We have characterized the positional specificity of the mammalian and yeast VIP/Diphosphoinositol pentakisphosphate kinase (PPIP5K) family of inositol phosphate kinases. We deployed a microscale metal dye detection protocol coupled to a high performance liquid chromatography system that was calibrated with synthetic and biologically synthesized standards of inositol pyrophosphates. In addition, we have directly analyzed the structures of biological inositol pyrophosphates using two-dimensional $^1$H-$^1$H and $^1$H-$^{31}$P nuclear magnetic resonance spectroscopy. Using these tools, we have determined that the mammalian and yeast VIP/PPIP5K family phosphorylates the 1/3-position of the inositol ring in vitro and in vivo. For example, the VIP/PPIP5K enzymes convert inositol hexaphosphatase to 1/3-diphosphoinositol pentakisphosphate. The latter compound has not previously been identified in any organism. We have also unequivocally determined that 1/3,5-(PP)$_2$IP$_4$ is the iso- meric structure of the bis-diphosphoinositol tetrakisphosphate that is synthesized by yeasts and mammals, through a collaboration between the inositol hexaphosphatase kinase and VIP/PPIP5K enzymes. These data uncover phylogenetic variability within the crown taxa in the structures of inositol pyrophosphates. For example, in the Dictyostelids, the major bis-diphosphoinositol tetrakisphosphate is 5,6-(PP)$_2$IP$_4$ (Laussmann, T., Eujen, R., Weisshuhn, C. M., Thiel, U., Falck, J. R., and Vogel, G. (1996) Biochem. J. 315, 715–725). Our study brings us closer to the goal of understanding the structure/function relationships that control specificity in the synthesis and biological actions of inositol pyrophosphates.

Signal transduction pathways frequently rely on a specific target protein recognizing a precise spatial arrangement of one or more phosphate groups on either another protein or a small metabolite. The six-carbon inositol ring offers what is arguably the most dramatic example of how even subtle modifications to phosphate topology can impart signaling specificity. The combinatorial manner in which phosphate groups can be arranged around the inositol skeleton creates a large family of phosphorylated molecules, many of which have individual, physiological roles (1). The inositol pyrophosphates, such as diphosphoinositol tetrakisphosphate (also known as PP-IP$_4$), PP-IP$_5$ (also known as IP$_7$) and (PP)$_2$IP$_4$ (also known as IP$_8$) (2, 3), are a specialized subgroup of the inositol-based signaling family that are distinguished by the presence of diphosphate groups. These particular molecules regulate a diverse range of cellular activities, including phosphate sensing, actin cytoskeleton dynamics, apoptosis, vesicle trafficking, transcription, and DNA repair (see Refs. 4 and 5 for reviews). The different isomers of inositol pyrophosphates can be distinguished by biological receptors (6, 7). Thus, there is great interest in understanding the structure/function relationships of protein interactions with the inositol pyrophosphate ligands.
Another reason to study the structure of the inositol pyrophosphates is to understand at a molecular level what factors dictate the positional specificity of the kinases and phosphatases that metabolize these polyphosphates. Furthermore, the structure of (PP)$_2$IP$_5$ has additional significance, because it can impact the degree of the free energy change during its hydrolysis (8). When one of the diphosphate bonds is cleaved, the ensuing relief of a portion of the electrostatic and steric congestion around the inositol ring will be substantially greater if the pyrophosphates in (PP)$_2$IP$_4$ are vicinal (8). This may have practical consequences; the higher the free energy of phosphate hydrolysis, the more thermodynamically favorable it becomes for the phosphate to be transferred to another cellular moiety (8).

In an earlier study (9), chromatographic comparisons of lysates from mammalian cell lines with synthetic PP-IP$_5$ standards indicated that 5-PP-IP$_5$ was the major isomer to accumulate. It has, therefore, generally been anticipated that the enzymes that phosphorylate IP$_6$ to PP-IP$_5$ would be 5-kinases. One group of these enzymes (IP6Ks; Kcs1 in Saccharomyces cerevisiae) was cloned by Snyder and co-workers (10, 11). Recombinant versions of these enzymes were recently used to generate milligram quantities of PP-IP$_5$ for analysis by one-dimensional (12) and two-dimensional (13) NMR spectroscopy; these experiments confirmed that the IP6Ks are indeed 5-kinases.

