The Significance of the Bifunctional Kinase/Phosphatase Activities of Diposphoinositol Pentakisphosphate Kinases (PPIP5Ks) for Coupling Inositol Pyrophosphate Cell Signaling to Cellular Phosphate Homeostasis*

Chunfang Gu1, Hoai-Nghia Nguyen2, Alexandre Hofer3, Henning J. Jessen4,1, Xuming Dai5, Huanchen Wang4, and Stephen B. Shears3,5

From the 4Laboratory of Signal Transduction, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina, 27709, 5Department of Chemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, 6Institute of Organic Chemistry, Albert Ludwigs University, Albertstrasse 21, 79104 Freiburg, Germany, and 7Division of Cardiology, McAllister Heart Institute, University of North Carolina, Chapel Hill, North Carolina 27599

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Proteins responsible for P_i homeostasis are critical for all life. In *Saccharomyces cerevisiae*, extracellular [P_i] is “sensed” by the inositol-hexakisphosphate kinase (IP6K) that synthesizes the intracellular inositol pyrophosphate 5-diposphoinositol 1,2,3,4,6-pentakisphosphate (5-InsP_7) as follows: during a period of P_i starvation, there is a decline in cellular [ATP]; the unusually low affinity of IP6Ks for ATP compels 5-InsP_7 levels to fall in parallel (Azevedo, C., and Saiardi, A. (2017) Trends. Biochem. Sci. 42, 219–231). Hitherto, such P_i sensing has not been documented in metazoans. Here, using a human intestinal epithelial cell line (HCT116), we show that levels of both 5-InsP_7 and ATP decrease upon [P_i] starvation and subsequently recover during P_i replenishment. However, a separate inositol pyrophosphate, 1,5-bisdiphosphoinositol 2,3,4,6-tetraakisphosphate (InsP_8), reacts more dramatically (i.e. with a wider dynamic range and greater sensitivity). To understand this novel InsP_8 response, we characterized kinetic properties of the bifunctional 5-InsP_7 kinase/InsP_8 phosphatase activities of full-length diposphoinositol pentakisphosphate kinases (PPIP5Ks). These data fulfill previously published criteria for any bifunctional kinase/phosphatase to exhibit concentration robustness, permitting levels of the kinase product (InsP_8 in this case) to fluctuate independently of varying precursor (i.e. 5-InsP_7) pool size. Moreover, we report that InsP_8 phosphatase activities of PPIP5Ks are strongly inhibited by P_i (40–90% within the 0–1 mM range). For PPIP5K2, P_i sensing by InsP_8 is amplified by a 2-fold activation of 5-InsP_7 kinase activity by P_i within the 0–5 mM range. Overall, our data reveal mechanisms that can contribute to specificity in inositol pyrophosphate signaling, regulating InsP_8 turnover independently of 5-InsP_7, in response to fluctuations in extracellular supply of a key nutrient.

Phosphate has multiple functions that direct the survival of all living organisms: in its organic form, P_i is a component of genomic material, it serves as an energy currency, and it is ubiquitous in cell signaling. Thus, P_i homeostasis is essential to life, but the mechanisms by which this occurs in humans and other metazoans are largely unknown (1, 2). Most of the previous work in this field of research has focused on yeast models (3–5).

In particular, recent studies with *Saccharomyces cerevisiae* have revealed a new function in P_i homeostasis for inositol pyrophosphates (5). The latter are soluble, intracellular signals that contain multiple phosphates and diphosphates; up to seven (InsP_7) or eight (InsP_8) phosphates in total are crammed around a six-carbon inositol ring (see Refs. 6–8 and Fig. 1). In *S. cerevisiae*, levels of one inositol pyrophosphate, 5-InsP_7, track perturbations to P_i homeostasis (5).

This P_i-sensing activity of 5-InsP_7 appears to reflect it being synthesized by a kinase class (kcs1 in yeast; IP6Ks in metazoans) that exhibits an unusually low affinity for ATP (9, 10). Consequently, cellular levels of 5-InsP_7, in yeast decrease in response to the drop in [ATP] that accompanies extracellular [P_i] depletion (5, 11). Furthermore, these ATP-driven changes in 5-InsP_7 levels appear to comprise a dynamic signaling response because 5-InsP_7 regulates proteins that maintain P_i homeostasis through interactions with their SPX domains (5). However, it is not known to what extent this signaling response is applicable to metazoan cells, which lack orthologs of many of the yeast genes that function in P_i sensing and P_i homeostasis (2).

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3 To whom correspondence should be addressed: Laboratory of Signal Transduction, NIEHS, National Institutes of Health, 111 T. W. Alexander Dr., Research Triangle Park, NC 27709. Tel.: 919-541-0793; E-mail: shears@niehs.nih.gov.

