Integration of Inositol Phosphate Signaling Pathways via Human ITPK1*

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Inositol 1,3,4-trisphosphate 5/6-kinase (ITPK1) is a reversible, poly-specific inositol phosphate kinase that has been implicated as a modifier gene in cystic fibrosis. Upon activation of phospholipase C at the plasma membrane, inositol 1,4,5-trisphosphate enters the cytosol and is inter-converted by an array of kinases and phosphatases into other inositol phosphates with diverse and critical cellular activities. In mammals it has been established that inositol 1,3,4-trisphosphate, produced from inositol 1,4,5-trisphosphate, lies in a branch of the metabolic pathway that is separate from inositol 3,4,5,6-tetrakisphosphate, which inhibits plasma membrane chloride channels. We have determined the molecular mechanism for communication between these two pathways, showing that phosphate is transferred between inositol phosphates via ITPK1-bound nucleotide. Intersubstrate phosphate transfer explains how competing substrates are able to stimulate each others’ catalysis by ITPK1. We further show that these features occur in the human protein, but not in plant or protozoan homologues. The high resolution structure of human ITPK1 identifies novel secondary structural features able to impart substrate selectivity and enhance nucleotide binding, thereby promoting intersubstrate phosphate transfer. Our work describes a novel mode of substrate regulation and provides insight into the enzyme evolution of a signaling mechanism from a metabolic role.

Cellular inositol phosphate metabolism is an intricate web of kinase and phosphatase reactions that produces a number of important signaling molecules (for review see Ref. 1). A now classic example of these signaling activities is the release of Ca2+ into the cytoplasm through an intracellular channel that is gated by inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) (2). Additional roles are continually being discovered: inositol phosphates have recently been shown to be critical to the activity of RNA-editing enzymes (3), to participate in telomere maintenance (4), and to be the phosphate donors in certain protein phosphorylation events (5). It is of critical interest, therefore, to establish the regulatory mechanisms that govern the metabolism of inositol phosphates.

An interesting feature of inositol phosphate metabolism is the promiscuity with which several key kinases phosphorylate multiple substrates (6). For example, ITPK1 (also known as inositol 1,3,4-trisphosphate 5/6-kinase) adds either a 5- or 6-phosphate to Ins(1,3,4)P3 and also attaches a 1-phosphate to Ins(3,4,5,6)P4 (6, 7) (inositol phosphate structures shown in Fig. 2). These reactions have been demonstrated to be reversible: ITPK1 can also dephosphorylate Ins(1,3,4,5,6)P5 back to Ins(3,4,5,6)P4 (8). An especially puzzling aspect of this phenomenon is that dephosphorylation of Ins(1,3,4,5,6)P5 by human ITPK1 is stimulated, rather than competitively inhibited, by one of its alternate substrates, Ins(1,3,4)P3 (8).

The fact that mammalian ITPK1 reversibly phosphorylates both Ins(3,4,5,6)P4 and Ins(1,3,4)P3 takes on special significance because the two substrates occur in separate branches of the metabolic pathway (9). Ins(3,4,5,6)P4 is formed in a metabolic cycle with Ins(1,3,4,5,6)P5, whereas Ins(1,3,4)P3 is formed by a kinase/phosphatase pathway as a consequence of receptor-dependent phospholipase C activity. The metabolic coupling of these two distinct pathways has been demonstrated in cells (10).

The metabolic interaction between ITPK1 substrates is of particular biological significance because Ins(3,4,5,6)P4 decouples calcium-activated chloride channels from activation by cytoplasmic Ca2+ (11). In this way Ins(3,4,5,6)P4 regulates a range of biological processes, including salt and fluid secretion, neurotransmission, and insulin secretion from pancreatic β-cells (8, 11–13). Recently it was shown that changes in ITPK1

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4 The abbreviations used are: Ins(1,4,5)P3, inositol 1,4,5-trisphosphate; Ins(1,3,4)P3, inositol 1,3,4-trisphosphate; ITPK1, inositol 1,3,4-trisphosphate 5/6-kinase; Ins(1,3,4,6)P4, inositol 1,3,4,6-tetrakisphosphate; Ins(1,3,4,5,6)P5, inositol 1,3,4,5,6-pentakisphosphate; eITPK1, E. histolytica ITPK1; hITPK1, H. sapiens ITPK1; HPLC, high pressure liquid chromatography; ALS, Advanced Light Source; gmITPK4, Glycine max ITPK1 homologue.
expression influence Ins(3,4,5,6)P$_4$ levels, possibly modulating the phenotypic severity of the cystic fibrosis condition (7). Changes in ITPK1 expression have also been reported to affect tumor necrosis factor-α-induced cell death (14).

