Structural features of human inositol phosphate multikinase rationalize its inositol phosphate kinase and phosphoinositide 3-kinase activities

Received for publication, June 14, 2017, and in revised form, September 1, 2017 Published, Papers in Press, September 7, 2017, DOI 10.1074/jbc.M117.801845

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Human inositol phosphate multikinase (HsIPMK) critically contributes to intracellular signaling through its inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) 3-kinase and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) 3-kinase activities. This catalytic profile is not conserved; orthologs from Arabidopsis thaliana and Saccharomyces cerevisiae are predominantly Ins(1,4,5)P3 6-kinases, and the plant enzyme cannot phosphorylate PtdIns(4,5)P2. Therefore, crystallographic analysis of the yeast and plant enzymes, without bound inositol phosphates, do not structurally rationalize HsIPMK activities. Here, we present 1.6-Å resolution crystal structures of HsIPMK in complex with either Ins(1,4,5)P3 or PtdIns(4,5)P2. The Ins(1,4,5)P3 headgroup of PtdIns(4,5)P2 binds in precisely the same orientation as free Ins(1,4,5)P3 itself, indicative of evolutionary optimization of 3-kinase activities against both substrates. We report on nucleotide binding between the separate N- and C-lobes of HsIPMK. The N-lobe exhibits a remarkable degree of conservation with protein kinase A (root mean square deviation = 1.8 Å), indicating common ancestry. We also describe structural features unique to HsIPMK. First, we observed a constrained, horseshoe-shaped substrate pocket, formed from an α-helix, a 3_10 helix, and a recently evolved tri-proline loop. We further found HsIPMK activities rely on a preponderance of Gln residues, in contrast to the larger Lys and Arg residues in yeast and plant orthologs. These conclusions are supported by analyzing 14 single-site HsIPMK mutants, some of which differentially affect 3-kinase and 6-kinase activities. Overall, we structurally rationalize phosphorylation of Ins(1,4,5)P3 and PtdIns(4,5)P2 by HsIPMK.

Considerable attention is focused on the enzymes that regulate the metabolism and hence the myriad cell signaling activities of the inositol phosphates and the inositol lipids. These are two physicochemically and functionally distinct groups of intracellular signals (1), which typically each rely on separate families of kinases for their synthesis. The sole exception is the inositol phosphate multikinase, initially named for its ability to phosphorylate inositol phosphates (2, 3), but later found to also phosphorylate PtdIns(4,5)P2 (4). This “dual-specificity” has endowed the inositol phosphate multikinase (IPMK) family with multiple biological activities. For example, IPMK is indispensable for connecting PLC-mediated Ins(1,4,5)P3 release to the generation of InsP6 (5–7); the latter is a precursor for InsP6 and the inositol pyrophosphates, which each have many cellular functions (1, 8). Activation of the inositol phosphate kinase activities of IMPK appears to be a key response in the Wnt/β-catenin signaling pathway (9). IPMK is mainly localized in the nucleus (4, 10, 11), where its kinase activities have been shown to mediate cellular differentiation programs (12), and transcript-selective mRNA export from the nucleus (13). Also in the nucleus, the PtdIns(4,5)P2 3-kinase activity of IPMK stimulates the transcriptional activity of the nuclear receptor steroidogenic factor 1 (14). In addition, mammalian IPMK has moonlighting functions, unrelated to its catalytic activities, which are mediated through interactions with a number of protein-binding partners, such as mTOR (mechanistic target of rapamycin) (15), p53 (16), and AMP-activated protein kinase (17).

The wide-ranging importance of the IPMKs is underscored by the observation that knock-out of the IPMK gene in mice is embryonic lethal (5). There are also some pathological consequences for genetic defects in human IPMK that have been associated with a reduction in its kinase activities. For example, a heterozygous, frameshift mutation in the human IPMK gene has been identified in six members of the same family who all developed small intestinal neuroendocrine tumors; these individuals also exhibited a reduction in InsP6 synthesis (18). Additionally, impaired IPMK transcription and a decrease in IPMK stability has been linked to the pathology of Huntington’s disease, by virtue of an attenuation of the PtdIns(3,4,5)P3/3-kinase activities of the PtdIns(3,4,5)P3/AKT signaling pathway (9).

2 The abbreviations used are: IPMK, inositol phosphate multikinase; AMP-PNP, adenylyl-imidodiphosphate; Ins(1,4,5)P3, inositol-1,4,5-trisphosphate; Ins(1,3,4,5,6)P5, inositol-1,3,4,5,6-pentaosio kinase; PIK3CA, PtdIns(4,5)P2 3-kinase 110-kDa catalytic subunit α; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; GroPtdIns(4,5)P2, glycero-phosphoinositol 4,5-bisphosphate; r.m.s. deviation, root mean square deviation; PDB, Protein Data Bank.
signaling cascade (19). Genome-wide association studies have described that decreased IPMK expression in brain tissue is associated with the pathogenesis of inflammation-associated neurodegeneration (20).

IPMK is a member of the so-called IP-kinase family that includes IP3Ks and IP6Ks (21). Characterization of the structures of each of these enzymes can rationalize their alternate catalytic specificities, assist in deciphering evolutionary relationships, and permits rational design of enzyme-specific inhibitors. Previous work has described crystal structures of an IP3K (21, 22) and an IP6K (23), each with substrates captured in the active site. Two previous studies have described the crystal structures of IPMK orthologues from *Saccaromyces cerevisiae* (24) and *Arabidopsis thaliana* (25), at resolutions of 2 and 2.9 Å, respectively. However, neither study captured inositol phosphate within the active site.

