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The Significance of the 1-kinase / 1-phosphatase Activities of the PPIP5K Family

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Abstract

The inositol pyrophosphates (diphosphoinositol polyphosphates), which include 1-InsP₇, 5-InsP₇, and InsP₈, are highly ‘energetic’ signalling molecules that play important roles in many cellular processes, particularly with regards to phosphate and bioenergetic homeostasis. Two classes of kinases synthesize the PP-InsPs: IP6Ks and PPIP5Ks. The significance of the IP6Ks - and their 5-InsP₇ product - has been widely reported. However, relatively little is known about the biological significance of the PPIP5Ks. The purpose of this review is to provide an update on developments in our understanding of key features of the PPIP5Ks, which we believe strengthens the hypothesis that their catalytic activities serve important cellular functions. Central to this discussion is the recent discovery that the PPIP5K is a rare example of a single protein that catalyses a kinase/ phosphatase futile cycle.

Introduction

Inositol pyrophosphates (PP-InsPs; Fig 1) are a specialized class of cell signaling molecules that boast a unique, functionally significant feature: the most crowded three-dimensional arrangement of phosphate groups that has been observed throughout Nature. Crammed around a six-carbon inositol scaffold are as many as seven or eight phosphates (for “InsP₇” and “InsP₈” respectively, see Fig 1), including diphosphate groups that are highly ‘energetic’ (Hand and Honek 2007; Shears 2015).

Yeasts and metazoan cells can phosphorylate InsP₆ to InsP₈ through two parallel pathways (Fig 1), which utilize two separate classes of enzymes to form diphosphate groups: the

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5-kinases (the IP6Ks (E.C.2.7.4.21); Draskovic et al. 2008; Saiardi et al. 1999) and the 1-kinases (the PPIP5Ks (E.C. 2.7.4.24); Lin et al. 2009; Wang et al. 2012). Consequently, cells contain two InsP₇ isomers, which are distinguished by whether the diphosphate is attached to either the 5- or 1-position on the inositol ring; InsP₈ possesses both of these diphosphates (Fig 1). The IP6Ks can also add a diphosphate group to Ins(1,3,4,5,6)P₅ (Draskovic et al. 2008; Saiardi et al. 2000), but with less positional specificity, since both the 1- and 5-positions can become diphosphorylated (Draskovic et al. 2008). These particular PP-InsPs are not well studied, and lie outside the main focus of this review which concerns the catalytic activities of the PPIP5Ks. A family of phosphatases - the DIPPs (E.C. 3.6.1.52) (Safrany et al. 1998) - hydrolyze both the 1- and 5-diphosphate groups.

In most studies that have analyzed cellular PP-InsP levels, the HPLC methodology that was used was unable to resolve 1-InsP₇ from 5-InsP₇, and so total InsP₇ has been recorded. Its levels in mammalian cells have generally been reported to lie in the 1 – 2 μM range (see (Ingram, Safrany, and Barnes 2003; Wilson, Livermore, and Saiardi 2013)). As for InsP₈, its concentration is typically around 10% that of total InsP₇.

To gain insight into the relative contributions from the two InsP₇ isomers, TNP (*N*2-(*m*-trifluoromethyl)benzyl) *N*6-(*p*-nitrobenzyl)purine), a cell permeant pan-IP6K inhibitor, was added to intact cells to attenuate 5-InsP₇ synthesis (Padmanabhan et al. 2009). This inhibitor reduced the size of the InsP₇ peak by around 90% (Padmanabhan et al. 2009). Thus, it was concluded that 1-InsP₇ accounts for no more than 10% of total InsP₇. A more direct approach -the introduction of an HPLC technique which can resolve the two InsP₇ isomers - has further reduced estimates of 1-InsP₇ levels to less than 2% of total InsP₇, at least in HCT116 cells (Gu et al. 2016).

The fact that 5-InsP₇ is the most abundant of the PP-InsPs has translated into its preeminence in the PP-InsP literature. Thus, 5-InsP₇ has been reported to regulate multiple, diverse biological processes: vesicle trafficking (Saiardi et al. 2000; Saiardi et al. 2002), apoptosis (Morrison et al. 2001; Nagata et al. 2005), autophagy (Nagata et al. 2010), repair of DNA repair by homologous recombination (Jadav et al. 2013; Luo et al. 2002), transcription of glycolytic genes (Szijgyarto et al. 2011), hemostasis (Ghosh et al. 2013), phagocytic and bactericidal activities of neutrophils (Prasad et al. 2011), craniofacial morphogenesis (Sarmah and Wenthe 2010), epigenetic modifications to chromatin (Burton et al. 2013), resistance to peroxide stress (Onnebo and Saiardi 2009), cell adhesion (Rao et al. 2015), dynein-driven vesicle transport (Chanduri et al. 2016) and exocytic insulin secretion (Illies et al. 2007). This extensive list of publications serves to underscore how little we understand concerning the specific significance of 1-InsP₇ and InsP₈. Against this backdrop, the purpose of this review is to draw attention to some important features of the PPIP5Ks, so as to make the case that their control over cellular levels of PP-InsPs must be functionally significant.