4 The abbreviations used are: InsP_7, diposphoinositol pentakisphosphate; 1-InsP_7, 1-diposphoinositol 2,3,4,5,6-pentakisphosphate; InsP_8, inositol hexakisphosphate; 5-InsP_8, 5-diposphoinositol 1,2,3,4,6-pentakisphosphate; InsP_9, 1,4-bispoxiphosphoinositol 2,3,4,6-tetakisphosphate; XPR1, xenotropic and polytropic retrovirus receptor 1; PPIP5K, diposphoinositol-tetakisphosphate kinase; SPX, SYG1/Pho81/XPR1 proteins; IP6K, inositol-hexakisphosphate kinase; InsP_6, inositol pentakisphosphate; DIPP, diposphoinositol-polyphosphate phosphohydrolase; CRISPR, clustered regularly interspaced short palindromic repeats.
In the current study, we have searched for links between phosphate homeostasis and inositol pyrophosphates in a human model system: the HCT116 intestinal epithelial cell line. This choice reflects the physiological relevance of both small and large fluctuations in $[\text{Pi}]$ within the gastrointestinal tract (12). One of our goals has been to investigate whether there are changes in extracellular $[\text{Pi}]$ that might cause intracellular $[\text{ATP}]$ and $[5-\text{InsP}_7]$ to co-vary, which as mentioned above is considered to be an IP6K-dependent phenomenon.

Perhaps as a consequence of $5-\text{InsP}_7$ being the most abundant of the inositol pyrophosphates, it has been the focus of much of the literature in this field (6, 8, 13, 14). In the current study, we also study a different inositol pyrophosphate, $\text{InsP}_8$ (Fig. 1). We describe some new features to $\text{InsP}_8$ turnover that solidify its own, independent cell signaling credentials. This information arises out of our focus on the PPIP5Ks (Fig. 2). The latter enzymes are of general interest; in addition to hosting a kinase domain that phosphorylates $5-\text{InsP}_7$ to $\text{InsP}_8$, PPIP5Ks possess a separate phosphatase domain that dephosphorylates $\text{InsP}_8$ back to $5-\text{InsP}_7$ (15–17).

That is, PPIP5Ks interconvert substrates and products in apparent “futile cycles” (Figs. 1 and 2). Kinase/phosphatase and other covalent modification cycles are a nexus for regulatory inputs into metabolic and signaling pathways (18); in fact this phenomenon is considered a core motif in the field of systems biology (19). However, in general, such competing catalytic activities are hosted by separate proteins for the purposes of compartmentalization and for promoting signaling fidelity (20). Only in rare cases have these apparent benefits been selected against in order that the mutually antagonistic catalytic activities co-exist within a single protein (19, 21, 22). The PPIP5K family is one of these exceptions; representatives from humans, yeasts, and plants contain kinase and phosphatase domains (16), indicating that this bifunctionality has survived at least 1.5 billion years of evolutionary pressure (23).

Among a number of proposed advantages of having competing catalytic activities in a single, bifunctional protein are the following: (a) preventing signaling incoherence that can otherwise arise due to stochastic fluctuations in the degrees of expression of two separate proteins; (b) robustness, i.e. invariance to quantitative changes of the system’s components, including substrate concentration; and (c) increased “parametric sensitivity,” that is, a situation in which signaling output is amplified following relatively small changes in the concentration of a particular parameter, such as an enzyme regulator (19, 21). However, the significance of these phenomena in vivo is dictated by the catalytic parameters of the mutually antagonistic domains (19, 21). Hitherto, we lacked this information. The full kinetic profile for PPIP5Ks has not previously been determined in the full-length versions of these enzymes. Moreover, there is no information in the literature describing the existence of a modulator of either the kinase or the phosphatase activity of any mammalian PPIP5K. The current study addresses these important gaps in our understanding of inositol pyrophosphate turnover. We demonstrate that $\text{Pi}$ regulates the catalytic activities of the PPIP5Ks. Furthermore, our data indicate that $\text{InsP}_8$ and $5-\text{InsP}_7$ each act through separate mechanisms to individually sense extracellular $\text{Pi}$ status.

In the current study, we have searched for links between $\text{Pi}$ homeostasis and inositol pyrophosphates in a human model system: the HCT116 intestinal epithelial cell line. This choice reflects the physiological relevance of both small and large fluctuations in $[\text{Pi}]$ within the gastrointestinal tract (12). One of our goals has been to investigate whether there are changes in extracellular $[\text{Pi}]$ that might cause intracellular $[\text{ATP}]$ and $[5-\text{InsP}_7]$ to co-vary, which as mentioned above is considered to be an IP6K-dependent phenomenon.

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FIGURE 1. Inositol pyrophosphate metabolism. The schematic describes all known mammalian enzyme classes that interconvert $\text{InsP}_6$ with $\text{InsP}_8$. Note that current thinking (28, 29) has the major route from $\text{InsP}_6$ to $\text{InsP}_8$ in mammalian cells progressing through $5-\text{InsP}_7$, rather than $1-\text{InsP}_7$.