Recently, Miller et al. (15) described the crystal structure of an ITPK1 homologue in Entamoeba histolytica (eITPK1). This structure revealed an unusually versatile catalytic cleft that possesses little or no stereospecific constraints. Ligand binding was demonstrated that this characteristic has evolved specifically in higher organisms; we show that Ins(1,3,4)P$_3$ does not activate Ins(1,3,4,5,6)P$_5$ dephosphorylation in ITPK1 homologues from soybean and in E. histolytica. Moreover, we describe the crystal structure of the human enzyme and identify structural features likely to contribute to this and other important differences in the catalytic activities of different homologues of ITPK1.

### Experimental Procedures

A rapid Escherichia coli expression, purification, and crystallization procedure for hITPK1 has been developed. Human ITPK1 was cloned into a MH4 vector (17) by PCR and expressed in the HK100 strain of E. coli. Briefly, 12 × 65-ml cultures were grown at 37 °C in a Genomics Institute of the Novartis Research Foundation fermenter (18), and hITPK1 expression was induced by the addition of 0.02% L-arabinose for 3 h. Proteins were extracted by sonication into 20 ml of 50 mM Tris, pH 8, 100 mM NaCl, 1 mM tri(2-carboxyethyl)phosphine hydrochloride (TCEP), 10 mM imidazole, with 1 Complete EDTA-free protease inhibitor tablet (Roche Applied Science). Cell debris was removed by centrifugation at 30,000 × g for 45 min. The recombinant enzyme was purified by nickel-nitrilotriacetic acid-agarose chromatography and eluted into a buffer composed of 20 mM Tris, pH 8, 100 mM NaCl, 10% glycerol, 0.5 mM TCEP, 100 mM imidazole. S-200 size exclusion chromatography followed. Selenomethionine-substituted protein was produced by inhibition of methionine biosynthesis (19) and subsequently purified in the same manner as the native protein.

Protein was concentrated in a Centriprep 15 10-kDa concentrator (Amicon) prior to crystal screening. Native protein was concentrated to 15–22 mg/ml for crystallization trials, whereas selenomethionine protein appeared insoluble beyond 12 mg/ml and was used at this concentration. 480 crystallization conditions were set up in Greiner low profile 96-well sitting drop vapor diffusion plates at two temperatures (4 and 20 °C) (conditions described in Ref. 17). Full-length ITPK1 failed to crystallize despite extensive coarse crystallization screening and showed some proteolytic degradation during purification.
and storage. Proteolytic products, presumably a consequence of the action of endogenous *E. coli* proteases, were analyzed by mass spectroscopy, and sequences corresponding to the truncated products were cloned back into the MH4 vector. The construct composed of residues 1 to 335 proved amenable to crystallization and was used in subsequent structural studies. Crystals grew in 2 weeks at 4 °C after mixing the protein solution 1:1 with, and subsequently equilibrating against, a reservoir solution of 0.2 M citrate, pH 5.6, 2.0 M (NH₄)₂SO₄, 0.2 M potassium/sodium tartrate. Further crystals were obtained by co-crystallization with either 1 mM ATP or AMPNP (Sigma), along with 5 mM MnCl₂. Crystals were cryo-protected in reservoir solution containing 20% glycerol.

Data were collected at the Advanced Light Source (ALS, Berkeley, CA) beamlines 5.0.2 and 5.0.3. Data were integrated, reduced, and scaled using HKL2000 (20) and the CCP4i suite (21). Crystallographic statistics are summarized in Table 1. ITPK1 was initially phased using a three-wavelength multiwavelength anomalous dispersion experiment on selenomethionine-derivative protein, and the structure was subsequently solved using Solve/Resolve (22). Subsequently, non-isomorphous structures were solved by molecular replacement using Phaser (23). Iterative rounds of manual model building in Coot (24) were followed by restrained refinement using Refmac5 (25). Ligand solvent accessibility was calculated using AREAIMOL (26) with a probe radius of 1.4 Å. The protein-protein interaction server was used to evaluate the hITPK1 crystal interactions (27). Figures were prepared using PyMOL.

