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IP6K structure and the molecular determinants of catalytic specificity in an inositol phosphate kinase family

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Inositol trisphosphate kinases (IP3Ks) and inositol hexakisphosphate kinases (IP6Ks) each regulate specialized signalling activities by phosphorylating either InsP₃ or InsP₆ respectively. The molecular basis for these different kinase activities can be illuminated by a structural description of IP6K. Here we describe the crystal structure of an *Entamoeba histolytica* hybrid IP6K/IP3K, an enzymatic parallel to a 'living fossil'. Through molecular modelling and mutagenesis, we extrapolated our findings to human IP6K2, which retains vestigial IP3K activity. Two structural elements, an α -helical pair and a rare, two-turn 3_{10} helix, together forge a substrate-binding pocket with an open clamshell geometry. InsP₆ forms substantial contacts with both structural elements. Relative to InsP₆, enzyme-bound InsP₃ rotates 55° closer to the α -helices, which provide most of the protein's interactions with InsP₃. These data reveal the molecular determinants of IP6K activity, and suggest an unusual evolutionary trajectory for a primordial kinase that could have favored efficient bifunctionality, before propagation of separate IP3Ks and IP6Ks.

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Phosphate is a universal device for imposing specificity in cell signalling. The phosphate group's very bulk establishes geometric constraints on ligand–protein and protein–protein interactions. In addition, the phosphate's negative charge at physiological pH bestows specificity through ionic and hydrogen bonding to only certain amino-acid residues. Signalling specificity is further enhanced through interactions that utilize multiple phosphates, such as those that are placed in various combinations around the six carbon inositol ring. Each of the resulting molecules—inositol phosphates (InsPs)—have a distinct three-dimensional phosphate pattern that can encode unique signalling properties. Within this family of signalling molecules, there is a subgroup that possess functionally significant, 'high-energy' diphosphate groups: the inositol pyrophosphates (also known as diphosphoinositol polyphosphates)^{1,2}. These molecules, particularly InsP₇ and InsP₈, are now viewed as being of fundamental importance in all eukaryotes². Inositol pyrophosphates play pivotal roles in several aspects of cellular and organismic metabolic homeostasis^{1,3–7}, and they regulate interferon transcription in response to viral infection⁸. Indeed, cell signalling by the inositol pyrophosphates is considered to be among the most evolutionarily ancient functions of the entire inositol phosphate family⁹. Thus, there is considerable interest in the kinases that synthesize InsP₇ and InsP₈.

For example, mammals express three isoforms of inositol hexakisphosphate kinases (IP6Ks)^{10,11} that each add a second phosphate group to a preexisting phosphate at the 5-position of the inositol ring. These kinases thereby convert InsP₆ to 5-PP-Ins(1,2,3,4,6)P₅ (5-InsP₇)^{12,13}. The type 1 and type 2 isoforms (IP6K1 and IP6K2, respectively) have been the subject of a number of genetic studies with startling phenotypes. Silencing of IP6K1 in pancreatic β -cells inhibits insulin secretion⁴. Gene disruption of murine IP6K1 increases the insulin sensitivity of target tissues¹⁴, reduces spermatogenesis¹⁴ and compromises haemostasis¹⁵. Furthermore, in MEFs prepared from IP6K1^{−/−} mice, DNA repair by homologous recombination is impaired¹⁶, the epigenetic program is altered¹⁷ and mitochondria are defective³. A reduction in IP6K2 expression through an antisense strategy reduces apoptosis¹⁸, while IP6K2^{−/−} mice show increased susceptibility to carcinogen-induced squamous cell carcinoma¹⁹. As IP6Ks have so many important functions, the determination of their structures is a key research goal: such information illuminates catalytic mechanisms, regulatory processes and evolutionary relationships with other kinases, while also offering templates for the rational design of selective inhibitors as research tools or for therapeutic applications. However, IP6Ks have been stubbornly resistant to structural analysis for many years. Our goal has been to fill this significant void in our structural and molecular understanding, so as to further our insight into the entire inositol phosphate kinase family.

Entamoeba histolytica express an intriguing IP6K homologue (Genbank: XP_648490.2) that, secondary to its InsP₆ kinase activity, also phosphorylates the 6-OH of Ins(1,4,5)P₃ (ref. 20)—an inositol phosphate multikinase (IPMK)-like activity^{10,21,22}. IPMK itself is positionally promiscuous in that it is a 3-, 5- and 6-kinase²³. Another nonspecific inositol phosphate kinase is ITPK1, which phosphorylates the 1-, 5- and 6-positions around the inositol ring²⁴. Within the active sites of IPMK and ITPK1, the plane of the inositol rings in all of the alternate substrates are proposed to occupy the same spatial orientation, enabling each substrate to interact with a common set of protein contacts^{25–28}. However, there has not previously been any direct structural confirmation of that hypothesis, because the published crystal structures of IPMK^{26,28} and ITPK1^{27,29} lack bound substrate. We therefore considered that structural analysis of the IP6K from

E. histolytica could generate new ideas concerning specificity determinants of the positional promiscuity of inositol phosphate kinases.

IP6Ks are members of a wider inositol phosphate kinase family (Pfam PF03770) that includes IPMKs and inositol trisphosphate kinases (IP3Ks); these enzymes all share a PxxxDxKxG ('PDKG') catalytic motif^{10,21}. Phylogenetic analysis⁹ has led to the hypothesis that this kinase family arose from a primordial IP6K precursor. However, such an evolutionary pathway would be highly unusual, at least according to current thinking³⁰. The issue of concern is that the original substrate (InsP₆) for the putative progenitor kinase is both larger and substantially more polar than the substrates for the descendant kinases: the InsP₃ and InsP₄ that are phosphorylated by IPMKs^{26,28} and the InsP₃ that is specifically phosphorylated at the 3-OH by IP3Ks^{31,32}. The traditional viewpoint is that improvements in the efficiency of catalysis of the smaller substrates (InsP₃/InsP₄) would evolve through the compression of the active site³⁰. However, that would generally be expected to reduce activity against the larger InsP₆ molecule—a 'negative trade-off'—that would impede organismic fitness and thereby select against the emergence of the independent InsP₃/InsP₄ kinase activities³⁰. Such restrictions would seem inevitable if all substrates interacted with common structural elements. Nevertheless, the significant InsP₃ kinase activity of *E. histolytica* IP6K²⁰ suggests it has somewhat overcome the constraints of negative trade-off; structural analysis could reveal how this was accomplished. We now describe several crystal complexes of the IP6K from *E. histolytica*, including those that contain either large (Ins(1,3,4,5,6)P₅/InsP₆) or small (Ins(1,4,5)P₃) substrates. Furthermore, we extrapolate our findings to mammalian IP6Ks by site-directed mutagenesis of the human IP6K2. We describe two structural elements, an α -helical pair and a rare, two-turn 3₁₀ helix, that together form a substrate-binding pocket with an open clamshell geometry. InsP₆ has substantial contacts with both structural elements. Relative to InsP₆, enzyme-bound InsP₃ rotates 55° closer to the α -helices, which provide most of the protein's interactions with InsP₃. These data provide insight into the molecular determinants of both IP3Ks and IP6Ks, and suggest an unusual evolutionary trajectory for propagation of these separate kinases.