Molecular modeling has generated a consensus view that IPMKs host a relatively spacious and conformationally flexible substrate-binding pocket, in which mobile side chains of Lys and Arg residues play major roles (24, 25). Here, we demonstrate that this model does not apply to HsIPMK; we describe a catalytic pocket that is more constrained than those of the plant and yeast orthologs. Also unique to mammalian IPMK is a catalytically important proline-loop, and a preponderance of Gln residues in the active site. These conclusions are drawn from our description, for the first time, of the crystal structure of HsIPMK. Moreover, we present the first structures of any IPMK in complex with inositol phosphate substrate: we describe two versions of Ins(1,4,5)P3 within the active site, first as a free inositol phosphate, and second as the headgroup of a soluble analogue of PtdIns(4,5)P2. This allows a structural rationalization of 3-kinase activity toward Ins(1,4,5)P3 and PtdIns(4,5)P2.

**Results and discussion**

**General structure of HsIPMK**

Previous work (3, 4, 11, 14) has shown that HsIPMK is both an inositol phosphate kinase and a PtdIns(4,5)P2 kinase. Here, we confirmed that recombinant HsIPMK uses 3-kinase activity to phosphorylate Ins(1,4,5)P3 to Ins(1,3,4,5)P4, and then phosphorylates the latter with a 6-kinase activity that yields Ins(1,3,4,5,6)P5 (Fig. 1, A–E). In addition, we used a decacylated, soluble version of Ptd[^3H]InsP(4,5)P2 (i.e. GroPtd[^3H]InsP3)

![Figure 1. The inositol phosphate and inositol lipid kinase activities of HsIPMK. A, graphic depicting the successive Ins(1,4,5)P3 3-kinase and Ins(1,3,4,5)P4 6-kinase activities of HsIPMK; filled circles represent phosphate groups. B, HPLC analysis of [^3H]Ins(1,4,5)P3, at zero time (solid line). C, HPLC analysis of the phosphorylation of [^3H]Ins(1,4,5)P3, for 15 min by 0.9 ng HsIPMK (solid line). Also shown is the elution of a [^14C]Ins(1,4,5,6)P4 standard (open circles; dotted line) obtained in parallel to a run by collecting fractions. D, HPLC analysis of the phosphorylation of [^3H]Ins(1,4,5)P3, for 15 min by 90 ng of HsIPMK (solid line). E, the elution of a [^14C]Ins(1,3,4,5,6)P5 standard (dotted line) obtained in parallel to an assay in which [^3H]Ins(1,4,5)P3 was phosphorylated to [^3H]Ins(1,3,4,5,6)P5 by HsIPMK (solid line). F, graphic depicting GroPtd[^3H]Ins(4,5)P2 3-kinase activity of HsIPMK. G, HPLC analysis of GroPtd[^3H]Ins(4,5)P2, at zero time. H, HPLC analysis of phosphorylation of GroPtd[^3H]Ins(4,5)P2 to GroPtd[^3H]InsP3, (elution peak = 38 min) by 4.5 ng of HsIPMK for 15 min. I, HsIPMK (9.5 ng) was incubated for 16 h with [^23P]ATP and dicro-InsP(4,5)P2; the dicro-InsP[^23P] was deacylated to GroPtd[^23P]InsP3, which was analyzed by HPLC (solid line). In a parallel HPLC run, we analyzed a GroPtd[^23P]InsP3 standard (dotted line), derived from PtdIns(4,5)P2 3-kinase 110-kDa catalytic subunit α (PIK3CA). The elution peak = 37.7 min. Full details are provided under “Experimental procedures.”
PtdIns(4,5)P2 to confirm the PtdIns(4,5)P2 3-kinase activity of recombinant HsIPMK (Fig. 1, F–I). Our next goal was to rationalize these various kinase reactions structurally, at an atomic level. We produced crystals of the core catalytic domain of the enzyme that contains residues 50 to 416, from which we deleted an internal domain comprising residues 263 to 377 (Fig. 2A); that deletion, which was necessary to obtain crystals, was replaced with a Gly-Gly-Ser-Gly-Gly linker (Fig. 2A).

Previous work has shown that this deletion does not compromise catalytic activity (26); this is a non-catalytic region of the protein. It contains a nuclear localization sequence, flanked by residues that host protein kinase phosphorylation sites that regulate nuclear localization sequence functionality (26).

The structure of the IPMK apoenzyme was determined by a molecular replacement approach using a model constructed from the template of ScIPMK (PDB accession code 2IF8). That information was then used for further elucidation of the structures of crystal complexes with ADP plus either Ins(1,4,5)P3 (Fig. 2, A–D) or dIC4-PtdIns(4,5)P2 (see below).

For each asymmetric unit, there is one molecule of IPMK in space group P42212 (Table 1). Analysis of the overall fold of HsIPMK (Fig. 2B) reveals domains that are similar to the so-
Table 1

| Data collection and structure refinement statistics | 3W2G | 5W2H | 5W2I |
|---------------------------------------------------|------|------|------|
| **Ligand** | Apo | ADP-Ins(1,4,5)P<sub>3</sub> | ADP-DiC4-6-P | PrdInsP<sub>2</sub>-Mg |
| **Data collection** | | | | |
| Space group | P4<sub>2</sub>1<sub>2</sub>2 | P4<sub>2</sub>1<sub>2</sub>2 | P4<sub>2</sub>1<sub>2</sub>2 | |
| Cell dimensions, a, c (Å) | 78.47, 85.65 | 78.02, 85.92 | 78.09, 86.32 | |
| Resolution (Å)<sup>a</sup> | 50–1.80 (1.83) | 50–1.9 (1.93) | 50–1.6 (1.63) | |
| R<sub>merge</sub> | 0.121 (0.857) | 0.116 (0.953) | 0.104 (0.782) | |
| C<sub>H</sub>/2 in the highest shell | 0.754 | 0.763 | 0.853 | |
| I<sub>ori</sub><sup>a</sup> | 15.5 (3.6) | 18.0 (3.3) | 22.1 (2.9) | |
| Completeness (%)<sup>a</sup> | 99.4 (99.9) | 99.9 (100.0) | 99.9 (100.0) | |
| Redundancy<sup>a</sup> | 8.7 (8.1) | 9.0 (8.9) | 7.7 (7.3) | |

<sup>a</sup> The numbers in parentheses are for the highest resolution shell.