FIGURE 2. Domain graphic for human PPIP5Ks. Domain graphics are shown for the human PPIP5Ks used in this study (type 1, BC057395.1; type 2, XM_005271938). For PPIP5K1, amino acid residues defining each domain are numbered as in a previous study, which also defined the intrinsically disordered domain (IDR) (49). These boundaries were matched to those of the corresponding domains in PPIP5K2 by sequence alignments using Clustal Omega. The aligned intrinsically disordered domain boundaries in PPIP5K2 are consistent with those independently predicted from the PSIPRED Protein Sequence Analysis Workbench. The percent sequence identities across each of the three domains are also indicated. Also indicated are the nature and the locations of our engineered mutations in the kinase and phosphatase domains.
Results and Discussion

The Effects of Extracellular [P$_i$] Starvation and Replenishment upon Levels of Inositol Pyrophosphates in HCT116 Cells—Previous work with S. cerevisiae (24) has shown that extracellular [P$_i$] is sensed by the IP6K that synthesizes the intracellular inositol pyrophosphate 5-InsP$_7$. During a period of P$_i$ starvation, there is a decline in cellular [ATP]. The unusually low affinity of IP6Ks for ATP compels 5-InsP$_7$ levels to fall in parallel (5). As noted in a recent review of this field (24), yeasts are the only organisms that have previously been used to study
5-InsP_7 turnover in response to fluctuations in extracellular Pi availability. We have addressed this gap in the field by using the HCT116 human intestinal epithelial cell line as a model system. We prelabeled cells with [3H]inositol for several days, and then we investigated the effects of removal and replenishment of extracellular Pi. We used HPLC to assay the intracellular levels of “higher” InsPs, including InsP_8, InsP_9, and the inositol pyrophosphates.

First (Fig. 3A), we analyzed cells that had been incubated under Pi-replete conditions (i.e. 2 mM [Pi] (25, 26)). As in all metazoan cells, levels of InsP_5 and InsP_6 are much higher than those of the inositol pyrophosphates (InsP_7 and InsP_8); thus for clarity, those regions of the chromatograms that include the inositol pyrophosphates are replotted on an expanded y axis (Fig. 3, A, B, C, and D). The depiction of InsP_8 as being the 5-isomer is based on previous work with HCT116 cells in which our HPLC procedures were shown to resolve 5-InsP_7 from 1-InsP_7; the latter comprises ~2% of total InsP_7 (27). Indeed, it is generally believed that most of the cell’s InsP_8 is synthesized from 5-InsP_7, rather than 1-InsP_8 (28, 29).

We next investigated the effects of perturbations to Pi homeostasis. We challenged cells with 6 h of Pi starvation (Fig. 3B). This procedure did not impact levels of InsP_5 or InsP_6 (Fig. 3, A, B, E, and F), but InsP_7 levels decreased by ~70% (Fig. 3, A, B, and G). This is the first demonstration, for any metazoan cell type, that levels of InsP_7 are sensitive to a change in extracellular Pi. Nevertheless, InsP_7 levels fell by 98%, which is a much greater response to the Pi depletion protocol (Fig. 3, A, B, and H). It has not previously been reported that InsP_7 levels are influenced by extracellular Pi in any organism (e.g. see Ref. 24). In other words, our data describe a new connection between nutrient status and the poise of a cell signaling cascade.

Next, we substituted the last 15 min of the Pi starvation protocol with the readdition of 2 mM Pi. The levels of 5-InsP_7 were not influenced by this 15-min period of Pi replenishment (Fig. 3, B, C, and G). In contrast, there was a substantial rescue of the InsP_8 peak (Fig. 3, B, C, and H). These data testify to an acute effect of extracellular Pi status upon InsP_8 synthesis that is independent of the supply of 5-InsP_7 (i.e. the major InsP_7 precursor for InsP_8; see above). We revisit this point below.

In further experiments, a 3-h period of Pi starvation was followed by 3 h of 2 mM Pi readdition. After this protocol, levels of 5-InsP_7 were similar to those found in cells incubated with 2 mM Pi, for 6 h (Fig. 3, A, D, and G). In contrast, levels of InsP_8 in Pi-starved/Pi-replenished cells were 80% higher than those of control cells (Fig. 3, A, D, and H), suggestive of a longer term adaptive response to a period of Pi starvation. Indeed, a major conclusion to draw from all of these experiments is that InsP_8 reacts more dramatically to fluctuations in extracellular Pi than does 5-InsP_7.

The Effects of Extracellular Pi Starvation and Replenishment upon Levels of Intracellular Pi and [ATP] in HCT116 Cells—How do intracellular inositol pyrophosphates sense short term fluctuations in extracellular Pi levels? As a first step toward answering that question, we investigated whether intracellular Pi tracked the imposed changes in extracellular Pi. We found that our 6-h Pi starvation protocol reduced intracellular Pi by 55% (Fig. 3D); only 15 min of Pi replenishment was sufficient to completely restore intracellular Pi (Fig. 3D). The recovery in InsP_8 lags behind that for Pi (Fig. 3, H and I); the rate of change in InsP_8 levels depends upon dynamic fluctuations in InsP_8 synthesis and metabolism (see below).