Results

Ins(1,4,5)P₃, InsP₅ and InsP₆ phosphorylation by EhIP6KA. In our study, a recently characterized IP6K expressed by *E. histolytica*²⁰ (Genbank: XP_648490.2) is referred to as EhIP6KA, in order to distinguish it from two other gene products of *E. histolytica* that are currently also annotated as IP6Ks in Genbank. High-performance liquid chromatography (HPLC) of the InsP₇ synthesized by EhIP6KA (solid line, Fig. 1a) found it to co-elute with the 5-InsP₇ produced by HsIP6K2 (ref. 12) (dotted line, Fig. 1a). A previous study²⁰ also concluded from HPLC that EhIP6KA synthesizes 5-InsP₇. Nevertheless, current HPLC methodology alone does not completely resolve all possible InsP₇ isomers^{33,34}. Furthermore, there have been reports that amoeba contain atypical isomers of inositol pyrophosphates³⁵. Considering that positional specificity is such a central feature of our study (see the Introduction), we employed nuclear magnetic resonance (NMR) for definitive characterization of the InsP₇ product^{12,13}.

To prepare sufficient quantities of pure InsP₇ for NMR analysis, we adopted a strategy that has proved useful in the past¹², namely, to analyse reaction mixtures (Fig. 1b). The ¹H chemical shifts of the unreacted InsP₆ and the product InsP₇ were assigned by comparison with reported InsP₆ chemical shifts¹³ and from an analysis of the 2D ¹H–¹H COSY spectrum of the reaction mixture (Supplementary Table 1). The proton resonances from

Figure 1 | HPLC and NMR analysis of the products of Ins(1,4,5)P₃ and InsP₆ phosphorylation by EhIP6KA. (a) HPLC analysis (Partisphere SAX, Gradient 1; see Methods) of InsP₆ phosphorylation by either EhIP6KA (57 ng, 20 min; solid line) or HsIP6K2 (7.6 ng, 20 min; dotted line). (b) NMR analysis of the products of the reaction between InsP₆ and ATP catalysed by EhIP6KA; the figure depicts an overlay of the 1D ¹H and 2D ¹H/³¹P HSQC spectra. The acquisition parameters are given under Methods. (c) HPLC analysis (Partisphere SAX, Gradient 2; see Methods) of the phosphorylation of ~9,000 d.p.m. [¹⁴C]Ins(1,3,4,5,6)P₅ by EhIP6KA (6 μg, 20 min, 50 μl; filled circles). Also shown are the elution of [³H]-labelled internal standards of PP-InsP₄ and [PP]₂-InsP₃, as well as InsP₆ (open circles). (d,e) depict representative HPLC assays (90 min; 200 μl) that contained ~10,000 d.p.m. [³H]Ins(1,4,5)P₃ plus 2 μg EhIP6KA. After quenching and neutralization, products (filled circles) were analysed by Q100 SAX HPLC (Gradient 3); d includes the elution positions of standards of [³H]Ins(1,2,4,5)P₄ and [³H]InsP₆ (open circles), determined in parallel HPLC runs; e zooms in on the InsP₄ region of the chromatograph; elution of internal [¹⁴C] standards of Ins(1,3,4,5)P₄ and Ins(1,4,5,6)P₄ are also shown (open circles).

InsP₆ and InsP₇ are labelled in the 1D ¹H spectrum (Fig. 1b). The integral from the InsP₆ protons in the 4/6 positions is approximately three times larger than the corresponding integral arising from the PPInsP₅ product, indicating that about 25% of the InsP₆ was phosphorylated. To verify the assignments and differentiate the InsP₆ and InsP₇ resonances, the 1D ¹H and ³¹P spectra were reacquired after spiking the sample with an additional 100 μl of 5 mM InsP₆. The major difference between the InsP₆ and InsP₇ in the new ¹H spectrum is that the resonance from H-5 is shifted to higher chemical shift and no longer overlaps the H-1,3 resonances as it does in InsP₆ (see also Supplementary Table 1). The remainder of the ¹H resonances exhibit less dramatic changes relative to the corresponding resonances in InsP₆. The H-1,3 and H-4,6 resonances of InsP₇ are equivalent due to the symmetry of molecule. These data are consistent with the site of phosphorylation being at the 5-position. With the ¹H chemical shift assignments in hand, the ³¹P chemical shifts of InsP₇ were assigned from a combined analysis of 2D ¹H-³¹P HSQC and ³¹P-³¹P COSY spectra (Supplementary Table 1). The HSQC spectrum correlates the ¹H shifts at each site to the ³¹P shifts of the phosphate on the same carbon, and the COSY spectrum correlates the ³¹P shifts of the two ³¹P nuclei in the diphosphate moiety. The H-2, H-1,3 and H-4,6 proton chemical shifts correlate to ³¹P chemical shifts from the monophosphate groups in InsP₇, and the H-5 proton shift

correlates to the ³¹P chemical shift of the α-phosphate of the only diphosphate group (Fig. 1b), so we conclude that the site of phosphorylation is the 5-position. Note the two other α-phosphate resonances arise from the ADP product and from unreacted ATP (Fig. 1b). The β-phosphate chemical shift of InsP₇ is assigned from its correlation to the α-phosphate chemical shift in the ³¹P-³¹P COSY spectrum. Thus, our NMR data reinforce the conclusion reached by HPLC that EhIP6KA, like mammalian IP6Ks, converts InsP₆ to 5-InsP₇.

Mammalian IP6Ks also phosphorylate Ins(1,3,4,5,6)P₅, yielding PP-InsP₄^{12,36}. We found that EhIP6KA likewise phosphorylates Ins(1,3,4,5,6)P₅, and the major product co-elutes with a PP-[³H]InsP₄ standard^{12,36}; no InsP₆ was formed (Fig. 1c). A small amount of [PP]₂-InsP₃^{12,36} also accumulated (Fig. 1c). The first order rate constant for Ins(1,3,4,5,6)P₅ phosphorylation (mean ± s.e.; 0.15 ± 0.006 s⁻¹ mg⁻¹, n = 3) is 40-fold lower than that for InsP₆ (6.1 ± 0.64 s⁻¹ mg⁻¹, n = 3). The rate of phosphorylation of Ins(1,4,5)P₃, another EhIP6K substrate²⁰, was 0.028 ± 0.005 s⁻¹ mg⁻¹ (n = 3), which is 220-fold slower than that for InsP₆. HPLC indicates that Ins(1,4,5,6)P₄ is the major InsP₄ product formed from Ins(1,4,5)P₃ (Fig. 1d,e)²⁰. We also observed the accumulation of an additional InsP₄ isomer that co-elutes with a standard of Ins(1,2,4,5)P₄ (Fig. 1d). The phosphorylation of an inositol phosphate at the 2-position has only previously been observed for IP5K^{26,37}. We also confirmed

that there were no pyrophosphorylated molecules associated with the InsP_4 products, because these were not dephosphorylated upon their incubation with DIPPI (Supplementary Fig. 1), an inositol pyrophosphate phosphatase that removes the β -phosphate from the diphosphate group^{36,38}.