Structure of human IPMK

Table 1

| Data collection and structure refinement statistics | 3W2G | 5W2H | 5W2I |
|---------------------------------------------------|------|------|------|
| **Ligand** | Apo | ADP-Ins(1,4,5)P<sub>3</sub> | ADP-DiC4-6-P | PrdInsP<sub>2</sub>-Mg |
| **Data collection** | | | | |
| Space group | P4<sub>2</sub>1<sub>2</sub>2 | P4<sub>2</sub>1<sub>2</sub>2 | P4<sub>2</sub>1<sub>2</sub>2 | |
| Cell dimensions, a, c (Å) | 78.47, 85.65 | 78.02, 85.92 | 78.09, 86.32 | |
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<sup>a</sup> The numbers in parentheses are for the highest resolution shell.

called N- and C-lobes that comprise the ATP-binding sites in the orthologs ScIPMK (24) and AtIPMK (25), as well as EhlIP6KA (23) and HsIP3KA (21). Superimposition structures of each of these proteins with HsIPMK reveals root mean square deviations (r.m.s. deviations) of 0.93 Å for ScIPMK (139 comparable Cα atoms), 1.37 Å for AtIPMK (149 comparable Cα atoms), 1.65 Å for EhlIP6KA (145 comparable Cα atoms), and 1.14 Å for HsIP3KA (144 comparable Cα atoms).

The N-terminal lobe in HsIPMK is designated by residues 65–135, which consist of four antiparallel β-strands and two short helices α1 and α2 and the β1 and β3 strands. Residues 66–69 lie within a β-strand that has not been observed in either ScIPMK or AtIPMK; we designated this the β0 strand. We designated the C-terminal lobe as comprising residues 136–149 and 175–416, which is an α+β-fold with five, central antiparallel β-strands including β4–6, β8, and β9, a pair of small antiparallel β-strands (β7 and β10), and three α-helices (α5–α7). Also in the C-lobe of HsIPMK, a 3<sub>10</sub> helix was observed between the β6 strand and α5 helix. An equivalent, albeit longer 3<sub>10</sub> helix is present in EhlIP6KA, where it is important for substrate binding (23).

Description of the nucleotide-binding region of HsIPMK

We soaked Ins(1,4,5)P<sub>3</sub>, ATP, and magnesium into the apoenzyme crystals. Nucleotide binding did not alter protein conformation. The ADP moiety was observed (Fig. 3A), but not the γ-phosphate of ATP. A similar result was obtained in an earlier study with ScIPMK (24). Perhaps in our study, the γ-phosphate was hydrolyzed, or alternately, disordered in the crystal. The latter explanation is feasible, because the terminal phosphate of adenylyl-imidodiphosphate (AMP-PNP) was also the only portion of that non-hydrolyzable ATP analogue that we could not visualize, after it was soaked into the crystal structure with Ins(1,4,5)P<sub>3</sub>.

HsIPMK clasps the nucleotide between the N- and C-lobes, which are linked by a hinge that comprises residues Asp<sup>133</sup> to Pro<sup>140</sup> (Figs. 2, A and C, and 3A). The N<sup>4</sup> and N<sup>6</sup> atoms of adenine both make hydrogen bonds with the polypeptide backbone: N<sup>1</sup> contacts the amide nitrogen of Val<sup>133</sup> from the hinge, and N<sup>6</sup> interacts with the carbonyl oxygen of Glu<sup>131</sup> from the N-lobe (Fig. 3A). The ATP-ribose group is loosely confined by several van der Waals contacts with Leu<sup>254</sup> and Ile<sup>384</sup>, plus one hydrogen bond with Asp<sup>144</sup>. The α-phosphate of the nucleotide forms a salt bridge with Lys<sup>75</sup> (Fig. 3A). Asp<sup>385</sup> interacts with two magnesium ions to make contact with the α- and β-phosphates of ADP. The particular importance of Asp<sup>385</sup> is reflected in the fact that it is not observed in either of the nucleotide-agonist crystal structures of IPMK (27). Furthermore, a high degree of conservation of the entire N-lobes was revealed by superimposition of the secondary structure elements of the HsIPMK structure upon those in PKA (Fig. 3D): a core r.m.s. deviation of 1.80 Å (51/69 comparable residues). The C-lobes (Fig. 3F) are less conserved: a core r.m.s. deviation of 3.35 Å (78/168 comparable residues). The major structural differences between the C-lobes of the two proteins reflects specialization of the alternative substrate-binding pockets. The C-lobe of PKA, and indeed protein kinases in general, contains a greater degree of helical structure, and a wider binding site to accommodate a polypeptide (27, 28) (Fig. 3F). These data confirm and extend the idea (21, 29) that protein kinases and the so-called IP-kinase family share an evolutionary ancestry.

Description of inositol phosphate binding

The successful soaking of Ins(1,4,5)P<sub>3</sub> into HsIPMK crystals (Fig. 2, B and C) has yielded the first description of any inositol phosphate substrate captured in the active site of an IPMK. Simulated annealing omit maps and 2<sub>Fc</sub> − F<sub>c</sub> maps clearly identify the inositol ring and individual phosphate groups for Ins(1,4,5)P<sub>3</sub> (Fig. 2B). The 2–5 axis of the Ins(1,4,5)P<sub>3</sub> substrate inserts vertically into a positively charged (at physiological pH) horseshoe-shaped pocket (Fig. 4A) constructed from (in anticlockwise rotation), a short 3<sub>10</sub> helix, the α3 helix, and a unique loop that is fabricated from three proline residues (Fig. 4B). Rigidity in the α3 helix is enhanced by virtue of a 2.8-Å hydrogen bond between the carboxyl and amine groups in the side chains of Gln<sup>163</sup> and Gln<sup>164</sup>, respectively (Fig. 5A). This constrains the conformations of the two side chains and introduces

<sup>3</sup>H. Wang, unpublished data.
planarity, resulting in a stacking effect between the inositol ring and the α3 helix.