How might inositol pyrophosphate levels be modified by changes in intracellular Pi? Previous work has indicated that Pi levels influence ATP production (30). Indeed, we found that our Pi starvation protocol reduced [ATP] by 66% (Fig. 3F), i.e. to a value of about 1.5 mM, based on the concentration of ATP normally being around 5 mM (31–33). Thus, the associated drop in 5-InsP_7 levels (Fig. 3, A, B, and G) may reflect the unusually low affinity (K_m approximately 1 mM) of IP6Ks for ATP (9, 10). Both ATP and 5-InsP_7 were at normal levels when a 3-h period of Pi starvation was followed by 3 h of replenishment with 2 mM Pi (Fig. 3, G and I).

On the other hand, there is no reason to suspect that synthesis of InsP_8 by the PPIP5Ks would be influenced by these changes in [ATP]. In contrast to IP6Ks, the PPIP5Ks exhibit a low K_m value for ATP (20 μM (29)), so their kinase activities would be saturated even by the reduced levels of [ATP] caused by 6 h of Pi starvation (Fig. 3F). We therefore searched for other mechanisms by which Pi might regulate InsP_8 turnover. There are precedents for Pi itself being a physiologically relevant regulator of both kinases and phosphatases (34, 35). Thus, we decided to study the synthesis and metabolism of InsP_8 by the human bifunctional PPIP5Ks in vitro and then investigate whether these catalytic activities are regulated by Pi. To our knowledge, no previous study has characterized the competing kinase and phosphatase activities of full-length PPIP5Ks purified from a human expression system.

Kinetic Analysis of the Bifunctional Human PPIP5Ks: the Interconversion of 5-InsP_7 and InsP_8—Mammals express two PPIP5Ks, type 1 and type 2 (Fig. 2). HEK cells were used as hosts for the expression of full-length, recombinant wild-type PPIP5K1 and PPIP5K2, each of which were engineered to possess an N-terminal FLAG tag to facilitate purification with anti-

**FIGURE 3.** The effects of Pi starvation and replenishment upon intracellular levels of inositol phosphates, ATP, and Pi in HCT116 cells. [3H]Inositol-labeled HCT116 cells were treated for different conditions of extracellular Pi availability as represented schematically by the horizontal bar at the top of each panel (filled bar, 2 mM Pi; empty bar, 0 mM Pi; A, 2 mM Pi, for 6 h; B, no Pi, for 6 h; C, no Pi, for 5.75 h followed by 2 mM Pi, for 0.25 h; D, no Pi, for 3 h followed by 2 mM Pi, for 3 h). Next, cells were quenched, and the levels of the indicated inositol phosphates (InsP_5, InsP_6, InsP_7, and InsP_8) were analyzed by HPLC (see "Experimental Procedures"). Data for InsP_5 and InsP_6 are replotted on an expanded y axis scale. A–D are representative of three biological replicates; scatter plots (error bars represent S.D.) are compiled from all three experiments in E (InsP_5), F (InsP_6), G (InsP_7), and H (InsP_8). The identification of InsP_8 as the 5-isomer is based on our previous work with HCT116 cells in which our HPLC procedures were shown to resolve 5-InsP_8 from 1-InsP_8; the latter is a minor isomer that comprises ~2% of total InsP_8 (27). The graphics below the x axes correspond to those described above that depict the various extracellular Pi conditions. Scatter plots (error bars represent S.D.) in I and J show total intracellular Pi (malachite green/molybdate method; n = 8–10) and ATP (n = 4–7), respectively, determined in parallel experiments with non-radiolabeled cells. The mean values for cell Pi content (nmol/mg of protein) from left to right are as follows (error bars represent S.D.): 62 ± 2.3, 28 ± 1.9, 62 ± 2.7, and 53 ± 2.2; these values closely match the Pi, levels that were obtained by an independent enzymatic method (60 ± 5.6, 32 ± 2.5, 59 ± 2.9, and 50 ± 2.7; n = 6).
Nevertheless, when these assays were performed with phosphatase as “no-transfection” controls for PPIP5K2 and vice versa. the concentrations of the PPIP5K constructs. The lanes containing PPIP5K1 domain (39) that were used as standards to calibrate through densitometry labeled with the quantities of the recombinant human PPIP5K2 kinase and, even if that had been present, it would have dephosphorylated InsP8 to InsP6 without appreciable accumulation of a "no-transfection" controls for PPIP5K2 and vice versa.

Thus, the phosphatase activities constrain but do not overwhelm kinase activities. In other words, it can be concluded that InsP8 turnover and not mass action effects due to fluctuations in 5-InsP7 supply.

We next verified that wild-type PPIP5K1 and PPIP5K2 both dephosphorylate InsP8 to 5-InsP7. (Fig. 5, D, E, and F). We also confirmed that the site mutation sites in the phosphatase domain nearly completely impaired InsP8 hydrolysis (Fig. 5, D, E, and F). This observation also usefully confirms that there is negligible contamination of our enzyme preparations with any contaminating 5-InsP7 kinase activities. Indeed, our enzyme preparations are free of any contaminating 5-InsP7 kinase activities.

Analysis of the wild-type kinase activities by HPLC confirmed that the InsP8 product accumulated (Fig. 5, A, B, and C). Nevertheless, these assays were performed with phosphatase mutants (PPIP5K1R399A and PPIP5K2R338A), a 2.4–4-fold larger net formation of InsP8 was observed (Fig. 5, A, B, and C). Thus, the phosphatase activities constrain but do not overwhelm kinase activities.