Indications that there is a separate class of enzymes capable of phosphorylating IP$_6$ to PP-IP$_5$ arose initially from experiments performed on a kcs1Δ/pp1Δ double gene deletion strain of S. cerevisiae (14). The genes that encode this IP$_6$ kinase activity have been identified in both S. cerevisiae (Vip1) (12) and in mammals (VIP1/PPIP5K1 and VIP2/PPIP5K2) (15, 16). These kinases also phosphorylate PP-IP$_5$ to (PP)$_2$-IP$_4$ (12, 15, 16). Indeed, the VIP/PPIP5K family appears to account for a novel PP-IP$_5$ kinase activity that was observed initially in experiments with cell lysates (17, 18) and subsequently in yeast mutants lacking Kcs1 (19). Previous one-dimensional $^{31}$P NMR studies demonstrated that yeast Vip1 phosphorylates IP$_6$ to a novel isomer of PP-IP$_5$ that is different from the 5-PP-IP$_5$ produced by recombinant human IP6K (12). However, the isomeric nature of this new PP-IP$_5$ isomer could not be unequivocally established by one-dimensional NMR (it was tentatively suggested to be 4/6-PP-IP$_5$ (12)). One of the goals of the current study was to unequivocally determine the structure of this novel PP-IP$_5$ isomer. This is a topic of current interest, because this new isomer regulates the transcriptional activity of a cyclin-cyclin kinase complex in S. cerevisiae (6, 7).

It was also our goal to address the lack of information concerning the position of the diphosphate groups on (PP)$_2$-IP$_4$. The structure of (PP)$_2$-IP$_4$ has previously only been determined in Dictyostelid cell extracts, since these organisms contain unusually high (300 μM) levels of this compound (20). For example, two-dimensional $^1$H/$^{31}$P NMR spectroscopy was used to demonstrate that the (PP)$_2$-IP$_4$ in Dictyostelium cell lysates has one diphosphate group at the 5-position and another at the 4/6-position (21). Another of the Dictyostelids, Polysphondylium, also contains 4/6,5-(PP)$_2$-IP$_4$ plus relatively minor quantities (12–17% of total) of an additional isomer, namely 1/3,5-(PP)$_2$-IP$_4$ (22). However, it is considerably more challenging to analyze the structures of inositol pyrophosphates in yeast and animal cells, which typically contain only 1–2 μM concentrations of these compounds (23). As a consequence, the structure of (PP)$_2$-IP$_4$ in mammalian cells has not previously been determined.

In the current study, we have developed a HPLC protocol to routinely identify the structures of the relatively low quantities of the inositol pyrophosphates that are present in most eukaryotic cells. Additionally, in experiments performed in vitro, we used recombinant kinases to generate sufficient quantities of (PP)$_2$-IP$_4$ for unequivocal structural analysis by two-dimensional NMR strategies. We show for the first time that yeast and mammalian VIP/PPIP5K enzymes are 1/3-kinases rather than having the 4/6-kinase activity that was tentatively proposed in an earlier study (12). Thus, the VIP/PPIP5K kinases phosphorylate IP$_6$ to 1/3-PP-IP$_5$. Finally, we demonstrate that VIP/ PPIP5K enzymes act in concert with IP6K proteins to synthesize (PP)$_2$-IP$_4$ that has diphosphates at the 5- and 1/3-positions.

**EXPERIMENTAL PROCEDURES**

**Preparation of Synthetic PP-IP$_5$ and (PP)$_2$-IP$_4$ Standards**—5,6-(PP)$_2$-IP$_4$ and 1/3,5-(PP)$_2$-IP$_4$ were isolated from Dictyostelium and Polysphondylium, respectively, and then purified as previously described (22). The synthesis of the 2,5-(PP)$_2$-IP$_4$ standard is described in the supplemental material. A mixture of PP-IP$_5$ and (PP)$_2$-IP$_4$ standards was prepared by a slight modification of previously published procedures (24, 25). Briefly, a 25-ml reaction was prepared that contained 0.5 mmol of IP$_6$, 10 mmol of phosphocreatine, and 50 mM NaCl. The pH was adjusted to 3.0 with HCl, and subsequently the mixture was dehydrated by overnight freeze-drying. The freeze-dried material was resuspended in 200 ml of water, and the mixture of PP-IP$_5$/ (PP)$_2$-IP$_4$ isomers were purified by Q-Sepharose chromatography.