The proline loop of HsIPMK (Fig. 4B) is a structural element that is absent from HsIP3K (Figs. 2D and 4C) and both the plant and yeast IPMKs (Fig. 4, D and E, and 5B). In fact, in our IPMK sequence alignment (Fig. 5B), there are gaps in the yeast and plant sequences in the region corresponding to the proline loop. Interestingly, the equivalent Drosophila IPMK sequence is Lys-Pro-Glu, suggesting that a nascent version of this loop is present in this, and perhaps, other invertebrates (Fig. 5B). Arg12 at the N terminus of the proline-loop makes 3 polar contacts with the 4- and 5-phosphates of Ins(1,4,5)P3, indicating its particular importance in substrate binding. Gln174, at the N terminus of the proline loop, interacts with two water molecules that coordinate with the β-phosphate of ADP and a magnesium atom (Fig. 4B).

We were unable to visualize the γ-phosphate of ATP within the catalytic center, apparently because it is disordered in the crystal (see above), but the ADP moiety is only 6.3 Å from the 3-OH of Ins(1,4,5)P3 that is phosphorylated (Fig. 5, A and C). Thus, ATP may phosphorylate the Ins(1,4,5)P3 substrate by direct, in-line transfer (Fig. 5C). There are also two magnesium ions that are in a position to stabilize the negative charge that would develop on the leaving γ-phosphoryl group. Additionally, His388 is also only 7.7 Å from the β-phosphate of the ADP moiety, and so it is possible that His388 may contribute to charge balance during catalysis. His388 can also hydrogen bond with the 4-phosphate of Ins(1,4,5)P3 (Fig. 5A).

Other residues that form polar contacts with Ins(1,4,5)P3 include Lys160, Gln163, Gln164, and Lys167 from the α3 helix, and Gln196 from the 310 helix (Fig. 5A). A contact between Gln164 and the axial 2-hydroxyl group appears to help locate the inositol ring near-parallel to the α3 helix (Figs. 4A and 5A). The interactions of Gln164, Lys167, and Gln196 with Ins(1,4,5)P3 may be particularly important for enforcing its phosphorylation at the 3-position. Thus, we have described a relatively constrained...
Table 2
Effects upon the catalytic activities of HsIPMK following mutation of key residues to Ala

Rate equations (see under “Experimental procedures”) were used to derive the individual first-order rate constants $k_1$, $k_2$, and $k_3$. Data are mean ± S.E. from 3 to 6 determinations.

| Mutant  | Location          | $k_1$ (s$^{-1}$) InsP$_3$ metabolism (3-kinase) | $k_2$ (s$^{-1}$) InsP$_3$ synthesis (6-kinase) | $k_3$ (s$^{-1}$) GroPIns(4,5)P$_3$ metabolism (3-kinase) |
|---------|-------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------------|
| None (WT) |                  | 56.1 ± 2.0                                    | 0.64 ± 0.02                                   | 11.3 ± 0.2                                          |
| Q78A    | Proline-loop      | 5.4 ± 0.6                                     | 0.17 ± 0.03                                   | ND                                                  |
| R82A    | Proline-loop      | 1.6 ± 1.0                                     | 0.01 ± 0.0005                                 | ND                                                  |
| K160A   | IP-helix          | 0.8 ± 0.08                                    | 0.01 ± 0.001                                  | ND                                                  |
| Q163A   | IP-helix          | 20.6 ± 1.65                                   | 0.36 ± 0.011                                  | ND                                                  |
| Q164A   | IP-helix          | 0.5 ± 0.04                                    | 0.006 ± 0.00003                              | -107 0.08 ± 0.002                                   | -141 |
| K167A   | IP-helix          | 7.0 ± 0.36                                    | 0.091 ± 0.004                                 | -7 6.7 ± 0.4                                      | -2  |
| Q196A   | 3$_2$ helix       | 14.0 ± 1.4                                    | 0.22 ± 0.012                                  | -3 6.5 ± 0.4                                      | -2  |
| H388A   | Catalytic center  | 0.1 ± 0.004                                    | 0.00031 ± 0.00006                           | -206 ND                                             |

* ND, not determined.

Table 3
Differential effects of mutagenesis of catalytically-important Gln residues upon 3-kinase and 6-kinase activities of IPMK

Data are mean ± S.E. from 3 to 6 determinations. There was no significant accumulation of Ins(1,4,5,6)P$_4$ in these experiments.

| Mutant  | (s$^{-1}$) InsP$_3$, metabolism (3-kinase) | Δ | (s$^{-1}$) InsP$_3$, synthesis (6-kinase) | Δ | $k_1/k_2$ |
|---------|------------------------------------------|---|-----------------------------------------|---|-----------|
| None (WT) |                                           | -fold |                                     | -fold |          |
| Q163K   | 63 ± 0.4                                  | -9 | 0.41 ± 0.04                             | -2 | 16        |
| Q163R   | 9.3 ± 0.9                                 | -6 | 0.68 ± 0.02                             | 0  | 14        |
| Q164K   | 3.1 ± 0.2                                 | -18| 0.31 ± 0.05                             | -2 | 10        |
| Q164R   | 8.3 ± 0.8                                 | -7 | 8.5 ± 1.06                              | 13 | 1         |
| Q196K   | 19.4 ± 1.1                               | -3 | 2.4 ± 0.1                               | 4  | 8         |
| Q196R   | 12.3 ± 1.4                               | -4.5| 3.1 ± 0.4                               | 5  | 4         |