We next verified that wild-type PPIP5K1 and PPIP5K2 both dephosphorylate InsP8 to 5-InsP7. (Fig. 5, D, E, and F). We also confirmed that the site mutations in the phosphatase domain nearly completely impaired InsP8 hydrolysis (Fig. 5, D, E, and F). This observation also usefully confirms that there is negligible contamination of our enzyme preparations with any other InsP8 phosphatase activities. In fact, the only other class of mammalian enzyme known to hydrolyze InsP8 is DIPP (40), and, even if that had been present, it would have dephosphorylated InsP8 to InsP6 without appreciable accumulation of 5-InsP7. (see below).

Clearly, the phosphatase mutants of the PPIP5Ks yield the more accurate rates of the 5-InsP7 kinase reactions (Figs. 5 and 6A). In this respect, we found that PPIP5K2 is 6-fold more active than is PPIP5K1. Our phosphatase mutants were also useful in confirming that zero-order conditions prevail when physiologically relevant concentrations of 5-InsP7 are phosphorylated by PPIP5Ks: reaction rates were not increased when the 5-InsP7 concentration was increased from 1 to 5 μM (representative examples: PPIP5K1R399A, 1.2 and 0.9 nmol/mg of protein/min, respectively; PPIP5K2R338A, 6.4 and 5.8 nmol/mg of protein/min, respectively).

In the assays of phosphatase activities toward InsP8 described above, we incubated the PPIP5Ks with 0.05 μM InsP6, which is the estimated concentration of this inositol pyrophosphate in HCT116 cells cultured in DMEM/F-12 (27). In this reaction condition, the InsP8 phosphatase activities (0.8–1.5 nmol/mg of protein/min; Fig. 6B) lie in a range that is similar to that for the kinase activities (determined by assaying the phosphatase mutants: 0.7–4.5 nmol/mg of protein/min; Fig. 6A). These same data further indicate that, for PPIP5K2, the 5-InsP7 kinase/InsP8 phosphatase ratio slightly favors the kinase activity. For PPIP5K1, the phosphatase activity is favored. Such subtle differences between the two PPIP5K isoforms may serve some as yet unsuspected physiological purpose.

Our next goal was to determine how the InsP8 phosphatase activities might respond to elevated concentrations of substrate. Therefore, we assayed phosphatase activities against 1 μM InsP6: the reaction rates for both PPIP5K5s are 15–20-fold higher than those observed with 0.05 μM InsP6 (Fig. 6B). Thus, we conclude that the phosphatase activities in vivo may be modulated by fluctuations to supply of substrate (i.e. InsP6). In contrast, the 5-InsP7 kinase activities proceed under zero-order conditions (see above and Ref. 29). Mathematical modeling of just such a situation for a kinase/phosphatase bifunctional protein (zero-order for the kinase; first-order for the phosphatase) has established that a biological outcome is a degree of concentration robustness for the kinase product (21), in this case InsP8. In other words, it can be concluded that InsP8 is inherently insensitive to changes in 5-InsP7 levels. This property of PPIP5Ks could promote specificity of inositol pyrophosphate signaling by stabilizing InsP8 levels during periods of stimulus-dependent regulation of 5-InsP7 levels. Conversely, such concentration robustness indicates that the response of InsP8 to changes in P_i homeostasis (Fig. 3) reflects active regulation of InsP8 turnover and not mass action effects due to fluctuations in 5-InsP7 supply.

There are three additional conclusions that can be drawn from our kinetic data (Figs. 5 and 6). First, these results provide the first direct demonstration that the phosphatase activity of mammalian PPIP5Ks has the potential to significantly restrict net kinase activity in the context of the full-length proteins. That is, we conclude that so-called futile cycling by bifunctional PPIP5Ks is a realistic physiological scenario. Second, the activity of the kinase domain in full-length PPIP5K2 is 30-fold below its potential maximal capacity (190 nmol/mg of protein/min; as recorded for the isolated kinase domain expressed in Escherichia coli (29)). This catalytic constraint upon the kinase domain, when expressed as a full-length protein in a human cell type, could reflect covalent modification and/or a conformational constraint enforced by the other protein domains. Irrespective of the mechanism, the submaximal kinase activity in the full-length protein can be viewed as a selective advantage: at steady-state levels of 5-InsP7 (1 μM) and InsP8 (0.05 μM), the
rates of the kinase and phosphatase reactions are similar, so in such a situation, the slower these individual rates, the lower the amount of ATP that must be expended to maintain a given cellular content of InsP₈. A third conclusion that emerges from the phosphatase and kinase activities of full-length PPIP5Ks being similar is that it maximizes the sensitivity with which a modulator of either activity could affect net flux through the cycle. This is a topic we return to later (see below).