Overall structure of *EhIP6KA*. The structure of *EhIP6KA* (Fig. 2a–c; Table 1) was determined through two approaches. First, the structure of the fusion protein MBP-*EhIP6KA* (apo-enzyme; residues 32–270, space group $P2_12_12_1$) was determined by a molecular replacement approach using MBP (1ez9) and IP3K (1w2c) as search models. That information was then used for further molecular replacement in order to solve the structures of crystal complexes that bound ATP plus either $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5,6)\text{P}_5$ or InsP_6 (residues 27–270, space group $I4_122$), and a crystal of the apo-enzyme (residues 20–270, space group

$P2_1$). In the latter, the amino-terminal residues 20–29 were disordered.

For each asymmetric unit, our data are consistent with one molecule of MBP-*EhIP6KA* in space group $P2_12_12_1$, one molecule of substrate-bound *EhIP6KA* in space group $I4_122$ and two molecules of apo-*EhIP6KA* in space group $P2_1$. The latter crystal structure was analysed with the protein interfaces, surfaces and assemblies service (<https://www.ebi.ac.uk/pdbe/pisa/>), which estimated an interface area of $814.7 \text{ \AA}^2$ and total gained free energy upon interface formation of $-10.8 \text{ kcal mol}^{-1}$. It was also assigned a complexation significance score of zero, indicating that the apo structure is a monomer. Furthermore, gel filtration indicates that apo-*EhIP6KA* is a monomer in solution.

Analysis of the overall fold of *EhIP6KA* (Fig. 2a,b) offers a description of some architectural elements that are shared with IPMKs and IP3Ks, while also revealing some molecular differences that may contribute to individual catalytic preferences.

Table 1 | Data collection and structure refinement statistics.

PDB accession IDs	4O4B	4O4C	4O4D	4O4E	4O4F
<i>EhIP6KA</i> residues	MBP-32–270	20–270	27–270	27–270	27–270
Ligand	Apo	Apo	ATP-Ins(1,4,5)P ₃	ATP-Ins(1,3,4,5,6)P ₅	ATP-InsP ₆
Data collection					
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁	I4 ₁ 22	I4 ₁ 22	I4 ₁ 22
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å), β (°)	117.63, 51.98, 117.33	43.42, 128.37, 52.53, $\beta = 114.3^\circ$	102.74, 102.74, 111.74	103.00, 103.00, 110.98	102.23, 102.23, 109.49
Resolution (Å)*	50–1.8 (1.83)	50–1.9 (1.93)	50–2.1 (2.14)	50–1.9 (1.93)	50–1.7 (1.73)
R _{sym} *	0.057 (0.457)	0.078 (0.235)	0.086 (0.551)	0.049 (0.527)	0.053 (0.540)
<i>I</i> / σ <i>I</i> *	39.6 (3.1)	25.0 (3.6)	29.1 (3.4)	42.9 (2.6)	56.7 (2.4)
Completeness (%)*	99.4 (99.4)	98.6 (93.3)	99.4 (100.0)	99.3 (93.9)	96.4 (71.6)
Redundancy*	5.0 (4.8)	3.7 (3.0)	6.7 (6.2)	8.1 (6.3)	9.8 (5.2)
Refinement					
Resolution (Å)*	1.8 (1.84)	2.2 (2.27)	2.1 (2.16)	1.9 (1.95)	1.7 (1.74)
No. reflections	64,104	26,476	16,743	22,265	29,589
<i>R</i> _{work} *	19.6 (27.7)	22.9 (24.3)	17.3 (20.2)	18.3 (25.4)	19.1 (28.4)
<i>R</i> _{free} *	22.1 (34.1)	28.6 (28.2)	22.7 (25.2)	21.9 (29.5)	22.2 (36.2)
No. atoms					
Protein	5,010	4,039	2,080	2,084	2,075
Ligand/ion			57	65	69
Solvent	654	328	198	200	200
B-factors (Å²)					
Protein	16.8	19.8	30.1	23.8	23.7
Ligand/ion			52.6	36.9	39.3
Solvent	47.1	29.7	46.3	41.5	42.1
R.m.s. deviations					
Bond length (Å)	0.006	0.007	0.012	0.011	0.01
Bond angle (°)	1.10	1.18	1.71	1.73	1.83

*The numbers in parentheses are for the highest resolution shell.

Among conserved elements, we identified domains in *EhIP6KA* that are similar to the so-called N- and C-domains that comprise the ATP-binding sites in both IPMK^{26,28} and *HsIP3KA*^{31,32}. First, an N-terminal lobe in *EhIP6KA* can be designated from residues 28–91, which consist of three antiparallel β -strands; the β 1 and β 2 strands are connected by two short helices α 1 and α 2. Second, we can designate a C-terminal lobe (residues 92–102 and 132–270), which is an $\alpha + \beta$ fold with five, central antiparallel β -strands including β 4–6, as well as β 8 and β 9 (connected by a 3-residue 3_{10} helix), a pair of small antiparallel β -strands (β 7 and β 10), and three α -helices (α 5– α 7). Also in the C-lobe of *EhIP6KA*, between the β 6 strand and the α 5 helix, is an unusually long (6-residue), two-turn 3_{10} helix (3_{10A} ; Fig. 2a) that is not present in either IPMK^{26,28} or *HsIP3KA*^{31,32}. A 3_{10} helix of this length occurs relatively rarely because, being thinner and more tightly wound than classical α -helices, it would usually be too unstable^{39,40}. However, as we describe below, the highly specialized nature and orientation of the amino-acid side chains in this 3_{10} helix not only stabilize this structural element, but also make it functionally integral to substrate binding by *EhIP6KA*.

Description of the nucleotide-binding region of *EhIP6KA*. We observed that ATP is sandwiched between the N- and C-lobes of *EhIP6KA* (Fig. 2a); nucleotide binding did not alter protein conformation. Within a conserved IDF tripeptide (Fig. 2c) is Asp231, which coordinates with two Mg ions and makes polar contacts with the nucleotide phosphates (Fig. 3a,b). The orientation of the nucleotide in *EhIP6KA* is superimposable upon its position in the other kinases that share the PDKG motif, except for the more flexible γ -phosphate of ATP (Fig. 3c; Supplementary

Fig. 2), which may contribute to the unusually low affinity of IP6Ks for this nucleotide⁴¹. The adenosine moiety of ATP is docked in a hydrophobic pocket in *EhIP6KA* that is surrounded by residues Leu36, Pro65, Met85, Asn87, Met89 from the N-lobe and Leu211 and Ile230 from the C-lobe (Figs 3a and 4a). The N¹ and N⁶ atoms of adenine are hydrogen bonded with the amide nitrogen and carbonyl oxygen of Leu88 and Glu86 respectively, which anchors the adenosine in the hydrophobic pocket (Figs 3a and 4a). The ATP-ribose group is loosely confined by several Van der Waals contacts with Val97, Asp99, Leu211 and Ile230. Lys38 forms two hydrogen bonds with oxygen atoms from the α - and β -phosphates (Figs 3a and 4a).

The molecular determinants of inositol phosphate binding. We have obtained crystal structures of *EhIP6KA* in complex with three different substrates: Ins(1,4,5)P₃, Ins(1,3,4,5,6)P₅ and InsP₆ (Figs 4a,b and 5a–d, Supplementary Fig. 3). Simulated annealing omit-maps and 2Fo – Fc maps show clear density of the substrate's inositol ring; the individual phosphate groups and their specific positions can each be identified (Supplementary Fig. 3).