Figure 4. The proline loop of HsIPMK. A, electrostatic surface plot of the catalytic site of HsIPMK. Ins(1,4,5)P$_3$ and ADP are depicted as cyan sticks. Magnesium atoms are shown in magenta. B, the proline loop that is unique to HsIPMK (orange schematic) is shown in a structural superimposition of that protein with HsIP3K (pink, PDB code 1W2C), Entamoeba histolytica IP6K (cyan, PDB code 404F), and ScIPMK (green, PDB code 2IF8). ADP and Ins(1,4,5)P$_3$ are shown in stick and ball models (green stick for carbon, red for oxygen, and orange for phosphorus atoms). C, electrostatic surface plot of the catalytic site of HsIP3K. Ins(1,4,5)P$_3$ and ADP are shown in cyan sticks. D and E, electrostatic surface plots of the catalytic sites of AtIPMK and ScIPMK, respectively. These crystal complexes do not contain an inositol phosphate in the active site. ADP is shown in cyan sticks.

catalytic pocket in which a preponderance of Gln residues make contact with substrate. This contrasts with the description of the active site that emerged from the modeling of substrates into the active sites of AtIPMK (25) and ScIPMK (24). The latter two studies described a less-enclosed and conformationally-flexible substrate pocket in which mobile side chains of Lys and Arg residues play major roles in ligand binding (Fig. 5, D and E). Two such residues in particular, Lys$^{153}$ and Arg$^{156}$ in AtIPMK, were proposed to form key contacts with substrate (25). In a structural alignment (Fig. 5E), the latter two residues correspond to His$^{197}$ and Arg$^{200}$ in HsIPMK (Fig. 5E), but these do not play any role in substrate-bonding in our crystal structures (Fig. 5, A and E).

As noted above, HsIPMK phosphorylates Ins(1,4,5)P$_3$ to Ins(1,3,4,5)P$_4$ and then to Ins(1,3,4,5,6)P$_5$. We were unsuccessful in our efforts to soak Ins(1,3,4,5)P$_4$ into the crystals, so we modeled this particular substrate into the active site (Fig. 6, A and B). We found we could accomplish this, such that the 6-OH of Ins(1,3,4,5)P$_4$ is the group closest to the active site, by a 180° flip of the 3–6 axis of the inositol ring of Ins(1,3,4,5)P$_4$, relative to that of Ins(1,4,5)P$_3$ (Fig. 6, A and B). We therefore conclude that HsIPMK is unlikely to processively phosphorylate Ins(1,4,5)P$_3$ to Ins(1,3,4,5,6)P$_5$; instead, it appears that the Ins(1,3,4,5)P$_4$ intermediate must dissociate and rebind in a different orientation.

In our model (Fig. 6B), the 4- and 5-phosphates of Ins(1,3,4,5)P$_4$ spatially mimic the orientation of the 5- and 4-phosphates of Ins(1,4,5)P$_3$, respectively. This model (Fig. 6) further predicts that the 3-phosphate of Ins(1,3,4,5)P$_4$ could gain up to two polar contacts with Gln$^{163}$ and Lys$^{167}$. Nevertheless, the overall conclusion is that, compared with Ins(1,4,5)P$_3$, the Ins(1,3,4,5)P$_4$ makes fewer total interactions with the protein. For example, the 1-phosphate of Ins(1,3,4,5)P$_4$ only makes 2 contacts with Lys$^{167}$ and Gln$^{163}$, whereas the 1-phosphate of Ins(1,4,5)P$_3$ makes 4 contacts with the same two residues. Second, Gln$^{163}$ interacts with Ins(1,4,5)P$_3$ at the axial 2-OH, but this group in Ins(1,3,4,5)P$_4$ does not directly interact with the
These comparisons suggest that, compared with Ins(1,4,5)P₃, IMPK may have a lower binding affinity for Ins(1,3,4,5)P₄; this may be the reason that we were unable to soak Ins(1,3,4,5)P₄ into the active site (see above). Differences in binding affinity may also explain why the rate of Ins(1,3,4,5)P₄ phosphorylation is 90-fold slower than that for Ins(1,4,5)P₃ (Table 2).

We do not exclude the possibility that the orientations of the amino acid side chains might be affected by the nature of the bound substrate. Nevertheless, no such movements were necessary for us to model Ins(1,3,4,5)P₄ into the active site, compared with their positions in the Ins(1,4,5)P₃-bound crystal complex. This situation contrasts with the conclusion that emerged after substrates were modeled into the plant and yeast IPMKs (24, 25). In the latter studies, it was proposed that conformational flexibility was likely an important aspect to accommodating the different substrates within a relatively spacious binding pocket. In those particular IPMK orthologs, such flexibility could be provided by the relatively long and mobile side chains of Lys and Arg (24, 25). In contrast, in the case of HsIPMK, the smaller side chains of Gln have a larger role in substrate-binding.

### Mutagenesis of HsIPMK

Elements of the nucleotide-binding domain of HsIPMK are well-conserved within the IP-kinase family (Fig. 2D) (21). In contrast, our structural analysis has revealed unique features of the inositol phosphate-binding site, which presumably enforce its own particular set of catalytic activities. We interrogated these new findings using site-directed mutagenesis. We mu-
of the ring flip (Fig. 6C). Moreover, a gain of function of Ins(1,3,4,5)P₄ 6-kinase activity could result from this substrate’s 6-OH and 3-phosphate groups making contact with the Lys or Arg replacement (Fig. 6C). Second, the relative spatial position of Gln₁⁶⁴ would be perturbed by mutation of Gln₁⁶³, because of elimination of the stabilizing electrostatic connection between the two Gln residues (Fig. 5A). Third, the substitution of Arg or Lys for Gln₁⁹⁶ would sterically disturb the latter’s favorable interaction with the proximal 1-phosphate of Ins(1,4,5)P₃ (Fig. 5A); in the Ins(1,3,4,5)P₄ model, the 1-phosphate is rotated further away from Gln₁⁹⁶, but this gap could be bridged by multiple contacts with the larger Lys or Arg, thereby potentially contributing to a 6-kinase gain of function (Fig. 6C). These data provide a foundation for the generation and utilization of substrate-selective HsIPMK mutations for a synthetic biology approach to understanding each of the individual biological activities of this multifunctional enzyme.