Kinetic Analysis of the Bifunctional Human PPIP5Ks: the Interconversion of InsP₆ and 1-InsP₇—PPIP5Ks are also capable of catalyzing a separate cycle of competing kinase and phosphatase activities that interconvert InsP₆ with 1-InsP₇ (Fig. 1). We next used the wild-type and phosphatase mutant constructs to determine the relative rates of these particular reactions. For the kinase assays (Fig. 6C), we set InsP₆ concentrations to 1 μM. Most estimates of cellular levels of InsP₆ are 10 μM or more (36–38), but the use of 1 μM increases assay sensitivity while still being more than sufficient to saturate PPIP5Ks with this substrate and obtain $V_{\text{max}}$ values (15, 29). For the phosphatase assays (Fig. 6D), we initially used a concentration of 0.05 μM 1-InsP₇ (because cellular levels of the latter are 2–10% of total InsP₇ (27, 28)). The maximum rates of the kinase activities toward InsP₆ (0.2–1 nmol/mg of protein/min; Fig. 6C) are 4–6-fold lower than the kinase activities toward 5-InsP₇ (Fig. 6A). Furthermore, the rates of the phosphatase activities toward 0.05 μM 1-InsP₇ (0.2–0.4 nmol/mg of protein/min; Fig. 6C) are 4-fold lower than the phosphatase activities toward InsP₈ (Fig. 6A). That is, kinetic parameters dictate that substrate cycling through InsP₆ and 1-InsP₇ is quantitatively less significant than is cycling through 5-InsP₇ and InsP₈. The latter conclusion is directly relevant to current thinking (6, 28) that the major route from InsP₆ to InsP₈ progresses through 5-InsP₇ rather than 1-InsP₇.

Although there is no direct evidence that the intracellular concentration of 1-InsP₇ in mammalian cells exceeds 0.05 μM (27), we also studied in vitro PPIP5K phosphatase activity toward this substrate at a concentration of 1 μM (Fig. 6D). At
Elevated Extracellular [P_i] Specifically Promotes InsP_8 Accumulation—Our main experimental paradigm has been to impose a nutritional challenge upon cultured cells: deprive and then restore the levels of P_i found in serum. Other groups have studied the impact upon signal transduction cascades when cells are incubated with “high” [P_i] (5–10 mM) (2, 30). We noted that such dramatic fluctuations in extracellular P_i concentration (up to 10 mM or more) are physiologically relevant for cells that line the gastrointestinal tract (12). We therefore used the HCT116 model to study the effects upon inositol pyrophosphate turnover of elevating extracellular P_i from 1 to 6 mM for periods of up to 1 h. This procedure did not significantly affect levels of either InsP_8, InsP_7, or 5-InsP_7 (Fig. 9, A, B, and C). The absence of an effect upon 5-InsP_7 illustrates how the reaction of inositol pyrophosphates to changes in P_i homeostasis may depend upon the nature of the experimental protocol. We propose that levels of 5-InsP_7 do not respond to the high [P_i] protocol because [ATP] and hence IP6K activity (see above) are also not altered (Fig. 9D).

In response to elevated extracellular [P_i], intracellular [P_i] trended higher (by 9–14%; Fig. 9E legend) but not with statistical significance. Nevertheless, InsP_8 levels responded dramatically: a 2.3-fold increase within 15 min and a 5-fold elevation after 1 h (Fig. 9, A, B, and F). This response of InsP_8 consolidates our discovery (see above) that it is more sensitive to changes in extracellular [P_i] than is 5-InsP_8. In view of how acutely and specifically InsP_8 responds to high [P_i], we sought further evidence for the participation of PPIP5Ks in this event. We gener-
ated an HCT116 cell line in which both PPIP5Ks have been knocked out using CRISPR (27). These cells do not synthesize InsP₈ irrespective of the concentration of extracellular [Pi] (Fig. 9).

Concluding Comments—Until quite recently, signal transduction cascades and metabolic circuits were not recognized to be intimately connected; now it is appreciated that a key aspect of cellular and organismal homeostasis is the acute regulation of cell signaling pathways by the levels of a particular metabolite or nutrient (41). In particular, inositol pyrophosphates have gained considerable attention for their actions that dovetail signaling with metabolism (6, 7, 24, 42). In our study, we add several new aspects to this important research topic by showing that InsP₈ levels sense and respond to fluctuations in the extracellular levels of a vital nutrient, Pi.

Our in vitro data describe physiologically relevant mechanisms by which Pi, may directly regulate the catalytic activities of the PPIP5Ks: inhibition of InsP₈ phosphatase (Fig. 7) and stimulation of 5-InsP₇ kinase (Fig. 8). Prior to this study, no regulators of either the kinase or phosphatase activities of mammalian PPIP5Ks had been described. Reciprocal regulation of any covalent modification cycle by a single modulator is a particularly efficient regulatory paradigm. Moreover, it is intuitive that the sensitivity of such a process is enhanced when the two opposing activities proceed at approximately equal rates as is the case for PPIP5K2 under physiologically relevant conditions (Fig. 6, A and B). Thus, there may be scenarios in which proportionately large changes in [InsP₈] may be promoted by quite small fluctuations in cytoplasmic [Pi] that are beyond the sensitivity of our assays, which record total intracellular [Pi] (Fig. 9E). A more
precise spatiotemporal understanding of the inter-relationships between $P_i$ and InsP$_8$ in vivo requires additional information that is not readily obtained: a quantitative resolution of their separation into different cellular pools (2, 43). Furthermore, differential levels of expression of PPIP5K1 versus PPIP5K2 could contribute to quantitative and qualitative cell type-specific differences in InsP$_8$ responses to extracellular $P_i$ because this anion only stimulates the kinase domain of PPIP5K2 and not that in PPIP5K1 (Fig. 8). It will be important to determine how widespread in other cell types is the impact of fluctuations in extracellular $P_i$ upon intracellular levels of InsP$_8$.