All *in vitro* assays of inositol phosphate metabolism were performed as previously described (28). For these experiments, recombinant hITPK1 was prepared after expression in Sf9 cells as previously described (28) except that the gene was first shuttled into the pDEST605 kinase vector, which also contains a green fluorescent protein reporter gene that was then used to generate bacmid insect viral DNA (Invitrogen). Site-directed mutagenesis was performed as previously described (28).

An expressed sequence tag clone encoding gmITPK4 was obtained from the Public Soybean Data Base (Biogenetic Services, Brookings, SD). PCR amplifications were performed with a 5′-primer containing a Smal restriction site and the 3′-primer containing XhoI. PCR products were digested and ligated into pGEX4T-1 (encoding an N-terminal glutathione S-transferase tag), which was used to transform competent TOP10 bacterial cells. Protein expression was induced with isopropyl-1-thio-D-galactopyranoside, cells were lysed, and protein was purified using glutathione S-transferase-Sepharose

![FIGURE 1. Intersubstrate phosphate transfer by hITPK1.](image)
The addition of non-radio-labeled Ins(1,3,4)P₃ to these reactions increased the rate of dephosphorylation of Ins(1,3,4,5,6)P₅ despite the fact that both are substrates of ITPK1 (Fig. 1A). This is a biologically significant phenomenon that enables Ins(1,3,4)P₃ to regulate Ins(3,4,5,6)P₄ accumulation in vivo (8), but it has not previously been rationalized at a molecular level. We now show that Ins(1,3,4)P₃ considerably reduced the quantity of [³²P]ATP that accumulated when [³²P-1]Ins(1,3,4,5,6)P₅ was dephosphorylated (Fig. 1, A and B). Instead, the [³²P]-1-phosphate that was released from Ins(1,3,4,5,6)P₅ was now transferred to Ins(1,3,4,5)P₃, thereby forming [³²P]InsP₄ (Fig. 1, A and B). It has been known for some time that ITPK1 shows both 5- and 6-kinase activities toward Ins(1,3,4)P₃ (29). Using [³H]InsP₄ we confirmed that two [³H]InsP₄ isomers were formed, Ins(1,3,4,5)P₄ and Ins(1,3,4,6)P₄ (Fig. 1, A and B). The above results imply that Ins(1,3,4)P₃ can bind to an enzyme-ATP state faster than ATP can be exchanged for ADP. Thus, the enzyme may return to its ADP state more quickly (ready for another cycle of Ins(1,3,4,5,6)P₅ dephosphorylation) if enzyme-bound ATP is used to phosphorylate Ins(1,3,4)P₃. Note also that a small amount of newly formed [³²P]InsP₄ was itself dephosphorylated to a [³²P]-labeled InsP₃, which consistently eluted one fraction after ATP (Fig. 1B).

The assays described above contained a physiologically relevant concentration of ADP (1 mm) (30) but no ATP. In further experiments, non-radioactive ATP was added to 5 mm and it is striking that there was only a 30% decrease in both the extent of Ins(1,3,4,5,6)P₅ dephosphorylation and the rate of [³²P] transfer from Ins(1,3,4,5,6)P₅ to Ins(1,3,4,6)P₄ (Fig. 1E) compared with incubations containing 1 mm ADP and no ATP. These results support our hypothesis that there is a relatively slow rate of exchange of bulk phase ATP with either enzyme-bound ADP or enzyme-bound [³²P]ATP. Clearly, the intersubstrate phosphate transfer activity of ITPK1 occurs even in the presence of physiologically relevant levels of adenine nucleotides (i.e. [ATP] > [ADP]) (30).

Simultaneous binding of both Ins(1,3,4)P₃ and Ins(1,3,4,5,6)P₅ to the same active site seems implausible because of the topological and electrostatic constraints imposed by the binding pocket (described below). In order for the same phosphate to be transferred between different substrates via the same binding site, a sequential reaction model has been proposed (Fig. 2A). The efficient operation of this proposed nucleotide-mediated