Structural Characterization of the PPIP5K kinase domain.

PPIP5Ks are distributed throughout eukaryotic kingdoms; the orthologs have various aliases: Asp1 in *Schizosaccharomyces pombe*, Vip1 in *Saccharomyces cerevisiae*, VIH in *Arabidopsis thaliana*. Yeasts and plants diverged from the animal kingdom 1.3 and 1.5

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billion years ago, respectively (see www.timetree.org), so it is apparent that PPIP5Ks - and hence 1-InsP₇ / InsP₈ - are evolutionarily ancient. Two *PPIP5K* genes are expressed in both mammals and plants (Choi et al. 2007; Fridy et al. 2007; Laha et al. 2015; Mulugu et al. 2007). Both the type 1 and 2 forms of mammalian PPIP5Ks are relatively large proteins - approximately 160 and 140 kDa respectively; under some circumstances, they may even oligomerize into even larger complexes (Choi et al. 2007). The kinase domain is self-contained at the N-terminal one-third of these proteins (Fridy et al. 2007; Mulugu et al. 2007; Pohlmann et al. 2014; Wang et al. 2012). This certainly facilitated the expression and preparation of the kinase domain of human PPIP5K2 to the concentration and purity required to solve its structure (Wang et al. 2012).

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This structural analysis was driven by the need to obtain an atomic level rationalization of some specialized features - for example, catalytic specificity: PPIP5Ks exhibit negligible activity towards 1-InsP₇, Ins(1,3,4,5,6)P₅ and 5-PP-Ins(1,3,4,6)P₄ (Wang et al. 2012) which, among the naturally-occurring inositol phosphates, are those that are the most similar to 5-InsP₇ and InsP₆. Another catalytic accomplishment is that the active site accommodates sterically hindered and intensely electronegative substrates - and yet adds to those ligands another bulky and negatively charged phosphate group. Furthermore, the substrates are metabolized with high affinity: initial kinetic analyses determined that the K_m values for InsP₆ and 5-InsP₇ are both in the 0.1 to 0.3 μM range (Choi et al. 2007; Fridy et al. 2007). Subsequent work derived an even lower K_m value for 5-InsP₇ (0.06 μM), perhaps in part reflecting technical improvements in the procedures for preparation of pure PP-InsPs (Weaver, Wang, and Shears 2013).

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Our structural data derived from the PPIP5K2 kinase domain were obtained at a resolution of 1.8 Angstroms. The conclusions that we drew likely also apply to human PPIP5K1, since the two kinase domains share 84% sequence identity, and residues that are dissimilar are confined to the protein surface, distal to the active site (Wang et al. 2012). Our results established that ligand specificity is in part enforced by the geometry of the substrate-binding pocket, which comprises two near-parallel grooves that form a staggered “H”-shape. This architecture makes a perfectly-tailored aperture for accommodating six phosphate/pyrophosphate groups attached to an inositol ring in its chair conformation (Wang et al. 2012). Specificity within the catalytic pocket is additionally imposed by an array of Lys and Arg residues that make polar contacts with every phosphate group in both InsP₆ and 5-InsP₇; Mg²⁺ ions also contribute to specificity by electrostatic bridging between certain phosphate groups (Wang et al. 2012). In fact, both a Mg²⁺ ions and Lys214 of human PPIP5K2 specifically interact with the 5-β-phosphate in 5-InsP₇, which of course is missing from InsP₆, thereby accounting for the latter’s lower catalytic efficiency (k_{cat}/K_m = 7.6 × 10⁴ M/s for InsP₆; 2.2 × 10⁶ M/s for 5-InsP₇; Weaver, Wang, and Shears 2013). Indeed, most current evidence points to the phosphorylation of 5-InsP₇ being the predominant activity of PPIP5Ks in vivo, while the phosphorylation of InsP₆ is generally considered to be a relatively minor activity (Padmanabhan et al. 2009; Wang et al. 2012; Weaver, Wang, and Shears 2013). Bearing that in mind, and the relatively low levels of 1-InsP₇ in cells, it is reasonable to propose that InsP₈ may be the more physiologically-important product.

By comparing crystals with and without substrates, we established that their occupation of the catalytic pocket is accompanied by induced fit motion of side-chains of three active-site residues, namely, Arg262, Arg281 and Lys329; such conformational dynamics are recognized to help define ligand specificity, while also reducing the degree of free-energy of activation (Herschlag 1988). Were there instead to be backbone rearrangements, the energetic demand would have been higher (Wang et al. 2012).