It is also possible that regulation of PPIP5K activity by $P_i$ is not the only cell signaling mechanism that regulates InsP$_8$ levels, particularly those that might be activated by high extracellular $P_i$ (2, 30). This would not be surprising considering the many interactions between inositol pyrophosphate signaling and metabolic homeostasis (6, 7, 24, 42) and the domain complexities of the PPIP5Ks (Fig. 2).

Our new data are also intriguing in light of recent data derived from yeast that show that 5-InsP$_7$ rheostatically regulates mechanisms of $P_i$ homeostasis mediated by SPX domains (5). The only human protein known to contain an SPX domain is XPR1, which transports $P_i$ out of cells (5). In the current study with a human cell type, we show [ATP] and [5-InsP$_7$] to co-vary in their response to $P_i$ starvation. This is the first time such a response has been observed in any metazoan model. Additionally, we show that InsP$_8$ senses changes in $P_i$ status by separate mechanisms that exhibit greater sensitivity as compared with 5-InsP$_7$. It could be useful to study whether InsP$_8$ is a homeostatic regulator of XPR1, perhaps adjusting the rate of cellular $P_i$ efflux depending upon organismal $P_i$ status. This would be a new direction for this field, which prior to our study had mainly focused on the biological actions of 5-InsP$_7$ (see Refs. 8 and 14). The possibility of there being other types of InsP$_8$ receptor should not be ignored. Finally, our results raise the possibility that dysregulation of inositol pyrophosphate signaling by genetic or environmental factors could conceivably contribute to mechanisms of toxicity of unbalanced $P_i$ homeostasis (1).

**Experimental Procedures**

**Cell Culture**—HEK293T cells and HCT116 cells were obtained from ATCC; HCT116 cells in which both PPIP5K1 and PPIP5K2 were knocked out using CRISPR were derived as described previously (27). The culture medium (Thermo Fisher Scientific) was DMEM/F-12 for HCT116 cells and DMEM for HEK293T cells, each supplemented with 10% fetal bovine serum (Gemini Bio Products) and 100 units/ml penicillin-streptomycin (Thermo Fisher Scientific) at 37 °C in 5% CO$_2$.

**Measurement of Intracellular Levels of $P_i$, ATP, and Inositol Phosphates**—For assays of intracellular ATP and $P_i$, 3 × 10$^5$ cells/well were seeded in a 12-well dish and cultured in DMEM/F-12 (containing 1 mM $P_i$) for 2 days at which point cultures were 70% confluent. For some experiments, an additional 5 mM $P_i$ was added (as a Na$_2$HPO$_4$/Na$_2$HPO$_4$ mixture (pH 7.4)), for the times indicated (see “Results and Discussion”). In other experiments, cells were washed three times with $P_i$-free DMEM (ThermoFisher Scientific) and then incubated in either $P_i$-free DMEM or DMEM plus 2 mM Na$_2$HPO$_4$ for the times indicated (see “Results and Discussion”). To terminate these assays, cells were washed three times in ice-cold buffer containing 20 mM HEPES (pH 7.2), 150 mM NaCl and then lysed by agitation in 1 ml of wash buffer containing 1% Triton X-100 for 5 min at 4 °C. The ATP was assayed using a commercial kit (Molecular Probes™, A22066). For the $P_i$ assays, the lysate was cleared of debris by a brief centrifugation, and then the resulting supernatant was centrifuged through an Amicon Ultra filter (10-kDa molecular mass cutoff; 5 min at 4 °C). The $P_i$ in the eluate was generally assayed with a standard malachite green/molybdate method (44). Similar data were obtained (see figure legends) using an enzymatic assay (Cell Biolabs, Inc., catalogue number STA-685) that avoids potential contamination through acid-dependent hydrolysis of organic phosphates. The data that were obtained were normalized to protein concentration, which was measured using the Pierce™ BCA protein assay kit (product number 23225).

To assay inositol phosphates, 1 × 10$^6$ cells were seeded in a 10-cm dish and cultured for 3 days in 7 ml of medium supplemented with 10 μCi/ml $[^3$H]inositol (American Radiolabeled Chemicals) at which point cultures were 70% confluent. Cells were then incubated in medium with various $P_i$ concentrations as described above. Cells were acid-quenched, and the inositol phosphates were extracted and analyzed by a 3 × 250-mm Carbopac™ PA200 HPLC column (ThermoFisher Scientific) all as described previously (27).