**RESULTS AND DISCUSSION**

**Intersubstrate Phosphate Transfer Activity of hITPK1**—We investigated the mechanism by which Ins(1,3,4)P₃ stimulates Ins(1,3,4,5,6)P₅ dephosphorylation (Figs. 1 and 2). Dephosphorylation assays used Ins(1,3,4,5,6)P₅ in which the 1-phosphate was [³²P]-labeled. When incubated with ADP, hITPK1 dephosphorylated [³²P-1]Ins(1,3,4,5,6)P₅ to yield unlabeled Ins(3,4,5,6)P₄. As Ins(3,4,5,6)P₄ was not detectable in the HPLC profiles shown in Fig. 1, the accumulation of the Ins(3,4,5,6)P₄ product was separately verified using [³H]Ins(1,3,4,5,6)P₅ as substrate (data not shown). Note that there was no release of [³²P]P₄ when [³²P-1]Ins(1,3,4,5,6)P₅ was dephosphorylated to Ins(3,4,5,6)P₄ (Fig. 1A). Instead, [³²P]-labeled ATP accumulated (Figs. 1, A and B), which was separately verified using a luciferase reporter assay (data not shown). This is the first demonstration that the 1-phosphate from Ins(1,3,4,5,6)P₅ is transferred to ADP rather than being released to the medium as inorganic phosphate by ITPK1.
Integration of Inositol Phosphate Signaling by hITPK1

A

B

C

FIGURE 3. Both eITPK1 and gmITPK4 dephosphorylate Ins(1,3,4,5,6)P5, but Ins(1,3,4)P3 does not stimulate the reaction. Either eITPK1, hITPK1, or gmITPK4 were separately incubated as described under “Experimental Procedures” with 1 mM ADP and 5 μM either [3H]Ins(1,3,4,5,6)P_5 (A and B) or [32P-1]Ins(1,3,4,5,6)P_5 (C) in either the presence (closed symbols) or absence (open symbols) of 5 μM Ins(1,3,4)P_3. Assays were analyzed either by HPLC (A and C) or by gravity-fed columns (B) (means ± S.E., n = 3). The elution gradient was steeper in the chromatograph described in panel C, hence the arrow marks the elution position of InsP_4 determined in an adjacent run.

inter-substrate phosphate transfer may be facilitated by the enzyme withholding the nucleotide from the bulk phase during this series of reactions (Fig. 2B).

Homologues of hITPK1 from Plant and Protozoa Dephosphorylate Ins(1,3,4,5,6)P_5 but Ins(1,3,4)P_3 Does Not Stimulate This Reaction—Although the inositol phosphate kinase activity of eITPK1 has been well documented in previous reports (15, 31), the data shown in Fig. 3A represent the first demonstration that eITPK1 can dephosphorylate Ins(1,3,4,5,6)P_5 to Ins(3,4,5,6)P_4. It is particularly notable that the rate of Ins(1,3,4,5,6)P_5 dephosphorylation was not stimulated by Ins(1,3,4)P_3 (Fig. 3A). This is an important functional difference between eITPK1 and hITPK1 and may represent a divergence in cellular function. Homologues of hITPK1 are also present in plants (32). Plant enzymes have higher homology with hITPK1 than does eITPK1 (Fig. 4C). The plant enzymes are also functionally more similar to hITPK1 in that neither show physiologically significant kinase activity toward Ins(1,4,5)P_3 (Table 2 and Ref. 32), unlike eITPK1 which shows robust Ins(1,4,5)P_3 3-kinase activity (31). We therefore felt useful information could be obtained by comparing some of the characteristics of hITPK1 with a plant homologue.

Candidate homologues of hITPK1 were identified in the Glycine max (soybean) Expressed Sequence Tag data base (gmITPK1 to gmITPK4), which we have numbered based on their similarity to known genes from Arabidopsis thaliana (32) and Zea mays (33). Recombinant gmITPK4 was expressed in E. coli as an N-terminal glutathione S-transferase fusion protein and was found to phosphorylate both Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 (data not shown), verifying its nature as a homologue of hITPK1. Additionally, both gmITPK4 and hITPK1 showed Ins(1,3,4,5,6)P_5 dephosphorylation activity when assayed under identical conditions. The soybean enzyme was up to 40-fold more active (Fig. 3B). However, Ins(1,3,4)P_3 did not stimulate Ins(1,3,4,5,6)P_5 dephosphorylation by gmITPK4 (Fig. 3B). Instead, Ins(1,3,4)P_3 was a slight inhibitor, which is what would be expected if the two substrates competed for the same active site in gmITPK4. Moreover, gmITPK4 did not show substantial inter-substrate phosphate transfer activity (Fig. 3C) compared with hITPK1 (Fig. 1).