The binding site for InsP₆, which lies in a shallow depression in the enzyme's surface, can be likened to an 'open clamshell'. One jaw is represented by the IP helices, the other jaw comprises the 3_{10A} helix; note that neither *HsIP3KA* nor IPMK possess an equivalent to the 3_{10A} helix (Fig. 5e). The IP helices and the 3_{10} helix are not directly connected, but they approach within a sufficient proximity of each other to provide a metaphorical 'hinge' to the clamshell. Here, the 1- and 6-phosphates of InsP₆ make contacts with Lys115, Lys118 and Arg119 in the α -3/ α -4 helical pair (Figs 4b and 5d). Nevertheless, the plane of the

Figure 3 | Nucleotide binding by *EhIP6KA*. (a) ATP is depicted as a stick and ball model. Two Mg atoms are depicted as magenta spheres. Polar contacts are shown with dashed lines. Amino acids are shown as stick models. (b) Metal coordination. Two Mg atoms are depicted as magenta spheres. Water molecules are depicted as red spheres. The structure of ATP and Asp231 is shown as stick models. Polar contacts to coordinate with Mg atoms are shown with dashed lines. (c) The orientation of the *EhIP6KA*-bound nucleotide (green for carbon, red for oxygen, blue for nitrogen and orange for phosphorus atoms) and Mg atoms (magenta spheres) are superimposed upon that for *HsIP3KA* (grey stick represent AMPNP (ATP analogue); grey spheres represent Mg), and *ScIPMK* (light blue stick and spheres represent ADP and Mg).

Figure 4 | Ligplots showing interactions of *EhIP6KA* with ATP and InsPs. Ligplots are shown for (a), ATP and Ins(1,4,5)P₃ and (b), InsP₆. Hydrogen bonds are shown in green dashed lines; bond distances are denoted in Angstroms. Residues that make hydrophobic interactions are depicted as grey eyelashes (cutoff distance is 3.9 Å). Atoms are shown white for carbon, red for oxygen, blue for nitrogen and orange for phosphorus.

Figure 5 | Inositol phosphate-binding site for *EhIP6KA*. (a) Binding of $\text{Ins}(1,4,5)\text{P}_3$ (stick and ball model; green for carbon, red for oxygen and orange for phosphorus atoms). The phosphate groups are numbered. The refined $2F_o - F_c$ electron density map is contoured at 1.3σ . (b) Overlay of InsP_6 (grey stick model with orange and red phosphate groups) and $\text{Ins}(1,4,5)\text{P}_3$ (green stick). (c) Overlay of InsP_6 and $\text{Ins}(1,3,4,5,6)\text{P}_5$ (cyan stick). (d) Binding of InsP_6 (stick and ball model; green for carbon, red for oxygen and orange for phosphorus atoms). The phosphate groups are numbered. Amino acids are shown as stick models. The refined $2F_o - F_c$ electron density map is contoured at 1.3σ . (e), Electrostatic surface plot with blue and red coloration to respectively indicate positive and negative electrostatic potentials at physiological pH. In the magnified surface representation, the positions of the 3_{10} helix and IP helices are highlighted. Bound InsP_6 is depicted as a stick model. Also shown are surface representation of $\text{Ins}(1,4,5)\text{P}_3$ in the active site of *HslP3K* (data from ref. 32) and the active site of *ScIPMK* (data from ref. 26; crystals did not contain substrate).

inositol ring of InsP_6 runs parallel to the contour of the 3_{10}A helix (Fig. 5e), enabling the 4- and 6-phosphates to interact with Arg152 and Tyr153 (Figs 4b and 5d). These contacts appear to be stabilized by cation- π interactions between Arg152 and Tyr153, which in turn are facilitated by the specific architecture of the 3_{10} helix (Supplementary Fig. 4).

$\text{Ins}(1,3,4,5,6)\text{P}_5$ is also a substrate for *EhIP6K* (Fig. 1c,d). Indeed, $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 occupy a similar orientation within the active site (Fig. 5c), indicating how both can be phosphorylated at the same position. IP6Ks can also phosphorylate 1- InsP_7 (ref. 13). Assuming that InsP_6 and 1- InsP_7 also occupy similar orientations within the active site, molecular modelling indicates that the latter's β -phosphate projects into the bulk phase and should not greatly influence substrate binding.

While it is the 5-phosphate group on InsP_6 that is diphosphorylated by *EhIP6KA* (Fig. 1b), it is either the 2- or the 6-OH of $\text{Ins}(1,4,5)\text{P}_3$ that is phosphorylated (Fig. 1d,e). Positional promiscuity in other inositol phosphate kinases has been rationalized by proposing alternative substrate-binding modes, in which the plane of the inositol ring occupies the same spatial orientation relative to the active site, enabling each substrate to share the same protein-contact points^{25–28}. This is not the case for *EhIP6KA*. Instead, a unique adaptation of *EhIP6KA* lies in the nature of its contacts with $\text{Ins}(1,4,5)\text{P}_3$ that tilt the plane of the inositol ring by 55° relative to that of InsP_6 (Fig. 5b). Thus, near the clamshell hinge, the 5-phosphate of $\text{Ins}(1,4,5)\text{P}_3$ somewhat overlaps the space that would be occupied by the 1-phosphate of InsP_6 (Fig. 5b). However, at the open end of the clamshell, the 1-phosphate of $\text{Ins}(1,4,5)\text{P}_3$ is positioned $7.5 \text{ \AA}$ away from the

location of the 3-phosphate of InsP_6 (Fig. 5b). In fact, steric restrictions prevent InsP_6 from occupying the same space as $\text{Ins}(1,4,5)\text{P}_3$ in the substrate-binding pocket.

The 3_{10} helix makes only limited contact with $\text{Ins}(1,4,5)\text{P}_3$; the 4-phosphate has just one hydrogen bond with Arg152 and there are weak Van der Waals interactions with Tyr153 (Figs 4a,5a). Instead, the plane of the inositol ring in $\text{Ins}(1,4,5)\text{P}_3$ is tilted closer to the IP helices, which are what this substrate mainly interacts with. Lys118 and Arg119 make contacts with the 4- and 5-phosphates of $\text{Ins}(1,4,5)\text{P}_3$ (Figs 4a and 5a). The 1-phosphate, 6-hydroxyl and 5-phosphate of $\text{Ins}(1,4,5)\text{P}_3$ also have Van der Waals interactions with Lys115 (Fig. 4a). The 2-OH of $\text{Ins}(1,4,5)\text{P}_3$ is positioned $3.7 \text{ \AA}$ from the γ -phosphate of ATP (Supplementary Fig. 5), which is close enough to permit an in-line phosphoryl-transfer reaction⁴². Indeed, our HPLC analysis revealed $\text{Ins}(1,2,4,5)\text{P}_4$ to be a product of $\text{Ins}(1,4,5)\text{P}_3$ phosphorylation (Fig. 1d,e).

Furthermore, we remodelled the orientation of the inositol ring in enzyme-bound $\text{Ins}(1,4,5)\text{P}_3$, by flipping it 180° across its $1/4$ axis, whereupon the 6-OH of $\text{Ins}(1,4,5)\text{P}_3$ was then positioned $2.9 \text{ \AA}$ from the γ -phosphate of ATP (Supplementary Fig. 5). This rationalizes the 6-kinase activity against $\text{Ins}(1,4,5)\text{P}_3$ by *EhIP6KA* (Fig. 1d,e). Thus, $\text{Ins}(1,4,5)\text{P}_3$ can be held in the active site of *EhIP6KA* in two alternative, productive orientations, yielding either $\text{Ins}(1,2,4,5)\text{P}_4$ or $\text{Ins}(1,4,5,6)\text{P}_4$ as products.