**Structural rationalization of PtdIns(4,5)P₂ 3-kinase activity**

The determination of the position of the 1-phosphate of Ins(1,4,5)P₃ in the substrate pocket is of particular interest for understanding why PtdIns(4,5)P₂ is also a substrate for HsIPMK. This 1-phosphate, which is doubly ionized (21), makes contacts with both Lys₁⁶⁷ and Gln₁⁹⁶ (Fig. 5A). In this configuration, a single uncharged oxygen is exposed to the bulk phase; the esterification of this particular oxygen to a diacylglycerol backbone would not be expected to impose any steric hindrance to substrate binding. To pursue that idea, we next soaked a soluble dC₄-analogue of PtdIns(4,5)P₂ (along with nucleotide and magnesium) into the HsIPMK apoenzyme crystal; the structure of the enzyme co-complex revealed that the Ins(1,4,5)P₃ headgroup of the inositol lipid was oriented in a near-identical configuration to that of free Ins(1,4,5)P₃ (Fig. 7). Furthermore, three mutations that compromised Ins(1,4,5)P₃ 3-kinase activity, Q₁⁶₄A, K₁⁶₇A, and Q₁⁹₆A, had quantitatively similar effects upon PtdIns(4,5)P₃ 3-kinase activity (Table 2). These data indicate that there has been co-evolution of Ins(1,4,5)P₃ and PtdIns(4,5)P₃ 3-kinase activities.

No electron density was observed for the C₄-diacylglycerol moiety of the PtdIns(4,5)P₂ analogue, indicating that its mobility is not constrained upon binding to HsIPMK. This leaves the natural diacylglycerol backbone free to embed itself either into membranes, or the hydrophobic pockets of certain proteins (14). The position in the crystal complex of the Ins(1,4,5)P₃ headgroup of dC₄-PtdIns(4,5)P₂ clarifies that it can make the same contacts with the protein as Ins(1,4,5)P₃ itself, with just the one exception that the diester phosphate of the lipid at position 1 is held less tightly, because it only carries one negative charge, in contrast to the two in Ins(1,4,5)P₃ (30). Indeed, GroPIns(4,5)P₂ is a 5-fold weaker substrate than Ins(1,4,5)P₃ (Table 2).

**Concluding comments**

We have described several novel structural features of HsIPMK that clearly distinguish it from the orthologs in Arabidopsis and S. cerevisiae that predominantly phosphorylate the 6-hydroxyl of Ins(1,4,5)P₃ (24, 25). First, the horseshoe-shaped catalytic site in the human enzyme is more physically constraining. Second, HsIPMK hosts a smaller substrate-binding
Structure of human IPMK

Figure 7. Surface representation of HsIPMK crystal complexes with the Ins(1,4,5)P3 headgroup of diC4-PtdIns(4,5)P2 superimposed upon free Ins(1,4,5)P3. The figure describes the near-perfectly superimposed (r.m.s. deviation = 0.111 Å) positions of the Ins(1,4,5)P3 headgroup of diC4- PtdIns(4,5)P2 (yellow carbon stick) and Ins(1,4,5)P3 itself (green carbon stick). Phosphate groups are numbered. The likely position of the diester attachment is described with a black arrow.

Pocket in which Gln residues play major roles, unlike the plant and yeast orthologs that are more reliant on the longer and more flexible side chains of Arg and Lys. Third, the proline loop is a unique structural feature of the human enzyme that orients Arg82 and Gln78 into functionally-important positions within the catalytic pocket. Our results indicate that these are all adaptations that optimize Ins(1,4,5)P3 phosphorylation predominantly at the 3-position. Our crystal complex data also demonstrate that the Ins(1,4,5)P3 headgroup of PtdIns(3,4,5)P3 is near-perfectly superimposed upon free Ins(1,4,5)P3 itself (Fig. 7). Thus, we propose that 3-kinase positional specificity toward Ins(1,4,5)P3 has co-evolved along with the functional significance of PtdIns(3,4,5)P3 3-kinase activity of HsIPMK. Indeed, AtIPMK does not express PtdIns(4,5)P3 3-kinase activity of HsIPMK. Moreover, AtIPMK toward [3H]Ins(1,4,5)P3 (American Radiolabeled Chemicals). In these reactions, HsIPMK sequentially phos-
phorylates Ins(1,4,5)P$_3$ to Ins(1,3,4,5,6)P$_5$ (see “Results and Discussions” and Ref. 11). We circumvented the problem that $[^{3}H]$Ins(1,3,4,5)P$_4$ is not commercially available, by generating first-order rate constants for the two sequential reactions (38), from time-dependent changes in levels of Ins(1,4,5)P$_3$, Ins(1,3,4,5)P$_4$, and Ins(1,3,4,5,6)P$_5$. We assayed the PtdIns(4,5)P$_2$-3-kinase activity of HsIPMK by using soluble GroPtd$[^{3}H]$Ins(4,5)P$_2$ (prepared by deacylation (39) of Ptd$[^{3}H]$Ins(4,5)P$_2$ (American Radiolabeled Chemicals)).

Kinase assays were performed by incubating either wild-type HsIPMK or mutants with trace quantities of either $[^{3}H]$Ins(1,4,5)P$_3$ or GroPtd$[^{3}H]$Ins(4,5)P$_2$ at 37 °C in 100 μl of buffer containing 1 mM EDTA, 100 mM KCl, 20 mM HEPES, pH 7.2, 3 mM MgSO$_4$$_7$ 0, 5 mM Na$_2$ATP plus 50,000 dpm of $[^{32}P]$ATP (MP Biomedicals); prior to HPLC analysis, the diC$_8$-PtdIns(4,5)P$_3$ product was deacylated (39).