Structural Overview of hITPK1—To rationalize the species differences in substrate regulation, the crystal structure of hITPK1 was determined. The structure of hITPK1 was initially solved by selenomethionine multiwavelength anomalous dispersion and has been subsequently refined in a variety of ligand-bound forms and crystal forms to a maximum resolution of 1.6 Å (Table 1). The entire catalytic domain is defined as far as residue 335. In both crystal forms the C-terminal residues form a small dimer interface. Although this interface corresponds to ~10% of the total surface area of the protein, other biophysical methods such as gel filtration and static light scattering indicate a monomeric state for ITPK1 (data not shown).

hITPK1 exhibits the same overall topology as the eITPK1 structure, which is related to the former by 24% amino acid sequence identity between the catalytic domains (Fig. 4C). Superposition of the hITPK1 structure with eITPK1 results in a root mean square deviation of 2.2 Å on 242 topologically equivalent Cα atoms (Fig. 4A). As would be expected for enzymes with variations in enzymatic function, structural diversity is exhibited in regions proximal to the active site.

ATP Binding Site—The ATP binding sites of eITPK1 and hITPK1 are each sandwiched between two sets of four-stranded anti-parallel β-sheets (Fig. 4A), a structure commonly described as an ATP-grasp fold (34, 35). In hITPK1, one β-sheet is composed of β11, β11, β11, and β14; the opposed sheet is composed of β12, β12, β12, and β19 (Fig. 4, A and C). A further two extended strands provide contacts to the purine ring of the bound nucleotide. Two well defined Mn^{2+} ions are present in the active site of the hITPK1 structure. One Mn^{2+} ion is coordinated by Asp-281 and Asp-295 and interacts with oxygens from both the β- and γ-phosphates of the bound ATP, adjacent to

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the scissile bond. The second Mn$^{2+}$ ion is coordinated by the side chains of Asp-295 and Asn-297. These Mn$^{2+}$ ions are structurally equivalent to the Mg$^{2+}$ ions in the eITPK1 structure (15).

It was previously noted that the nucleotide binding site of eITPK1 encloses much of the ligand; only 22% was accessible to solvent (15). There is only one entry and exit site for nucleotides in both eITPK1 and hITPK1 structures that would not require considerable rearrangement of the protein backbone, and this opening occurs next to active site residues Asp-295 and Asn-297. In eITPK1, one face of the entry/exit site is formed by a turn in the polypeptide chain just after the conserved structural element β12 and is followed by two helices (eITPK1 residues 212 to 236) arranged anti-parallel to each other and oriented roughly parallel to β12 (Fig. 4B). However, the structure of hITPK1 diverges after β12, with a 14-residue deletion relative to the eITPK1 sequence. Instead, hITPK1 substitutes a 310 helix (3104), composed of residues 232 to 236, in a lateral orientation to the ATP phosphates (Fig. 4B). The two crystal forms (Table 1) each contain two unique hITPK1 molecules in the asymmetric unit, and in each molecule form these helices vary in the degree to which helix 3104 encloses the nucleotide, indicating flexibility within this motif. In the conformation in which the nucleotide is most enveloped, the side chain of His-233 is able to reach hydrogen bonding distance to the AMPPNP γ-phosphate, although the equivalent distance is 6 Å in the second molecule, beyond interacting range. Nevertheless, in both cases the entry/exit site for nucleotide is more restricted for hITPK1 than eITPK1, with solvent accessibilities of only ~5% for AMPPNP (Fig. 5, A and B).

The 3104 helix also offers several extra interactions with the bound nucleotide that are not conserved in eITPK1, with solvent accessibilities of only ~5% for AMPPNP (Fig. 5, A and B). The 3104 helix also offers several extra interactions with the bound nucleotide that are not conserved in eITPK1, with solvent accessibilities of only ~5% for AMPPNP (Fig. 5, A and B).