As for understanding how the catalytic cycle moves from the substrate-bound ground state to the transition state, a crystal structure that was complexed with a MgF^{3-} transition-state mimic revealed that conformational dynamics of both enzyme and substrate are involved (Wang et al. 2012). There are conformational changes in five amino-acid side chains in particular – Lys54, Arg213, Lys214, Arg262 and Lys329 – that advance the 1-alpha-phosphate of the substrate towards the gamma-phosphate of ATP. During this process, the 1-alpha-phosphate rotates almost 20° . That is, PPIP5Ks exploit a specific property of a phosphoester bond - its ability to rotate. Such a transition is not available for inositol phosphate kinases that phosphorylate the hydroxyl groups, which lack conformational flexibility.

Another feature of catalysis by PPIP5Ks is that an extensive negative charge balance by positively charged amino-acid residues facilitates a partly-associative, in-line phosphoryl transfer mechanism (Wang et al. 2012). That is, the new P-O bond can be formed by nucleophilic attack of the acceptor oxygen, before the original P-O bond with the donor oxygen is broken. Finally, in the active site of the kinase the inositol ring of $InsP_6$ and 5- $InsP_7$ is presented perpendicular to the plane of the nucleotide's beta-phosphate, which avoids steric and electrostatic clashing between the nucleotide and the non-reacting oxygens on the 1-phosphate of the substrate (Wang et al. 2012). This innovative topology distinguishes PPIP5Ks from all of the other inositol phosphate kinases that phosphorylate hydroxyl groups (Wang et al. 2012).

Nevertheless, these structural data also begged some questions: ATP is enveloped between two sets of anti-parallel beta-sheets, so that less than 10% of the nucleotide is predicted to be solvent accessible. Thus, it can be anticipated that the enzyme may undergo considerable conformational changes when admitting ATP and releasing ADP. Unfortunately, the nature of these rearrangements is unknown, as the structure of the apo-enzyme has not yet been solved. A second issue is, perhaps, more subtle: considering how highly constraining is the structure of the substrate-binding pocket, that will only admit ligand presented in the appropriate orientation - i.e., a direct hit - could that limit the enzyme's ability to attract ligand that rotates and diffuses randomly through the bulk phase? To illustrate the point about the need for a "direct hit", consider the usual outcome of a classic carnival game as an analogy: the high failure rate when attempting to throw a table tennis ball into the narrow neck of a distant goldfish bowl.

Further work has uncovered a near-unique mechanism by which PPIP5Ks circumvent the possibility of such a "high failure rate". Through X-ray analysis of several synthetic PP-InsP analogues in crystal complexes with the PPIP5K2 kinase domain, together with biochemical assays and mutagenesis, it has been shown that this class of enzyme utilizes

a surface-mounted, ligand-binding site that is adjacent to the catalytic pocket (Wang et al. 2014). It has been concluded that this unusual ligand-binding site facilitates substrate capture from the bulk phase, prior to its transfer into the catalytic pocket; a “catch-and-pass” reaction mechanism (Wang et al. 2014).

Once again, a structural rationalization of one question raised another - and this one remains to be resolved: why does ligand occupation of the substrate-capture site stimulates an intrinsic, non-productive ATPase activity (Wang et al. 2014)? Intrinsic ATPase - reactivity of the gamma phosphate of ATP with water instead of substrate - appears to be an inherent property of most, if not all, kinases. However, the catalytic cycle of the kinase domain of PPIP5K is apparently unique in that the ATPase activity is stimulated by the substrate itself, specifically, during its occupation of the capture site (Wang et al. 2014; Weaver, Wang, and Shears 2013). It has been estimated that during the phosphorylation of 5-InsP₇ by human PPIP5K2, an accompanying substrate-stimulated ATPase activity accounts for 17% of the total ATP that is consumed (Weaver, Wang, and Shears 2013). The proportion is much higher during InsP₆ phosphorylation: 50% (Weaver, Wang, and Shears 2013). It seems reasonable to speculate that this ATP hydrolysis is not simply ‘wasted’, but instead has some significance for the catalytic cycle. For example, transfer of ligand from the capture site to the catalytic pocket involves not only a lateral migration of 7 Angstroms, but also a ring flip, and a 100 degree rotation (Wang et al. 2014). Such molecular gymnastics are an appropriate candidate for assistance from the free energy of ATP hydrolysis.

Beyond the Kinase Activities: The Phosphatase Domain.