All assays were analyzed by ion-exchange HPLC, using an Adsorbosphere Q100 column. The elution gradient was Buffer B (Buffer A plus 2 M NH$_4$H$_2$PO$_4$, pH 3.9, with H$_3$PO$_4$) as follows: 0–5 min, 0% B; 5–10 min, 0–16% B, 10–60 min, 16–36% B, 60–61 min, 36–70% B. Radioactivity was measured either with an in-line counter using UltimaFlo AP (PerkinElmer Life Sciences), or by collecting 1-ml fractions, which were analyzed for dpm).

Acknowledgments—We thank Traci M. T. Hall and Chen Qiu for critical reading of the manuscript and the NIEHS Collaborative crystallography group, the Advanced Photon Source (APS) SE Regional Collaborative Access Team (SER-CAT) 22-ID and 22-BM beam lines for assistance with crystallographic data collection.

References

1. Shears, S. B. (2017) Intimate connections: inositol pyrophosphates at the interface of metabolic regulation and cell signaling. J. Cell Physiol. 10.1002/jcp.26017
2. Odom, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000) A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. Science 287, 2026–2029
3. Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., and Snyder, S. H. (1999) Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr. Biol. 9, 1323–1326
4. Resnick, A. C., Snowman, A. M., Kang, B. N., Hurt, K. J., Snyder, S. H., and Saiardi, A. (2005) Inositol polyphosphate multikinase is a nuclear PI3-kinase with transcriptional regulatory activity. Proc. Natl. Acad. Sci. U.S.A. 102, 12783–12788
5. Frederick, J. P., Mattiske, D., Wofford, J. A., Megosh, L. C., Drake, L. Y., Chiu, S. T., Hogan, B. L., and York, J. D. (2005) An essential role for an inositol polyphosphate multikinase, lpk2, in mouse embryogenesis and second messenger production. Proc. Natl. Acad. Sci. U.S.A. 102, 8454–8459
6. Leyman, A., Pouillon, V., Bostan, A., Schurmans, S., Emeux, C., and Pessen, X. (2007) The absence of expression of the three isoenzymes of the inositol 1,4,5-trisphosphate 3-kinase does not prevent the formation of inositol pentakisphosphatase and hexakisphosphate in mouse embryonic fibroblasts. Cell Signal. 19, 1497–1504
7. Fuji, M., and York, J. D. (2005) A role for rat inositol polyphosphate kinases, rlpk2 and rlpk1, in inositol pentakisphosphate and inositol hexakisphosphate production in Rat-1 cells. J. Biol. Chem. 280, 1156–1164
8. Barker, C. J., and Berggren, P.-O. (1999) Inositol hexakisphosphate and b-cell stimulus-scretion coupling. Anticancer Res. 19, 3737–3741
9. Gao, Y., and Wang, H. Y. (2007) Inositol pentakisphosphate mediates Wnt/β-catenin signaling. J. Biol. Chem. 282, 26490–26502
10. El Bakoury, M., Dibois, E., and Messenguy, F. (2000) Recruitment of the yeast MAD5-box proteins, ArgRI and Mcm1 by the pleitropic factor ArgRII is required for their stability. Mol. Microbiol. 35, 15–31
11. Nałaskowski, M. M., Deschermeier, C., Fanick, W., and Mayr, G. W. (2002) The human homologue of yeast ArgRII protein is an inositol phosphatase multikinase with predominantly nuclear localization. Biochem. J. 366, 549–556
12. Ramazzotti, G., Faenza, I., Fiume, R., Billi, A. M., Manzoli, L., Mongiorgi, S., Ratti, S., McCubrey, J. A., Suh, P. G., Cocco, L., and Follo, M. Y. (2017) PLCβ1 and cell differentiation: an insight into myogenesis and osteogenesis. Adv. Biol. Regul. 63, 1–5
13. Wickramasinghe, V. O., Savill, J. M., Chavali, S., Jonsdottir, A. B., Rajendra, E., Gruner, T., Laskey, R. A., Babu, M. M., and Venkitaraman, A. R. (2013) Human inositol polyphosphate multikinase regulates transcript-selective nuclear mRNA export to preserve genome integrity. Mol. Cell. 51, 737–750
14. Blind, R. D., Suzawa, M., and Ingraham, H. A. (2012) Direct modification and activation of a nuclear receptor-PIP2 complex by the inositol lipid kinase IPMK. Sci. Signal. 5, ra44
15. Kim, S., Kim, S. F., Maag, D., Maxwell, M. J., Resnick, A. C., Juluri, K. R., Chakraborty, A., Koldobskiy, M. A., Cha, S. H., Barrow, R., Snowman, A. M., and Snyder, S. H. (2011) Amino acid signaling to mTOR mediated by inositol polyphosphate multikinase. Mol. Biol. Cell. 22, 616–620
16. Xu, R., Sen, N., Paul, B. D., Snowman, A. M., Rao, F., Vaidiver, M. S., Xu, J., and Snyder, S. H. (2013) Inositol polyphosphate multikinase is a coactivator of p53-mediated transcription and cell death. Sci. Signal. 6, ra22
17. Bang, S., Kim, S., Dailey, M. J., Chen, Y., Morin, T. H., Snyder, S. H., and Kim, S. F. (2012) AMP-activated protein kinase is physiologically regulated by inositol polyphosphate multikinase. Proc. Natl. Acad. Sci. U.S.A. 109, 616–620
18. Sei, Y., Zhao, X., Forbes, J., Szymczak, S., Li, Q., Trivedi, A., Voellinger, M., Sei, Y., Zhao, X., Forbes, J., Szymczak, S., Li, Q., Trivedi, A., Voellinger, M.
19. Ahmed, I., Sbodio, J. I., Harraz, M. M., Tyagi, R., Grima, J. C., Albacarys, V., Venkatesan, A. M., Chandrasekharappa, S. C., and Venkatesan, A. M., Chandrasekharappa, S. C., and Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., and Snyder, S. H. (1999) Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr. Biol. 9, 1323–1326
20. Yokoyama, J. S., Wang, Y., Schork, A. J., Thompson, W. K., Karch, C. M., Cruchaga, C., McEvoy, L. K., Witoelar, A., Chen, C. H., Holland, D., Brewer, J. B., Franke, A., Dillon, W. P., Wilson, D. M., Mukherjee, P., et al.