**TABLE 2**

| Substrate | Reaction | WT | H162D |
|-----------|----------|----|-------|
| Ins(1,4,5)P3 | Phosphorylation | 0.026 ± 0.001 | 0.0113 ± 0.003 |
| Ins(1,3,4,5,6)P5 | Phosphorylation | 671 ± 60 | 3.3 ± 0.4 |
| Ins(1,3,4,5)P3 | Dephosphorylation | 309 ± 57 | 43 ± 7 |
| Ins(1,3,4,5,6)P5 | Dephosphorylation | 53 ± 3.3 | 135 ± 13 |
| [32P]-Ins(1,3,4,5,6)P5 | Phosphate transfer: InsP4/ATP | 2.9 ± 0.4 | 0.96 ± 0.04 |

Inositol Phosphate Binding Site of hITPK1—A particularly interesting feature of the eITPK1 structure is that there are no direct interactions between inositol hydroxyl groups and the protein, reflecting a limited capability for stereochemical substrate discrimination (15). In contrast, experiments with hITPK1 indicate a much greater ability to discriminate between substrates, including those that are stereoisomers (16). The inositol phosphate binding site of hITPK1 can be visualized as a strongly electropositive cleft that is stationed at the entrance to the ATP binding pocket (Fig. 5). The hITPK1 inositol phosphate binding site is composed of residues from helices α1, α3, and 3104, Arg-212 from sheet β11, and loop regions between β14-α9 and β7-β8 (Fig. 4A and C). Despite repeated attempts, we have been unable to co-crystallize or soak inositol phosphates into the hITPK1 crystals, possibly due to the highly polar nature of the binding site in combination with the presence of ammonium sulfate in the crystallization buffer. Sulfate molecules are, in fact, bound in the inositol phosphate binding site (Fig. 5C).

In comparing the inositol phosphate binding sites of hITPK1 and eITPK1, structural rearrangements and amino acid substitutions contribute to a considerable reduction in binding pocket volume for hITPK1 (Fig. 5, A and B). Helix 3104 in hITPK1 is followed by a loop composed of residues Ser-236 to Ser-258 where the eITPK1 binding site is open to solvent. The consequences of this loop are the further encapsulation of the bound nucleotide, the constriction of the inositol phosphate binding site, and the introduction of residues capable of mediating novel interactions with the bound inositol phosphate. For example, Lys-237 reaches from this loop into the inositol binding pocket where it would be able form a bond with either a phosphate group or with a hydroxyl group (Fig. 5, B and C). From the opposite face of the inositol phosphate binding cleft, two additional lysine residues, Lys-17 and Lys-21, both project from the α1 helix into the inositol phosphate binding pocket (Fig. 5, B and C). Lys-21 is a substitution for Thr-20 in eITPK1 and in hITPK1 could form a bond with a phosphate or hydroxyl group on an inositol ring modeled into the binding site. Lys-17 is also capable of interacting with inositol phosphate groups (Fig. 5C). A kink in the loop formed from residues Ile-296 to

![Figure 4. Sequence and structural comparisons between hITPK1 and eITPK1. Panels A and B show a structural alignment of hITPK1 in complex with AMPPNP (blue) and eITPK1 (orange). Panel B highlights topological differences proximal to the ATP binding site. Selected hITPK1 residues are shown in stick representation and selected hydrogen bonds as broken lines. AMPPNP is from the hITPK1 structure and is shown in stick form, with carbons shown in yellow. Panel C shows a sequence alignment of hITPK1, eITPK1, and gmITPK4. Secondary structural elements from hITPK1 are shown in blue above the alignment, and eITPK1 secondary structure is shown in orange below the alignment. αX, βX, and 310X refer to α helices, β-sheets, and 310 helices, respectively, numbered in order from the N terminus. The position of His-162 is indicated by an asterisk.](image-url)
His-162 is equivalent to Gln-141 in eITPK1, which also hydrogen bonds with the 4-phosphate of Ins(1,3,4)P3 (15). Interestingly, when Ins(1,3,4)P3 is modeled into hITPK1 in the same amine of residue Lys-59 at a distance of 3.4 Å, which lines the inositol binding site (Fig. 5, B and C). Helix α3 is composed of residues 60–69 and corresponds to a loop region in eITPK1. In hITPK1, Asp-62 protrudes from α3 and forms a tight polar interaction with His-162 from the loop between β6 and β7 (2.6 Å, Fig. 5C). The side chain of His-162 is positioned over the side chain amine of residue Lys-59 at a distance of 3.4 Å, which lines the inositol phosphate binding site below the imidazole ring. Interestingly, when Ins(1,3,4)P3 is modeled into hITPK1 in the same binding mode as eITPK1, the imidazole ring of His-162 clashes with the 4-phosphate group of the ligand (Fig. 5, B and C). His-162 is equivalent to Gln-141 in eITPK1, which also hydrogen bonds with the 4-phosphate of Ins(1,3,4)P3 (15). The conformation of α3 in hITPK1 leads to a shift of 5.0 Å for Glu-307, and a substitution from a glycine in eITPK1 to a glutamate at position 303 in hITPK1, closes one further face of the inositol phosphate binding site (Fig. 5, B and C).