Another interesting observation is that, within the ligand-binding pocket of *EhIP6KA*, the orientation of the plane of the inositol ring of $\text{Ins}(1,4,5)\text{P}_3$ shows a remarkable degree of spatial conservation with that for *HslP3K* (Fig. 5a,e, Supplementary

Fig. 5);³² one inositol ring is simply flipped 180° across its 2,3/5,6 axis relative to the other so that, in *HsIP3KA*, the 3-OH that is phosphorylated lies 4.6 Å from the γ -phosphate of ATP (Supplementary Fig. 5), which is within the distance that permits an in-line, phospho-transfer reaction⁴². Thus, our data suggest that *EhIP6KA* be considered an enzymatic parallel to a 'living fossil' that illuminates the potential nature of a primordial $\text{InsP}_6/\text{InsP}_3$ kinase. In this putative protein, the open clamshell topology of the active site provides a partial diversification of specificity determinants for $\text{Ins}(1,4,5)\text{P}_3$ and InsP_6 , prior to its propagation into separate enzymes that could specialize in either of the two catalytic activities. We used mutagenesis to pursue this idea (see below).

Molecular modelling and mutagenesis of human IP6K2. So as to consolidate our structural work with *EhIP6KA*, and also to extrapolate it to mammalian IP6Ks, we turned to *HsIP6K2* as a model for mutagenic studies. Interestingly, we found that *HsIP6K2* harbours a vestigial $\text{Ins}(1,4,5)\text{P}_3$ kinase activity, some five orders of magnitude weaker than the InsP_6 activity (Fig. 6a). The products of $\text{Ins}(1,4,5)\text{P}_3$ phosphorylation are $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5,6)\text{P}_4$ (Fig. 6b). Although the $\text{Ins}(1,4,5)\text{P}_3$ kinase activity was relatively low, it was sufficient to permit us to pursue the specificity determinants for each substrate.

For a positive control in the mutagenic experiments, we prepared a Lys222Ala mutant of *HsIP6K2*. This residue (equivalent to Lys101 in *EhIP6KA*; Figs 2c, 4b and 5a,d), lies within the conserved PxxxDxKxG catalytic center (Fig. 2c). A *HsIP6K2* construct in which this residue was mutated to Ala has previously been used as a kinase-impaired control when studying the effects of overexpression of wild-type enzyme in intact cells⁴³. Nevertheless, to our knowledge, the degree to which this

mutation inhibits recombinant enzyme activity has not previously been published. We found that the K222A mutant of *HsIP6K2* exhibits negligible catalytic activity (Fig. 6a).

Our structural analysis of *EhIP6KA* indicates that Lys115 in the IP helices of *EhIP6KA* has multiple interactions with both substrates (Figs 4 and 5). We mutated the corresponding Lys236 in *HsIP6K2* (Fig. 2c) to Ala; catalytic activity against both substrates was strongly reduced (Fig. 6a). Lys118 and Arg119 are two other residues in the IP helices of *EhIP6KA* that make contact with both substrates (Figs 4 and 5). These residues are not precisely replicated in *HsIP6K2*, according to both our sequence alignments (Fig. 2c) and our molecular model of the mammalian kinase (Supplementary Fig. 6A). Instead, our modelling indicates that Lys243 in *HsIP6K2* has functionally significant interactions with the 6-phosphate of InsP_6 (Supplementary Fig. 6A). Indeed, an Lys243Ala mutation in *HsIP6K2* decreased InsP_6 kinase activity 600-fold (Fig. 6a) without affecting $\text{Ins}(1,4,5)\text{P}_3$ phosphorylation. These data speak to the specialization of the InsP_6 kinase activity of *HsIP6K2*.

We next used mutagenesis to test our hypothesis drawn from structural data that the 3_{10}A helix in IP6Ks is particularly important for binding InsP_6 . Arg152 and Tyr153 in the 3_{10}A helix of *EhIP6KA* align with Lys274 and Tyr275 in the equivalent region of *HsIP6K2* (Fig. 2c). We found that Lys274Ala and Tyr275Ala mutants of *HsIP6K2* exhibited a decrease in InsP_6 kinase activities of 55- and 8-fold, respectively; the fact that these two mutations did not affect $\text{Ins}(1,4,5)\text{P}_3$ kinase activities (Fig. 6a) confirms our conclusion that they play only a minor role in binding $\text{Ins}(1,4,5)\text{P}_3$. Interestingly, the 3_{10}A helix of *HsIP6K2* contains an Arg residue at position 278 for which there is no equivalent in *EhIP6KA* (Fig. 2c). Molecular modelling of *HsIP6K2* suggests Arg278 might contribute positive charge to the substrate-binding pocket, and/or stabilize the 3_{10} helix

Figure 6 | HPLC analysis of the kinase activities of wild-type and mutant IP6Ks. (a) InsP_6 kinase (black bars) and $\text{Ins}(1,4,5)\text{P}_3$ kinase (grey bars) activities of wild-type *HsIP6K2* and the indicated mutants (means $\pm$ s.e., $n \geq 3$). (b) Shows representative HPLC analysis (Partisphere SAX; Gradient 2) of $\text{Ins}(1,4,5)\text{P}_3$ kinase activities of wild-type *HsIP6K2* (8 μg , 7 h). The inset to b shows HPLC analysis (Q100 SAX; Gradient 3) of the InsP_4 products formed by the wild-type enzyme (closed circles) together with the elution of internal standards of [^{14}C]- $\text{Ins}(1,3,4,5)\text{P}_4$ and [^{14}C]- $\text{Ins}(1,4,5,6)\text{P}_4$ (open circles). (c) Shows a representative HPLC analysis (Partisphere SAX; Gradient 2) of $\text{Ins}(1,4,5)\text{P}_3$ kinase activity of a Cys257Glu mutant of *HsIP6K2* (5 μg , 1 h). (d) Shows a representative HPLC analysis (Q100 SAX; Gradient 3) of the InsP_4 products formed by a Cys257Glu mutant of *HsIP6K2* (5 μg , 45 min). (e) Shows a representative HPLC analysis of $\text{Ins}(1,4,5)\text{P}_3$ kinase activity of a Gln240Met mutant of *HsIP6K2* (11 μg , 7 h). WT, wild type.

through cation- π interactions with Tyr274 (Supplementary Fig. 6B). Consistent with these ideas, an Arg278Ala mutant of HsIP6K2 exhibited a ninefold reduction in InsP₆ kinase activity compared with wild-type enzyme (Fig. 6a). Interestingly, the Arg278Ala mutant also showed a dramatic increase in Ins(1,4,5)P₃ kinase activity (Fig. 6a). It is possible Arg278 has a negative impact on Ins(1,4,5)P₃ phosphorylation by attracting it into a nonproductive orientation.