**Preparation of Recombinant Proteins**—Recombinant human DIPPI and the PPIP5K2 kinase domain were prepared as described previously (17, 39). We also constructed pDEST515 plasmids (45) hosting wild-type hPPIP5K1 (BC057395.1) and hPPIP5K2 (NM_001345875), each with an N-terminal FLAG tag. Based on previous identifications of conserved catalytically essential residues (16, 46), we used the Q5 site-directed mutagenesis kit (New England Biolabs) to prepare constructs encoding single site mutant PPIP5Ks. The paired primers were as follows (mutagenic nucleotides are bold and underlined):
FIGURE 9. The effects of elevated extracellular [P_i] upon phosphate homeostasis and inositol phosphate levels in HCT116 cells. HCT116 cells were labeled with [3H]inositol in medium containing 1 mM P_i and then an additional 5 mM P_i (as a NaH_2PO_4/Na_2HPO_4 mixture (pH 7.4)) was added for intervals of up to 60 min. Next, cells were quenched, and the levels of the indicated inositol phosphates (InsP_5, InsP_6, InsP_7, and InsP_8) were analyzed by HPLC (see "Experimental Procedures"). A and B show representative HPLC chromatographs for the 0- and 60-min time points; data for InsP_5 and InsP_8 are replotted on an expanded y axis scale. Data are representative of three biological replicates. C shows time course data (InsP_5, InsP_6, and InsP_7; mean ± S.E., n = 3) from all experiments. D and E show time course data (error bars represent S.E.) for intracellular levels of ATP and P_i (malachite green/molybdate method), respectively, determined in parallel experiments with non-radiolabeled cells (n = 6–7) incubated for the indicated times with 6 mM extracellular [P_i]. The data for cell P_i content (nmol/mg of protein) at 0, 15, 30, 45, and 60 min (error bars represent S.E.; 56 ± 4.3, 59 ± 4.7, 58 ± 3.3, and 60 ± 1.8) closely match the P_i levels that we obtained by an independent enzymatic method (54 ± 3.5, 57 ± 3.7, 56 ± 2.7, and 62 ± 4.3; n = 6–7). F shows time course data for InsP_8 (*, p < 0.05; **, p < 0.02; paired t test). G shows representative HPLC chromatographs from the region in which InsP_8 elutes (as indicated by the elution profile (open circles) for an [3H]InsP_8 standard from a parallel run); cell extracts (closed circles) were derived from [3H]inositol-labeled PPIPSK^-/- HCT116 cells (see Ref. 27) that had been incubated for 1 h with either 1 or 6 mM P_i, as indicated.
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hPPIP5K1: forward, TTTGTGTGCTGTGAAAT-C; reverse, GGAATGACATGGCAAGC; R399A: forward, TGCAATTGTGGCTGTGGACGTGGTA; reverse, ATGCCACACGAGATTTGC; hPPIP5K2: D321A: forward, CTATGGCTGCTATGCAGC; reverse, GACTGTCCATGGCAGG; R388A: forward, AGCTTATAGCTATGGGATGCAAAC; reverse, ATGACACATCTAAGTTCT-ATC. All constructs were confirmed by DNA sequencing.

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Kinase and Phosphatase Assays—PPIP5K kinase activities were routinely measured at 37 °C in 1–2 h incubations comprising 100 µl of assay buffer containing 1 mM Na2EDTA, 50 mM KCl, 20 mM HEPES (pH 7.2), 7 mM MgCl2, 5 mM ATP, 0.5 mg/ml BSA, and either 1 mM InsP6 (Calbiochem) or 1 mM chemically synthesized 5-InsP7 (47). PPIP5K phosphatase activities were measured at 37 °C in 30-min incubations comprising 100 µl of assay buffer containing 1 mM Na2EDTA, 50 mM KCl, 20 mM HEPES (pH 7.2), 2 mM MgCl2, 0.5 mg/ml BSA, and either 1 or 0.05 mM chemically synthesized 1-InsP6 (48) or InsP8 (30). Each incubation also contained ~2000 dpm of the corresponding 3H-labeled substrate. [3H]InsP6 was purchased from PerkinElmer Life Sciences; the [3H]inositol pyrophosphates were each prepared as described elsewhere (29).

DIPP1 phosphatase activity was measured at 37 °C in 30-min incubations comprising 100 µl of assay buffer containing 33 ng of enzyme, 1 µM [3H]InsP4 (approximately 4500 dpm), 1 mM Na2EDTA, 50 mM KCl, 20 mM HEPES (pH 7.2), 2 mM MgCl2, 0.5 mg/ml BSA, and, where indicated, 0.1–5 mM KH2PO4. All assays were quenched by addition of 0.2 volume of 2 M perchloric acid plus 1 mg/ml InsP6 and then neutralized (29). Reactions were then analyzed by HPLC chromatography using on a 4.6 × 125-mm Partisphere strong anion exchange HPLC column as previously described (29).

Author Contributions—C. G. performed most of the experiments and analyzed the results. H.-N. N. and H. W. also performed experiments and analyzed results. A. H. and H. J. J. synthesized essential reagents. C. G., H.-N. N., H. W., X. D., and S. B. S. contributed to project conception and the design of experiments. S. B. S. wrote most of the paper with contributions from all of the other coauthors.
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