Off-switches for cell-signaling are as vitally important as the on-switches. Until recently, the off-switches for the PP-InsPs were thought to be the prerogative of the DIPPS family (Kilari et al. 2013; Safrany et al. 1998). Indeed, the DIPPS seem to have one feature that makes them especially well suited to this role: highly activate rates of PP-InsP dephosphorylation (Safrany et al. 1998). Nevertheless, their lack of specificity for a particular PP-InsP is not consistent with a need to regulate the levels of an individual PP-InsP that may have distinct functions.

Against that puzzling backdrop, one recent finding brings a new dimension to our understanding of the pathways of PP-InsP turnover: PPIP5Ks host a phosphatase domain (Fig. 2). This remarkable feature was originally recognized by John York’s laboratory (Fridy et al. 2007; Mulugu et al. 2007). The amino-acid sequence of the active site bears similarities to the relatively non-specific phytase subgroup of histidine acid phosphatases (Fridy et al. 2007; Mulugu et al. 2007). That observation belies the actual positional specificity of the PPIP5K phosphatase domain: only the 1-beta-phosphate from either 1-InsP₇ or 1,5-InsP₈ is hydrolyzed (Wang et al. 2015). In other words, the PPIP5K family catalyzes 1-kinase/1-phosphatase futile cycles. It has been shown that InsP₈ is the preferred of the two substrates; InsP₈ is dephosphorylated about 10-fold faster than is 1-InsP₇ (Wang et al. 2015). Since it is also the case that the kinase domain prefers 5-InsP₇ over InsP₆ (see above), we can conclude that, in vivo, flux through the 5-InsP₇ / InsP₈ cycle should exceed that through the InsP₆ / 1-InsP₇ cycle (see Fig 1). It should be interesting to establish from a structural analysis how this exquisite specificity is enforced; data obtained from Asp1 are

particularly illuminating - its phosphatase domain does not hydrolyze either InsP₆, or the 2-, 3-, 4-, 5-, or 6-isomers of InsP₇ (Wang et al. 2015).

It is very rare that a 'futile' kinase/phosphatase cycle can be catalyzed by a single protein at two separate catalytic sites. Understanding what are the particular advantages of such kinase/phosphatase bifunctionality is a topic of considerable general interest (Dasgupta et al. 2014). In the context of cell signaling, a substrate cycle that is driven by separate proteins is susceptible to incoherent output within a particular tissue, due to stochastic cell-to-cell variation in the expression of each of the individual proteins; such random variability in protein expression is even observed within populations of genetically identical cells cultured under highly similar conditions (Atay and Skotheim 2014). This potential for incoherent output is avoided when there is co-expression of the kinase and phosphatase - such as is inevitable when the two proteins are fused together (Dasgupta et al. 2014). Of course, for the maintenance of coherent output to be a benefit to arise from bifunctionality of the PPIP5Ks, their regulation of 1-InsP₇ / InsP₈ levels must fulfil some important biological functions.

Another potential advantage of bifunctionality could be its offering an opportunity for coordinated, reciprocal regulation of the competing kinase/phosphatase activities in PPIP5Ks; this could form the basis for a sensitive mechanism of control over 1-InsP₇ / InsP₈ synthesis and metabolism. However, as yet, this idea is purely speculative; no such regulatory process has been demonstrated for any metazoan PPIP5K. On the other hand, there has been a description of a mechanism for regulating the phosphatase activity of Asp1. This protein hosts an Fe-S cluster with a [2Fe-2S]²⁺ arrangement that inhibits phosphatase activity and thereby promotes net kinase activity (Wang et al. 2015). This is an unprecedented role for an Fe-S cluster: despite their being widely distributed throughout Nature, no others have previously been found to have a direct catalytic influence upon a phosphatase activity. Data obtained by electron paramagnetic resonance indicate the cluster is not redox active (Wang et al. 2015). It has been proposed that the role of the cluster in Asp1 may be to couple PP-InsP turnover to Fe availability (Wang et al. 2015). Indeed, it is intriguing that the environmentally controlled switch to an alternative morphology for *S. pombe* - pseudohyphal invasive growth - is influenced by both Fe (Prevorovsky et al. 2009) and the Asp1 phosphatase domain (Pohlmann et al. 2014). In any case, the regulatory impact of altered [2Fe-2S]²⁺ cluster assembly upon PP-InsP metabolism might be expected to be a relatively long-term adaptive process, since the mechanism by which the [2Fe-2S]²⁺ cluster inhibits phosphatase activity appears not to be readily reversible, at least *in vitro* (Wang et al. 2015).

