]. These experiments also verify the nature of the [^{3}H]Ins(1,4,5,6)P_4 substrate used in the experiment described in Fig. 6A.

3.3. Is Ins(4,5,6)P_3 a substrate of hITPK1?

Ins(4,5,6)P_3 is not a physiologically relevant inositol phosphate in mammalian cells. However, it is useful for studying the importance of ring stereochemistry in determining substrate specificity, because it is a *meso*-compound, i.e., a plane of symmetry runs through the ring (between the 2- and 5-positions). We found Ins(4,5,6)P_3 to have only weak affinity for hITPK1; 200 μM Ins(4,5,6)P_3 inhibited Ins(3,4,5,6)P_4 phosphorylation by only 25% (Fig. 7A). Nevertheless, Ins(4,5,6)P_3 was phosphorylated by hITPK1 (Fig. 7B). A single InsP_4 peak was detected, at the elution position of the Ins(3,4,5,6)P_4/Ins(1,4,5,6)P_4 enantiomeric pair. Since the InsP_4 was not further phosphorylated to InsP_5, we conclude the InsP_4 is Ins(1,4,5,6)P_4, since that is not a physiological substrate of hITPK1 (Fig. 6).

Despite Ins(4,5,6)P_3 being a *meso*-compound, our data reveal that hITPK1 shows a preference for phosphorylating the 1-hydroxyl rather than the 3-hydroxyl. Thus, hITPK1 can clearly distinguish between enantiotopic groups in this substrate. This result offers further testimony to the stereochemical preferences of this enzyme.

3.4. Concluding comments

The structural characterization of the ITPK1 from *E. histolytica* led to the generation of a model of the active site in which the hydroxyl groups, and their orientation, and the stereochemical properties of the molecule, all do not contribute significantly to substrate specificity [14]. It was further proposed that the model could be applied to mammalian ITPK1 [14]. An investigation of the latter proposal was one of the goals of the current study. We have used three different inositol phosphates to show that, in each case, hITPK1 recognizes the stereochemistry of its substrates at key positions around the inositol ring, including the hydroxyl groups. Miller et al. [14] have further suggested that hITPK1 mimics the ability of ITPK1 from *E. histolytica* to phosphorylate either of two adjacent hydroxyls on a single substrate, even if one of these hydroxyl groups is axial to the plane of the inositol ring. Our new data (Figs. 4 and 7) indicate that hITPK1 can only phosphorylate a single, equatorial hydroxyl on either Ins(3,5,6)P_3 or Ins(4,5,6)P_3. The same is true of Ins(3,4,5,6)P_4 [9]. It seems that evolutionary pressure has narrowed the substrate specificity of the mammalian enzyme [14,35]. For example, the human enzyme has lost the ability to phosphorylate Ins(1,4,5)P_3, which is a notable characteristic of ITPK1 from *E. histolytica* [14,35]. This difference may be attributed to Ser-295 in the amoeboid enzyme being replaced by a glycine residue in hITPK1 [14].

Our studies not only advance our insight into the nature of the active site of mammalian ITPK1, but they also increase our understanding of the metabolism of Ins(1,4,5,6)P_4, which is reportedly a biologically active constituent of eukaryotic cells [16,17,19]. Our data demonstrate that it is IPMK, and not ITPK1, that has the capacity to regulate the cellular levels of Ins(1,4,5,6)P_4 in mammalian cells. This conclusion should assist our understanding of the cell biology of Ins(1,4,5,6)P_4.

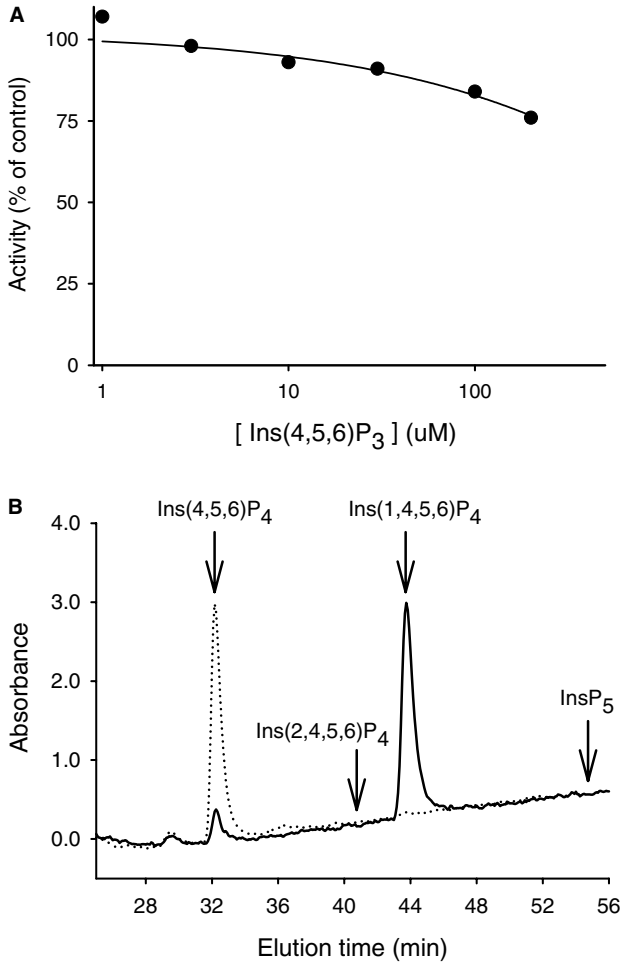

Fig. 7. Ins(4,5,6)P_3 is regioselectively phosphorylated by hITPK1. Panel A. Recombinant hITPK1 (24 ng) was incubated in 100 μl assay buffer for 30 min with 5 μM [^{3}H]Ins(3,4,5,6)P_4 as described in Section 2, plus the indicated concentrations of Ins(4,5,6)P_3. Samples were acid quenched, neutralized, and analyzed by gravity-fed columns as described in Section 2. Data shown are from a representative experiment performed in duplicate (typical of three). Panel B. Recombinant hITPK1 (3400 ng) was incubated in 200 μl assay buffer for 0 (dotted trace) or 4 h (solid trace) with 50 μM of Ins(4,5,6)P_3 as described in Section 2. Samples were quenched by boiling and analyzed by an on-line mass detection HPLC technique that uses an acidic gradient (see Section 2). The absorbance at the 30 min elution time was arbitrarily set to zero. Note that a small contaminant that was not metabolized eluted 2 min prior to Ins(4,5,6)P_3; the contaminant is Ins(3,4,5)P_3 (see text and Fig. 2). The elution positions of the named InsP_4 isomers are arrowed; these were determined using genuine standards [11].

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