A further reduction of the inositol phosphate binding pocket occurs as a consequence of structural changes at hITPK1. Ins(1,3,4)P3 is shown modeled into the hITPK1 based on structural alignment with eITPK1 and exhibits a steric clash with His-162. A solvent-accessible surface is shown for each molecule. The position of the ATP γ-phosphate is indicated by “*”. Metal ions are shown as green spheres. Panel C is a stereodiagram showing structural superposition of hITPK1 with eITPK1. hITPK1 is shown with blue carbons atoms, with a bound sulfate shown in gold and red. Residues capable of carrying a phosphate that are proximal to the ATP γ-phosphate are shown in yellow. eITPK1 in complex with Ins(1,3,4)P3 is shown with orange carbon atoms for protein, magenta carbons for the inositol.

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A further reduction of the inositol phosphate binding pocket occurs as a consequence of structural changes at hITPK1. Ins(1,3,4)P3 is shown modeled into the hITPK1 based on structural alignment with eITPK1 and exhibits a steric clash with His-162. A solvent-accessible surface is shown for each molecule. The position of the ATP γ-phosphate is indicated by “*”. Metal ions are shown as green spheres. Panel C is a stereodiagram showing structural superposition of hITPK1 with eITPK1. hITPK1 is shown with blue carbons atoms, with a bound sulfate shown in gold and red. Residues capable of carrying a phosphate that are proximal to the ATP γ-phosphate are shown in yellow. eITPK1 in complex with Ins(1,3,4)P3 is shown with orange carbon atoms for protein, magenta carbons for the inositol.
phosphate transfer activity of hITPK1, in part by promoting the phosphorylation of \text{Ins}(1,3,4)P_3 and also regulating ATP exchange with the bulk phase.

**Concluding Comments**—This study provides a molecular basis for understanding how hITPK1 couples steady-state levels of \text{Ins}(3,4,5,6)P_4 to a metabolically separate pool of inositol phosphates, including \text{Ins}(1,4,5)P_3 and \text{Ins}(1,3,4)P_3, that arise from receptor-regulated phospholipase C activity (9). During metabolic coupling, a phosphate is transferred between inositol phosphates by hITPK1, but not by eITPK1 or gmITPK4. Although our studies reveal that ADP does act as a phosphate carrier, we do not exclude the possibility that a phosphoencezyme intermediate might also play a role in the catalytic cycle. hITPK1 residues capable of carrying a phosphate and that are proximal to the ATP γ-phosphate are shown in yellow in Fig. 5C.

We propose that these differences between ITPK1 homologues represent the evolution in mammals of a mechanism for regulating the signaling activities of \text{Ins}(3,4,5,6)P_4. This function may be superimposed upon a more general role of ITPK1 homologues in eukaryotes: contributing to the synthesis of higher inositol polyphosphates such as \text{Ins}P_6 (33, 37). However, the extent to which ITPK1 contributes to higher order inositol phosphate synthesis in mammals remains to be demonstrated in a range of conditions and cell types. For example, it has been shown that two other inositol kinases, IPK1 and IPK2, are sufficient to recapitulate \text{Ins}P_6 synthesis from \text{Ins}(1,4,5)P_3 (38, 39).

In contrast, the function of gmITPK4 in soybean is likely restricted to the synthesis of higher inositol polyphosphates. Furthermore, \text{Ins}(1,3,4)P_3 has not been identified in any plant, nor has an enzyme that can synthesize it.

It seems likely the capacity for inositol phosphate signal integration has co-evolved as \text{Ins}(3,4,5,6)P_4 developed into an intracellular signal that, through its control over \text{Cl}^- channel conductance, regulates many biological processes, including epithelial salt and fluid secretion, insulin secretion, and likely smooth muscle contraction and neurotransmission. Together, the biochemical and structural studies on ITPK1 provide an example how evolution can transform a metabolic enzyme into one with a signaling function. The characterization of the structure of hITPK1 and its mechanism of substrate regulation together offer new avenues for intervention in the inositol phosphate metabolic pathway.

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