It remains to be established whether or not there are Fe-S clusters in mammalian PPIP5Ks. Reliable predictions have not been obtained from multiple sequence alignments, in part because Cys-based consensus motifs for [2Fe-2S] centers are so diverse (Wang et al. 2015). Furthermore, towards the C-terminus of Asp1, where the Fe-S cluster is most likely to be hosted, the amino-acid sequence diverges substantially from those in PPIP5K1 and PPIP5K2, which in turn diverge considerably from each other at their own C-termini (Wang et al. 2015). In fact, even the identity of the Cys residues that co-ordinate the Fe-S cluster in Asp1 has not yet been unequivocally established (Wang et al. 2015). A systematic approach (individual mutation of the twelve candidate Cys residues to Ser) revealed six - residues

607, 663, 864, 868, 879 and 905 - that each led to a reduction in the Fe content of the Asp1 phosphatase domain (Wang et al. 2015). However, no more than four Cys residues are required to ligate a $[2\text{Fe}-2\text{S}]^{2+}$ cluster (Beinert, Holm, and Munck 1997; Johnson and Smith 2005). Thus, these mutagenic data raise the possibility that two of these six mutations alter protein structure, and thereby *indirectly* impact Fe-S cluster assembly. Another possibility to consider is that there are alternate cluster ligation states. This lack of clarity does not help us predict which Cys residues in mammalian PPIP5Ks might co-ordinate any Fe-S cluster. A final confounding issue is that the biophysical techniques that can unequivocally detect Fe-S clusters require considerable (i.e. mg) quantities of highly-pure enzyme; it has not yet proved possible to satisfy that requirement, due to difficulties expressing and purifying such large proteins that are also rather labile (Choi et al. 2007).

Beyond the Kinase Activities: Other Motifs and Domains in the PPIP5Ks

Within the phosphatase domain of the PPIP5Ks there is a cryptic PH domain, and it has especially high affinity for $\text{PtdIns}(3,4,5)\text{P}_3$ (Gokhale et al. 2013; Gokhale, Zaremba, and Shears 2011). In some cells, stimulus-dependent activation of $\text{PtdIns}(3,4,5)\text{P}_3$ synthesis has been associated with a degree of relocalization of PPIP5K1 from the cytoplasm to the plasma membrane (Gokhale et al. 2013; Gokhale, Zaremba, and Shears 2011). We have proposed that the significance of PPIP5K translocation lies in it regulating the activity of the protein kinase AKT. The latter is a classic example of a PH-domain containing protein that is recruited to the plasma membrane and activated by binding to $\text{PtdIns}(3,4,5)\text{P}_3$ (Cantley 2002). Both InsP_6 and 5-InsP_7 are competitive inhibitors of AKT activation, in large part by competing with $\text{PtdIns}(3,4,5)\text{P}_3$ binding (Chakraborty et al. 2010; Luo et al. 2003). We (Gokhale et al. 2013) have suggested this to be a necessary process for preventing any non-sustained, stochastic increases in $\text{PtdIns}(3,4,5)\text{P}_3$ from inappropriately recruiting and activating AKT and other signaling proteins with PH-domains. The aberrant $\text{PtdIns}(3,4,5)\text{P}_3$ -signaling that underlies much of cancer biology is a good illustration of why PH-domain recruitment is so carefully regulated (Cantley 2002).

We have proposed that translocation of PPIP5K1 to the plasma membrane will locally deplete levels of InsP_6 and 5-InsP_7 ; moreover, the products of this catalytic activity, 1-InsP_7 and InsP_8 , have 10 to 20-fold lower affinity for AKT (Gokhale et al. 2013). That is, PPIP5K kinase activity will attenuate the inositolphosphate-based inhibition of $\text{PtdIns}(3,4,5)\text{P}_3$ signaling through PH domains. Thus, optimum signaling by the PI3K cascade may depend upon coincidence detection: the translocation of both PPIP5K *and* a PH-domain protein. This raises a rather ironic conclusion (at odds with the tone of the rest of this review) that one aspect of PPIP5K functionality rests on the relative biological *inactivity* of InsP_8 and 1-InsP_7 , at least as PH-domain ligands.

Another functionally significant region that is present in both PPIP5Ks is an unusually long, intrinsically disordered region (IRD) that lies at their C-termini (Machkalyan et al. 2016). Such domains are typically considered as molecular scaffolds for promoting binding of other proteins (Machkalyan et al. 2016). A proteomic-based search for protein partners of PPIP5K1 yielded many that participate in trafficking of intracellular vesicles, particularly those in the exocyst complex that tether post-Golgi vesicles to plasma membranes

(Machkalyan et al. 2016). Additionally, there is evidence that PPIP5K1 interacts with proteins that participate in lipid metabolism and cytoskeletal organization (Machkalyan et al. 2016). Thus, it appears likely that further delving into scaffolding functions for PPIP5Ks will be a productive future research direction.