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J. Biol. Chem. (2017) 292(44) 18192–18202 18201

Author contributions—H. W. performed all of the experiments and analyzed the results. H. W. conceived and designed the experiments. H. W. and S. B. S. wrote the paper.

Acknowledgments—We thank Traci M. T. Hall and Chen Qiu for critical reading of the manuscript and the NIEHS Collaborative crystallography group, the Advanced Photon Source (APS) SE Regional Collaborative Access Team (SER-CAT) 22-ID and 22-BM beam lines for assistance with crystallographic data collection.
Association between genetic traits for immune-mediated diseases and Alzheimer disease. *JAMA Neurol.* 73, 691–697

21. González, B., Schell, M. J., Letcher, A. J., Veprintsev, D. B., Irvine, R. F., and Williams, R. L. (2004) Structure of a human inositol 1,4,5-trisphosphate 3-kinase; substrate binding reveals why it is not a phosphoinositide 3-kinase. *Mol. Cell* 15, 689–701

22. Miller, G. J., and Hurley, J. H. (2004) Crystal structure of the catalytic core of inositol 1,4,5-trisphosphate 3-kinase. *Mol. Cell* 15, 703–711

23. Wang, H., DeRose, E. F., London, R. E., and Shears, S. B. (2014) IP6K structure and the molecular determinants of catalytic specificity in an inositol phosphate kinase family. *Nat. Commun.* 5, 4178

24. Holmes, W., and Jogl, G. (2006) Crystal structure of inositol phosphate multikinase 2 and implications for substrate specificity. *J. Biol. Chem.* 281, 38109–38116

25. Endo-Streeter, S., Tsui, M. K., Odom, A. R., Block, J., and York, J. D. (2012) Structural studies and protein engineering of inositol phosphate multikinase. *J. Biol. Chem.* 287, 35360–35369

26. Meyer, R., Nalaskowski, M. M., Ehm, P., Schröder, C., Naj, X., Brehm, M. A., and Mayr, G. W. (2012) Nucleocytoplasmic shuttling of human inositol phosphate multikinase is influenced by CK2 phosphorylation. *Biol. Chem.* 393, 149–160

27. Madhusudan, Akamine, P., Xuong, N. H., and Taylor, S. S. (2002) Crystal structure of a transition state mimic of the catalytic subunit of cAMP-dependent protein kinase. *Nat. Struct. Biol.* 9, 273–277

28. Stout, T. J., Foster, P. G., and Matthews, D. J. (2004) High-throughput structural biology in drug discovery: protein kinases. *Curr. Pharm. Des.* 10, 1069–1082

29. Cheek, S., Ginalska, K., Zhang, H., and Grishin, N. V. (2005) A comprehensive update of the sequence and structure classification of kinases. *BMC Struct. Biol.* 5, 6

30. Kooijman, E. E., King, K. E., Gangoda, M., and Gericke, A. (2009) Ionization properties of phosphatidylinositol polyphosphates in mixed model membranes. *Biochemistry* 48, 9360–9371

31. Maag, D., Maxwell, M. J., Hardesty, D. A., Boucher, K. L., Choudhari, N., Hanno, A. G., Ma, J. F., Snowman, A. S., Pietropaoli, J. W., Xu, R., Storm, P. B., Saiardi, A., Snyder, S. H., and Resnick, A. C. (2011) Inositol polyphosphate multikinase is a physiologic PI3-kinase that activates Akt/PI3K. *Proc. Natl. Acad. Sci. U.S.A.* 108, 1391–1396

32. Rodríguez-Escudero, I., Roelants, F. M., Thorner, J., Nombela, C., Molina, M., and Cid, V. J. (2005) Reconstitution of the mammalian PI3K/PTEN/Akt pathway in yeast. *Biochem. J.* 390, 613–623

33. Kim, E., Beon, J., Lee, S., Park, J., and Kim, S. (2016) IPMK: A versatile regulator of nuclear signaling events. *Adv. Biol. Regul.* 61, 25–32

34. Otwinowski, Z., and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* 276, 307–326

35. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2126–2132

36. Winn, M. D., Murshudov, G. N., and Papiz, M. Z. (2003) Macromolecular TLS refinement in REFMAC at moderate resolutions. *Methods Enzymol.* 374, 300–321

37. Stevenson-Paulik, J., Chiou, S. T., Frederick, J. P., dela Cruz, J., Seeds, A. M., Otto, J. C., and York, J. D. (2006) Inositol phosphate metabolomics: merging genetic perturbation with modernized radiolabeling methods. *Methods 39*, 112–121

38. Davies, D. D. (1961) Intermediary Metabolism in Plants, Cambridge University Press, Cambridge, United Kingdom

39. Shears, S. B. (1997) Measurement of inositol phosphate turnover in intact cells and cell-free systems. In Signalling by Inositides: a Practical Approach (Shears, S. B., ed) pp. 33–52, Oxford University Press, Oxford, UK

40. Gu, C., Nguyen, H. N., Hofer, A., Jessen, H. J., Dai, X., Wang, H., and Shears, S. B. (2017) The significance of the bifunctional kinase/phosphatase activities of PPIP5Ks for coupling inositol pyrophosphate cell-signaling to cellular phosphate homeostasis. *J. Biol. Chem.* 292, 4544–4555

41. Craxton, A., Erneux, C., and Shears, S. B. (1994) Inositol 1,4,5,6-tetrakisphosphate is phosphorylated in rat liver by a 3-kinase that is distinct from inositol 1,4,5-trisphosphate 3-kinase. *J. Biol. Chem.* 269, 4337–4342