The electronegative Glu333 in the C-lobe of HsIP3KA³² (Supplementary Fig. 6C) is replaced by Ser136 in EhIP6KA and Cys257 in HsIP6K2 (Fig. 2c). Structural modelling of this region of HsIP6K2 (Supplementary Fig. 6B,C) indicates a Cys257Glu replacement in HsIP6K2 would cause electrostatic repulsion of InsP₆ but not Ins(1,4,5)P₃. Indeed, we prepared a Cys257Glu mutant of HsIP6K2 and it showed 14-fold lower InsP₆ kinase activity than wild-type enzyme (Fig. 6a). Moreover, this mutant showed a 33-fold increase in the rate of phosphorylation of Ins(1,4,5)P₃ (Fig. 6a), specifically at the 3-OH group (Fig. 6c,d). These results indicate the importance of Glu333 in the evolution of catalytic specificity of HsIP3KA.

Finally, a hydrophobic patch in the IP helices in HsIP3KA and ScIPMK (Tyr315/Met316 in HsIP3KA, Met151 in ScIPMK; Fig. 2c), against which the inositol ring is proposed to rest, is hypothesized to enhance Ins(1,4,5)P₃ binding³². This hydrophobic patch is missing from both EhIP6KA and HsIP6K2 (Fig. 2c). We introduced HsIP3KA-like hydrophobic residues into the corresponding region of the IP helices of HsIP6K2 by preparing a single Gln240Met mutant and a tandem Asn239Tyr/Gln240Met mutant. These two mutant enzymes both exhibited a 50-fold increase in the rate of Ins(1,4,5)P₃ phosphorylation (Fig. 6a). Moreover, the variety of products that were formed was significantly increased (compare Fig. 6b and e), perhaps by favoring multiple ligand-binding modes. Arg242Ala is another mutation that increases the hydrophobicity of the IP helices; this elicited a threefold increase in Ins(1,4,5)P₃ phosphorylation (Fig. 6a). Significantly, InsP₆ kinase activity of HsIP6K2 was not substantially affected by these mutations to Asn239, Gln240 or Arg242 (Fig. 6a). These mutagenic data confirm the structural information that indicate InsP₆ and Ins(1,4,5)P₃ have non-overlapping orientations in the substrate-binding site. InsP₆ cannot interact with the synthetic hydrophobic patch since the plane of its inositol ring is tilted away from the plane of the IP helices, unlike that of Ins(1,4,5)P₃ (Fig. 5b,e). These mutagenic experiments also offer evolutionary insight by providing proof-of-principle of the nature of mutations to a progenitor kinase that could have increased Ins(1,4,5)P₃ kinase activity without negative trade-off from a compromised InsP₆ kinase activity.

Discussion

In this study, we describe the crystal structure of a bifunctional IP6K/IP3K from *E. histolytica* (EhIP6KA), and we extrapolate our findings to a human IP6K with mutagenic and biochemical experiments. The most important new ideas that we draw from this information are as follows: first, the geometry of the substrate-binding site is revealed to exhibit a relatively spacious, open clamshell geometry; one jaw is represented by a pair of α -helices, the other jaw is an unusually long 3₁₀ helix. Second, we show that InsP₃ and InsP₆ have non-overlapping orientations within the substrate pocket. Thus, relatively large InsPs (Ins(1,3,4,5,6)P₅/InsP₆) form contacts with both the α -helices and the 3₁₀ helix. In contrast, the smaller InsP₃ molecule is rotated 55° closer to the α -helices; the latter provide most of the protein's interactions with InsP₃. Third, we describe an unusual evolutionary trajectory that could have favored efficient

bifunctionality in a primordial kinase, prior to propagation of separate, dedicated IP3Ks and IP6Ks.

Previous ideas concerning how specialized enzymes diverged from promiscuous progenitors are based on the paradigm that the native catalytic activity should generally involve a substrate that is smaller than the emerging substrate, without a change in its degree of polarity³⁰. For the putative primordial IP6K⁹, the original, preferred substrate (InsP₆) would have been both larger and substantially more polar than the InsP₃ substrates for the emerging kinases (IPMK and IP3K). Usually, the evolution of increased catalytic activity against smaller substrates depends upon compression of the active site³⁰. That development would be expected to reduce activity against the larger InsP₆ molecule—a 'negative trade-off'—that would impede organismic fitness and thereby select against the emergence of the InsP₃/InsP₄ kinase activities³⁰. However, our substrate-bound structures of EhIP6KA uncover a partial separation of the binding sites for Ins(1,4,5)P₃ and InsP₆. It is because of these non-overlapping substrate orientations that the insertion of a hydrophobic patch into the α -helices can enhance IP3K activity without affecting IP6K activity: an avoidance of negative trade-off that would have favored efficient bifunctionality. It appears that *E. histolytica* may be a particularly appropriate model for gaining insight into evolution of a hybrid IP6K/IP3K, since current annotations of this organism's genome indicate that it does not encode a specialized Ins(1,4,5)P₃ 3-kinase. Thus, we conclude that EhIP6KA is the enzymatic equivalent of a 'living fossil' that is suggestive of the nature of a primordial IP6K prior to gene duplication. After the latter event had occurred, it would appear that evolutionary gain of function of Ins(1,4,5)P₃ phosphorylation would then have involved atrophy of the 3₁₀ helix and consolidation of the role of the IP helices in substrate binding (Fig. 5e). In HsIP3KA, the IP helices have been replaced with a distinct, highly structured 63-residue inositol-phosphate-binding domain: four helices with greater structural constraints that limit substrate selectivity to Ins(1,4,5)P₃ alone^{31,32}. The 3₁₀ helix has also been lost from IPMK (Fig. 5e), but the substrate-binding pocket remains relatively spacious, thereby retaining a degree of positional promiscuity.

Each of the inositol phosphate kinases that share the PDKG motif—IP6Ks, IPMKs and IP3Ks—are specialized to synthesize unique InsPs with differing biological actions. Our molecular understanding of these catalytic specializations has been incomplete, since among these proteins, only HsIP3KA has previously been co-crystallized with substrate³². In contrast, the published crystal structures of IPMK^{26,28} lacked bound substrate. Thus, our atomic level description of a substrate-bound IP6K fills a significant void in our structural and molecular understanding of the determinants of catalytic activity in the entire PDKG motif kinase family. This information is also relevant to IP5K—previously thought to be the only inositol phosphate kinase capable of phosphorylating the 2-OH. Nevertheless, in crystals of substrate-bound IP5K, the orientation of the axial 2-OH of Ins(1,3,4,5,6)P₅, relative to ATP, is very similar to the orientation of the equatorial 3-OH of Ins(1,4,5)P₃, relative to ATP, in crystals of HsIP3KA⁴⁴. Furthermore, the current study shows that the inositol ring of Ins(1,4,5)P₃ occupies very similar relative positions in the active sites of EhIP6KA and HsIP3KA (Fig. 5; Supplementary Fig. 5). This spatial conservation of substrate binding between these different inositol phosphate kinases is also matched by spatial conservation of some key active-site residues⁴⁵. Such observations help to rationalize our observation that EhIP6KA phosphorylates Ins(1,4,5)P₃ at the 2-position (Fig. 1d,e; Supplementary Fig. 5). Finally, our IP6K structure provides the missing template necessary for the rational design of inhibitors that might selectively target each of the

different kinases that contain the PDKG motif, both for research tools and therapeutic applications.