Within the IDR of PPIP5K2 (but not PPIP5K1) is a penta-arginine nuclear localization sequence (NLS) (Yong et al. 2015). The location of this NLS sequence is unlikely to be coincidental; such sequences typically reside in relatively unstructured regions of proteins (Bauer, Doetsch, and Corbett 2015). It is through interactions of the NLS with specialized molecular chaperones - karyopherins - that the host protein gains access to the nucleus (Bauer, Doetsch, and Corbett 2015). This NLS lies in a sequence of 63 amino-acid residues in PPIP5K2 that we have named as a CPA (C_{ontains} P_{enta}-A_{rginine}) region (Fig 2). This domain is rigorously conserved throughout metazoans, which presumably testifies to its importance (Yong et al. 2015). However, mRNA transcripts containing this CPA domain (e.g., XM_005277536.1) are relatively poorly expressed in human cells (or at least those that we have examined), possibly as a consequence of rare, alternate splicing. Nevertheless, this NLS is functional: we expressed a CPA-containing version of GFP-tagged, human PPIP5K2 in HEK cells, and a distinct nuclear pool of this protein was observed (at a concentration that was approximately 30% of that found in the cytoplasm (Yong et al. 2015)). Mutagenesis of the NLS (RRRRR to RAAAR) reduced the degree of nuclear localization almost 4-fold (Yong et al. 2015).

A Ser residue that lies immediately to the C-terminus of the penta-arginine is phosphorylated by an as yet unknown protein kinase (Yong et al. 2015); analysis of consensus motifs (Phosida database; <http://141.61.102.18/phosida/index.aspx>) indicate that candidates include PKA, CaMK2 and AKT. The mutation of this Ser residue to Ala increases the nuclear concentration of the PPIP5K2 by 60% (Yong et al. 2015). These data indicate that phosphorylation of the native Ser inhibits the functionality of the NLS. Such a regulatory process presumably supervises a specific, nuclear function for 1-InsP₇ and/or InsP₈. That conclusion indicates it would be valuable to systematically investigate which cell types might express isoform(s) of PPIP5K2 that contain the CPA domain.

Potential Signaling functions for 1-InsP₇ and InsP₈

While the PPIP5Ks synthesize 1-InsP₇ and InsP₈, the latter is at least 5 to 10-fold more abundant (see above). Despite 1-InsP₇ earning a booby-prize among PP-InsPs for its low relative abundance, evidence of its biological significance accompanied the initial description of a protein responsible for 1-kinase activity - the Vip1 ortholog in *S. cerevisiae* (Lee et al. 2007; Mulugu et al. 2007); depriving this yeast of inorganic phosphate was reported to substantially activate 1-InsP₇ synthesis, which was proposed to drive adaptive transcriptional responses (Lee et al. 2007). On the other hand, subsequent studies have described the opposite response of InsP₇ levels to phosphate starvation: a decrease in its concentration (Lonetti et al. 2011; Wild et al. 2016). An explanation for these inconsistent results has not been forthcoming, but it has previously been noted that phosphate deprivation is associated with a decline in cellular ATP levels, upon which all PP-InsP synthesis depends (see Saiardi 2012; Shears 2015).

1-InsP₇ has also been proposed to mediate certain innate immune responses - specifically, RIG-1 dependent transcription of interferon- β in response to viral invasion (Pulloor et al. 2014). Evidence for a mechanism underlying this response was obtained in vitro: 1-InsP₇ enhances the degree of phosphorylation of IRF3, which promotes its dimerization and entry into the nucleus, where it stimulates interferon- β transcription (Pulloor et al. 2014). In those experiments, 5-InsP₇ was found to be ineffective, while 1-InsP₇ acted more potently than did InsP₈. Again, interpreting whether 1-InsP₇ over InsP₈ is the more active molecule in vivo requires us to take their relative cellular levels into account.

Considering the apparent role of PPIP5Ks in innate immunity in mammalian cells (see above) it is intriguing that the VIH2 ortholog in *A. thaliana* also regulates innate immune responses (Laha et al. 2015) - and in this case it is InsP₈ that is suggested to be the functional signal. In this particular plant, cellular levels of InsP₈ are elevated by methyl-jasmonate, one of a group of plant hormones that helps protect plants from herbivorous insects and necrotrophic fungi (Laha et al. 2015). This protective response is impaired in lines of *A. thaliana* in which the kinase domain of VIH2 is disrupted by insertional mutagenesis (Laha et al. 2015). It has been speculated that InsP₈ may augment transcriptional activation by methyl-jasmonate (Laha et al. 2015); we await further developments in this area with interest.

A typical characteristic of an intracellular signal is stimulus-dependent changes in its cellular levels. The only published report that 1-InsP₇ levels are regulated is the aforementioned study with phosphate-deprived yeast, which others have failed to reproduce. On the other hand, stimulus-dependent regulation of InsP₈ concentration has been demonstrated in several studies. For example, hyperosmotic stress substantially elevates InsP₈ levels (Choi et al. 2008; Padmanabhan et al. 2009; Pesesse et al. 2004). This response has the hallmark of an adaptive response to an environmental stress, although as yet no functional significance has been attributed to it. Thermal challenges - both heat and cold - also stimulate InsP₈ synthesis (Choi, Mollapour, and Shears 2005), although it is unclear if this is a regulated stress response, or merely differential effects of temperature changes upon the competing activities of PP-InsP kinases and phosphatases. Finally, InsP₈ has been implicated as being a sensor of bioenergetic health in mammalian cells (Choi et al. 2008); levels of this PP-InsP decrease following relatively mild bioenergetic challenges, such as those known to elevate AMP, although AMP-dependent protein kinase appears not to play a role in regulating InsP₈ turnover (Choi et al. 2008). To date, a mechanistic basis for this phenomenon has not been derived, neither has a functional consequence been established. Nevertheless, these data indicate that InsP₈ serves some important homeostatic function(s).

Future Directions

Genetically-modified animal models have frequently illuminated biological functions of intracellular signaling cascades. At the time of writing, there are no published phenotypes for knock-out of either PPIP5K1 or PPIP5K2 in any animal model. Such information is likely to be available soon, since a PPIP5K2 knock-out mouse strain has been produced, according to the International Mouse Phenotyping Consortium (<http://www.mousephenotype.org>). The relative ease with which CRISPR can be used to

genetically modify cells in culture can also accelerate molecular studies into PP-InsP biology.

Nevertheless, although it is incontestable that genetic approaches have substantially contributed to our understanding of the biological roles of inositol phosphate kinase (e.g. Chakraborty et al. 2010; Ghosh et al. 2013; Prasad et al. 2011; Scoumanne et al., 2016; Szigyarto et al., 2011), such experiments do have certain drawbacks: the long term nature of such experiments introduces the possibility that the nature of a particular phenotype arising from gene deletion may be confounded by secondary genetic changes. Thus, pharmacological approaches are often a useful complement to genetic work. For example, it would be useful to have cell-permeable inhibitors of the kinase and phosphatase activities of the PPIP5Ks. To this end, we recently began a program to develop inhibitors of the kinase activity of PPIP5Ks. We have screened the human PPIP5K2 kinase domain against a library of almost 5000 molecules that have been curated because of their known - or predicted - actions as inhibitors of ATP-binding to protein kinases (Baughman et al. 2016). Some promising lead molecules have been identified (Baughman et al. 2016) which we intend to develop further.

It would be useful to be able to specifically modify levels of a particular PP-InsP in intact cells without genetic modification of their metabolic pathways. To this end, methods are also being introduced that circumvent the impermeability to the plasma membrane of highly charged molecules such as the PP-InsPs. For example, guanidinium-rich molecular transporters have been developed (Pavlovic et al. 2016). This method was recently used to deliver caged 5-InsP₇ into HeLa cells; the 5-InsP₇ was subsequently released upon cell exposure to UV-light. Furthermore, metabolically stable PP-InsP analogues are now available (Riley et al. 2012; Wu et al. 2013).

As we learn more about the unexpected and sometimes complex features of PPIP5Ks and their orthologs, it seems entirely reasonable to anticipate that the major product of these enzymes - InsP₈ - is serving important cellular roles. Recent developments (see above) make us optimistic that the shroud of uncertainty covering the biological significance of InsP₈ will shortly be removed.