Methods

Protein expression and purification. In this study, we use *EhIP6KA* as an abbreviation for the *E. histolytica* IP6K with Genbank accession number XP_648490.2, so as to distinguish it from two other gene products of that are also annotated as IP6Ks. A previous study²⁰ named this enzyme as *EhIPK1*. A codon-optimized cDNA for expression in *Escherichia coli* of *EhIP6KA* was synthesized by Genscript 5'-ATGAACACGAAAATCAAACGCGAACACATGAGTACGAAGAA AAGCCAAATGGGTCCAAGCAGGTGGTCCGTCGCAACAACTGCAT CCGGATGGCCAGTACCTGCTGAAACCGTGCCTGTCCCATCGTGAACGCG ACTTTTATCTGCACATTAAAGATGACAAGGAATGGACCGGCACGGGTA TTATCCCGAAATCTACGGCGTGCAGACTGCACGAATTTGGCTTCGGTGA ACTGGAATTTATCCGTATGAAAACCTGATGTACAAATACAAGCGCC CGTTCGTTCTGGATCTGAAAATCGGCACCCAAACGTGGGACCCGGAA ACCGCGAGCAGCAAAATGAAAAAGCGTCTGGTGGTTGATTCGACCAGCA CCACGACCTCTCTGGCGTGGCTTTTATGGTATGGAACGCAATATTG GCGAAGAAAACCGGATCCTGTATTTCGGCTTACCTGTGTACCCATGAAG TTAACACGCGGATAGCCTGAAGGAATACATCAAGCTGTTTTCAACGA CGGTAAAAAGTACCGTAAGGAATGGTTCCTGACTTTATTAGCCAGCT GGATAAAATGATCGAAGTCATGAAAAAGCGCGAATATAAGATGTTT AGTTCCTCAGTCTGTTCGTGTACGATTCTACGACCACGTGGAAGACA AGAAGTACAACGTCAAGATGATCGATTTCGCCACAATTGGATTCTGTG CAGAAGAAGATGTACCGTGAAGATGGCTTTCTGTTCCGTCTGAACA ATCTGAAATCCATTCTGGAAGACATCGAAAACGAATTAAGTCACT GTAA-3'.

The Gateway Expression System (Invitrogen) was used to subclone *EhIP6KA* into the pDest-566 vector. This vector encodes a 6 × His tag, a maltose-binding protein tag and TEV protease cleavage site at the N terminus. ArcticExpress (DE3)-competent cells (Stratagene) were transformed with the resultant plasmid. An overnight culture of *E. coli* cells carrying the pDest-566-*EhIP6KA* was inoculated into 2 × yeast extract tryptone medium at pH 7.5 and grown at 37 °C to $A_{595} = 0.7$. Isopropyl β-D-thiogalactopyranoside (0.1 mM) was then added and cultures were continued at 15 °C for 1 day. The cells were disrupted (Constant Systems) at 20 kpsi. Recombinant *EhIP6KA* proteins were purified with a Ni-NTA agarose column (Qiagen) followed by a HiTrap Q FF column (GE Healthcare). The bound proteins were released by TEV protease in the presence of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1.5 mM DTT, 10 mM ATP, 20 mM MgCl₂, then applied to a HiTrap Q FF column. In the case of the MBP fusion protein, this TEV cleavage step was omitted. As a final step, a Superdex 200 gel filtration column (GE Healthcare) was used with a running buffer of 50 mM NaCl and 20 mM Tris-HCl, pH 7.5.

Human IP6K2 cDNA was ordered from American Type Culture Collection (I.M.A.G.E. Clone ID 6162048, ATCC number 10437631). The Gateway expression system (Invitrogen) was used to subclone the kinase into the pDest-566 vector. Lemo21(DE3)-competent *E. coli* cells (New England Biolabs) were transformed with the resultant plasmid. An overnight culture of *E. coli* cells carrying the pDest-566-*HsIP6K2* was inoculated into 2 × yeast extract tryptone medium supplemented with 0.6 mM L-rhamnose at pH 7.5 and grown at 37 °C to $A_{595} = 0.7$. Isopropyl β-D-thiogalactopyranoside (0.1 mM) was then added and cultures were continued at 14 °C for 2 days. The cells were disrupted using a constant cell disruption system (Constant Systems) under 20 kpsi. Recombinant *HsIP6K2* was purified with a Ni-NTA agarose column (Qiagen) followed by a HiTrap Heparin HP column (GE Healthcare), TEV protease cleavage and a HiTrap Q FF column (GE Healthcare). As a final step, a Superdex 200 gel filtration column (GE Healthcare) was used with a running buffer of 150 mM NaCl and 20 mM Tris-HCl, pH 7.5. Mutant proteins were purified similarly. For site-directed mutagenesis, the 5' version of the complementary primers was as follows (mutagenic nucleotides are emphasized):

K222A, 5'-GGTGCCTTGTGTCCTTGACCTCGCGATGGGCACACGA CAACATGGTG-3'
K236A, 5'-CAACATGGTGATGATGCTTCAGAGGAGCGCGCAGCCAAC CAGATCCGAAAATG-3'
Q240M, 5'-CTTCAGAGGAGAAGGCAGCCAACATGATCCGAAAAAT GTCAGCAGAGCAGATC-3'
N239YQ240M, 5'-GCTTCAGAGGAGAAGGCAGCCTACATGATCCGAAA ATGTCAGCAGAGCAGATC-3'
R242A, 5'-GGAGAAGGCAGCCAACCCAGATCGCAAAATGTCAGC AGAGCAGCATCTGCAG-3'
K243A, 5'-GAAGGCAGCCAACCCAGATCCGAGCATGTCAGCAGAGCA CATCTGCAGTC-3'
K273A, 5'-GTGGGCAGCTCATGTTTCATGAACGCGTACCATGGAC GGAAGCTATCGG-3'
Y274A, 5'-GGGCAGCTCATGTTTCATGAACAAGGCCCATGG ACGGAAGCTATCGGTG-3'
C257E, 5'-GTCATTGGTGTGCGTGTGGAGGGCATGCAGGTGTACCA AG-3'
R278A, 5'-GTTTCATGAACAAGTACCATGGAGCGAAGCTATCGGTGC AG-3'

Recombinant DIPPI was expressed and purified as previously described⁴⁶ with an additional, heparin-affinity purification step. The purity of all proteins was estimated to be > 95% as judged by SDS-polyacrylamide gel electrophoresis. The purified proteins were concentrated and stored at -80 °C.

Sample preparation for NMR analysis. Full length *EhIP6KA* (1–3 mg) was incubated in 3 ml medium containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.0), 2 mM MgCl₂, 3 mM ATP and 3 mM InsP₆ at 37 °C for 1 day and then 25 °C for 3 days. The reaction sample was centrifuged and filtered to remove protein, and then vacuum dried at 45 °C for 30 min.