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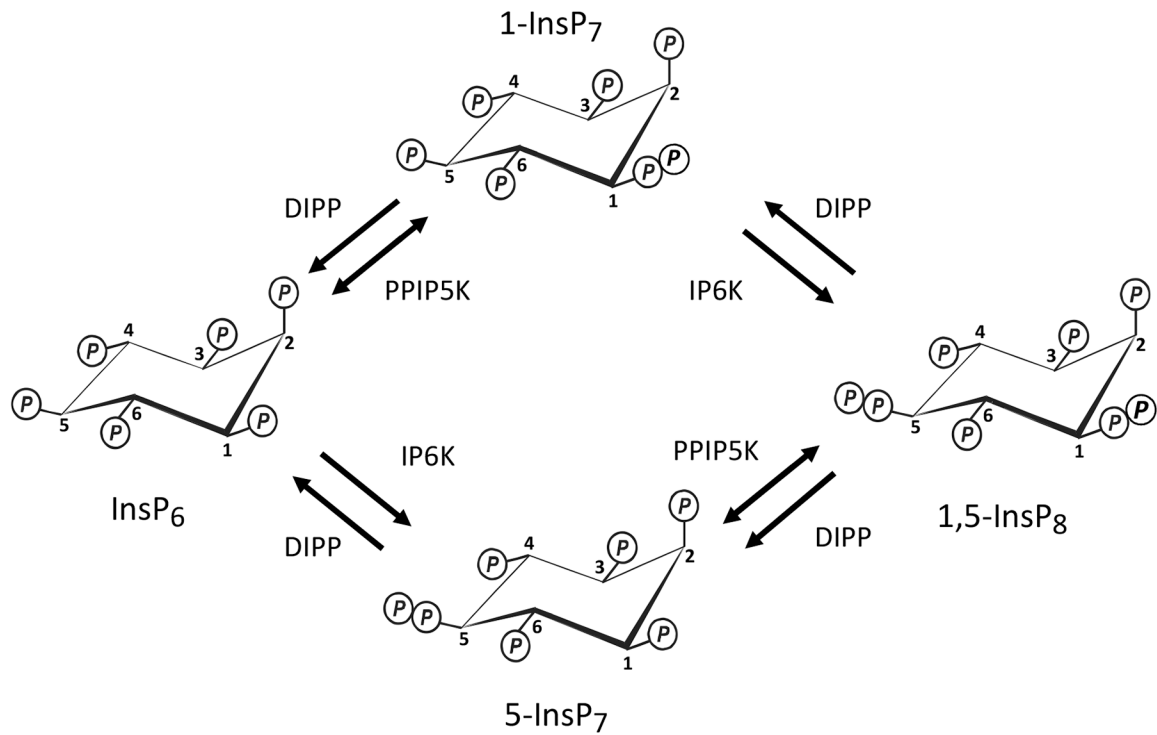


Figure 1. Inositol Pyrophosphate Turnover and the PPIP5Ks.

The schematic denotes the contributions to inositol pyrophosphate turnover of the 5-kinase activities of IP6Ks, the 1-kinase and 1-phosphatase activities of PPIP5Ks, and the DIPP phosphatases.

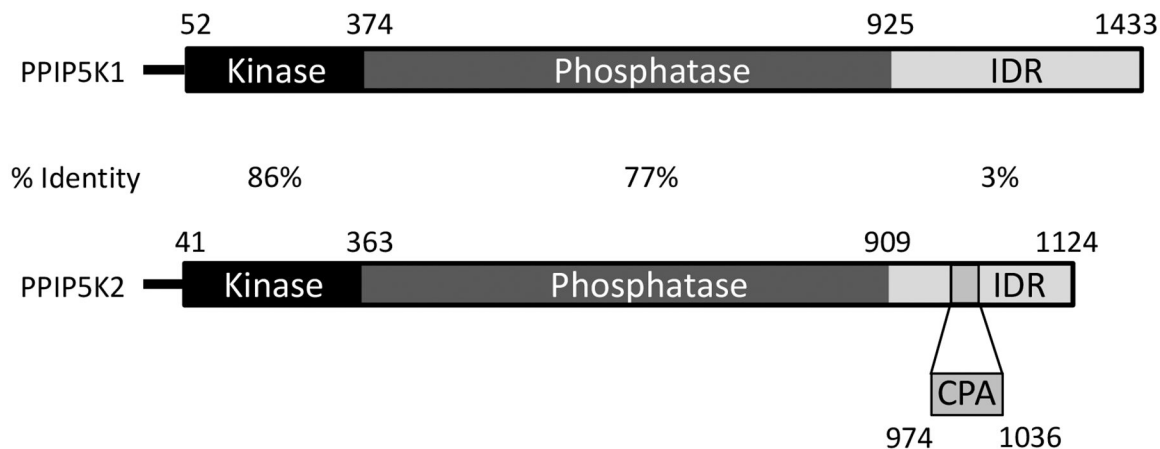


Figure 2. Domain graphic for PPIP5K1 and PPIP5K2

The figure depicts a domain graphic for human PPIP5K1 (BC057395.1) and human PPIP5K2 (XM_005271938). The so-called CPA (Contains Penta-Arginine) region in PPIP5K2 is rigorously conserved throughout metazoans; the eponymous pent-arginine is a functional nuclear localization sequence (Yong et al. 2015). However, transcripts containing this CPA domain (e.g., XM_005277536.1) are relatively poorly expressed in human cells (or at least those that we have examined), possibly as a consequence of rare, alternate splicing. In the figure, the amino acid residues defining each domain in PPIP5K1 are numbered as in a previous study, which also defined the intrinsically disordered domain (IDR) (Machkalyan et al. 2016). These boundaries were matched to those of the corresponding domains in PPIP5K2 by sequence alignments using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The aligned IDR boundaries in PPIP5K2 are consistent with those independently predicted from the PSIPRED Protein Sequence Analysis Workbench (<http://bioinf.cs.ucl.ac.uk/psipred/>). The % sequence identities across each of the three domains are also indicated.