The sample was prepared by taking up the reaction mixture in D₂O to a volume of 600 μl and adjusting the pH to 6.2 using NaOH and HCl. In addition, sodium CDTA was added to a concentration of 20 mM to alleviate broadening of the ³¹P resonances due to chemical exchange of the phosphate groups with the high concentration of Mg²⁺ in the sample. All ¹H and ³¹P chemical shifts were referenced to ~5 μM internal DSS or to external 85% phosphoric acid, respectively. All NMR experiments were carried out at 25 °C using a Varian Inova 600 MHz NMR spectrometers, equipped with either a 5 mm broad band NMR probe tuned to ³¹P on the observe channel and ¹H on the decoupler channel, or a Varian 5 mm ¹H{¹⁵N,¹³C} Cold Probe (Agilent, Santa Clara CA). The 1D ¹H spectra were acquired with sweep widths of 12 p.p.m., with an acquisition time of 1 s, using a 1-s relaxation delay and with presaturation of the residual water resonance during the relaxation delay. The 1D ³¹P spectrum was obtained using a 50 p.p.m. sweep width, with an acquisition time of 1.6 s, using a 1-s relaxation delay and with ¹H decoupling during acquisition. The 2D ¹H-³¹P HSQC spectrum was acquired with sweep widths of 12 p.p.m. and 25 p.p.m. in the ¹H and ³¹P dimensions, respectively, with 142.3 ms and 42 ms acquisition times in these dimensions and with ¹H decoupling during the acquisition. The 1/4J delay in the HSQC experiment was set to 31.25 ms, corresponding to a ¹H-³¹P scalar coupling of 8 Hz. The ¹H-¹H and ³¹P-³¹P COSY spectra were acquired using gradient-selected absolute value mode COSY experiments. The proton COSY experiment was acquired using 12 p.p.m. sweep widths in both dimensions, with acquisition times of 342 ms and 71 ms in the directly and indirectly detected dimensions, respectively, and with a 1-s relaxation delay, during which time, presaturation of the residual water signal was applied. The phosphorus COSY experiment was acquired using 25 p.p.m. sweep widths in both dimensions, with acquisition times of 512 ms and 32 ms in the directly and indirectly detected dimensions, respectively, and with a 1-s relaxation delay. The ³¹P-³¹P COSY spectrum was also acquired with ¹H decoupling in both dimensions. All spectra were processed using Varian Vnmrj 2.2D software (Agilent) on the Varian Inova spectrometer.

Enzyme assays and HPLC analysis. Kinase assays were performed by incubating enzyme preparations with either [³H]Ins(1,4,5)P₃ (American Radiolabeled Chemicals, Saint Louis, MO), [¹⁴C]-Ins(1,3,4,5,6)P₅ (isolated from [¹⁴C]-labelled parotid acinar cells⁴⁷) or [³H]InsP₆ (Perkin-Elmer Waltham MA) at 37 °C in buffer containing 150 mM KCl, 20 mM HEPES pH 7.0, 12 mM MgSO₄, 10 mM ATP, 1 mM EDTA. After either 20 min (InsP₆ phosphorylation) or 0.5–7 h (Ins(1,4,5)P₃ phosphorylation) reactions were acid quenched and neutralized⁴⁸, and analysed by HPLC using either a 4.6 × 125 mm Partisphere 5 μm SAX column or a Q100 SAX column. The Partisphere column was eluted with one of two ammonium phosphate elution protocols: Gradient 1 (ref. 48) or Gradient 2 (ref. 49). The Q100 column was eluted with a gradient generated from Buffer A (1 mM Na₂EDTA) and Buffer B (Buffer A plus 2 M NH₄H₂PO₄ pH 3.9 with H₃PO₄) in the following manner: 0–5 min, 0% B; 5–100 min, 0–42% B, 110–140 min, 42–70% B (Gradient 3). Radioactivity was measured either with an in-line counter, or after collecting 1 ml or 0.5 ml fractions. Other radiolabelled InsPs used for HPLC standards included [³H]Ins(1,2,4,5)P₄ (prepared by heating [³H]Ins(1,3,4,5)P₄ in 0.5 ml 1 M HCl at 90 °C for 20 min⁵⁰), [¹⁴C]-Ins(1,3,4,5)P₄ and [¹⁴C]-Ins(1,4,5,6)P₄ (isolated from carbachol-stimulated, [¹⁴C]-labelled parotid acinar cells⁴⁷), PP-[³H]InsP₄ and [PP]₂-[³H]InsP₃ (both prepared by hIP6K2-mediated phosphorylation of [³H]-Ins(1,3,4,5,6)P₅³⁶). Data for the kinase assays are presented as mean values ± s.e.

Crystallization. The MBP fusion protein of *EhIP6KA* (residues 32–270) was crystallized by hanging drop vapour diffusion against a well buffer of 12% (w/v) PEG 3350, 50 mM NaH₂PO₄, pH 5.5 at 18 °C. The protein concentration was > 60 mg ml⁻¹. The core catalytic domain of *EhIP6KA* (residues 20–270) was crystallized by hanging drop vapour diffusion for 1 week against a well buffer of 8% (w/v) PEG 3350, 100 mM Na₃Citrate, pH 5.2 at 25 °C, followed by dilution microseeding (Seed Bead kit, Hampton Research) for 2 weeks with a well buffer of 8% (w/v) PEG 3,350, 100 mM Na₃Citrate, pH 5.2, 8% ethylene glycol at 25 °C. The crystals were displayed as a thin plate and were anisotropic. Crystals of *EhIP6KA* (residues 27–270) emerged under 0.4 M NaH₂PO₄ in the presence of 10 mM ATP, plus either 10 mM InsP₆, 5 mM Ins(1,3,4,5,6)P₅ or 5 mM Ins(1,4,5)P₃/20 mM MgCl₂. To increase occupancy of InsPs and remove the effect of phosphate ions, the crystals were further soaked under 22% (w/v) PEG 3350, 10 mM MgCl₂, 10 mM ATP, 0.1 M sodium acetate, pH 5.2 and 20 mM InsP₆, 10 mM Ins(1,3,4,5,6)P₅ or 10 mM Ins(1,4,5)P₃ for 3 days. Cryosolvent was prepared by adding 33% ethylene glycol into the soaking buffer.

Data collection and structure determination. Diffraction data were collected using APS beamlines 22-ID and 22-BM. All data were processed with the programme HKL2000 (ref. 51). Initial phases for the structure of MBP-EhIPK1 were determined by molecular replacement using MBP (1e29) and HsIP3KA (1w2c) as searching models. Then the first structure was manually rebuilt with COOT⁵² and refined with REFMAC from the CCP4 package⁵³. The other crystal structures were determined either by molecular replacement if the space group was different, or by using rigid body and direct Fourier synthesis, and refined with the equivalent and expanded test sets. A Cartesian-simulated annealing protocol was performed in the first round of refinement. Ligand topology and parameter files were prepared using the incorporated PRODRG programme from the CCP4 package⁵³. The molecular graphics representations were prepared with the programme PyMol (Schrödinger, LLC).

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Author contributions

H.W., E.F.D. and S.B.S. performed experiments. All authors designed experiments and wrote the paper.

Additional information

Accession codes: Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession codes: 4O4B, *EhIP6KA* (residues 32–270) tagged with maltose-binding protein at the N terminus; 4O4C, apo-*EhIP6KA* (residues 20–270); 4O4D, *EhIP6KA* (residues 27–270) in complex with ATP and Ins(1,4,5)P₃; 4O4E, *EhIP6KA* (residues 27–270) in complex with ATP and Ins(1,3,4,5,6)P₅; 4O4F, *EhIP6KA* (residues 27–270) in complex with ATP and InsP₆.

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