**Preparation of Biological Isomers of PP-IP$_5$ and (PP)$_2$-IP$_4$**—Several different enzyme preparations were used to prepare inositol pyrophosphates for analysis by MDD-HPLC. In one series of experiments, (PP)$_2$-[H]IP$_4$ was prepared from full-length recombinant forms of human FLAG-tagged VIP1/ PPIP5K1 and, separately, from human FLAG-tagged VIP2/ PPIP5K2, both of which were expressed and immunopurified as described previously (15). These enzymes were individually incubated with approximately 4000 dpm of PP-[H]IP$_4$ (15) in 50–200 μl of a medium that was designed to maximize product yield from an impure enzyme preparation (e.g. it included an ATP regeneration system and NaF to inhibit phosphatase activity (26)): 1 mM EDTA, 50 mM KCl, 20 mM HEPES, pH 7.0, 12 mM MgSO$_4$, 10 mM NaATP, 20 mM phosphocreatine, 0.04 mg/ml creatine kinase, 0.6 mg/ml bovine serum albumin, 1 mM dithiothreitol, and 10 mM NaF. Reactions were quenched by boiling (4 min).

In a separate series of experiments, (PP)$_2$-IP$_4$ was prepared by incubating IP$_6$ with full-length recombinant human GST-IP6K1 plus either GST fusion constructs comprising residues 1–387 of the human VIP1/PPIP5K1 kinase domain or residues 1–535 of the S. cerevisiae Vip1 kinase domain; all of these enzymes were bacterially expressed and purified as previously described (12,
16). The incubation buffer contained 50 mM HEPES (pH 6.2), 50 mM NaCl, 5 mM MgCl₂, 30 mM phosphocreatine, 50 units of creatine kinase, 5 mM ATP, and 200 μM IP₆. Reactions were quenched by boiling (10 min). Parallel reactions were performed with 2-[³²P]IP₆ to monitor the progress of the reactions (16).

PP-IP₆ for NMR was prepared using recombinant GST-tagged ScVip1 kinase domain. In a 5-ml reaction, 10 μmol of IP₆ was incubated with 375 μg of GST-ScVip1 in buffer containing 50 mM HEPES (pH 7.0), 50 mM NaCl, 1.5 mM ATP, 3 mM MgCl₂, 24 mM creatine phosphate, and 250 units of creatine kinase. After 16 h at 37°C, the reaction was boiled for 10 min, and 350 μl of 1.5 M Tris-HCl (pH 8.8) was added along with 60 μl of 0.5 M EDTA (pH 8.0). The sample was centrifuged (12 min at 4,100 rpm), and the supernatant was passed through a 0.22-μm filter. The sample was then loaded onto a Mono Q HR 5/5 column (GE Life Sciences) and run with the following gradient: 0–10 min, 0–100% buffer B (0.5 M HCl, 15 mM YCl₃); gradient 1 was as follows: 0–2 min, 3–45% B; 2–2.5 min, 45–58% B; 2.5–2.8 min, 58–59% B; 2.8–9 min, 59–65% B; 9–12.5 min, 65–80% B; 12.5–13 min, 80–100% B; 13–16 min, 100% B; and 16.1–20 min, 3% B. Gradient 2 was as follows: 0–1 min, 3% B; 1–2 min, 3–50% B; 2–2.5 min, 50–58% B; 2.5–3 min, 58–59% B; 3–10 min, 59–60% B; 10–10.6 min, 60–65% B; 10.6–13.5 min, 65–80% B; 13.5–17 min, 80–100% B; 17–18.5 min, 100% B; and 18.6–23 min, 3% B. The flow rate was 0.5 ml/min.

The eluate from the column was mixed in-line with half of the volume of a buffer containing 300 μM 4-(2-pyridyldiazore)resorcinol in 1.6 M triethanolamine (pH 9.0 with HCl), and the degree of the postcolumn complexometric reaction was recorded by measuring absorbance at 546 nm (SPD-10Avvp; Shimadzu). Data were stored in a polarity-switched format (multiplied by -1) and exported into SigmaPlot for imaging. All samples were routinely mixed with our mixture of standards (see above) and rechromatographed; in no case did this cause any changes to the elution time of any of the peaks in the standards. In some experiments (gradient 2 only), the YCl₃ was omitted from the elution buffers; the 4-(2-pyridylazo)resorcinol was replaced by water; and 20 s fractions were collected and mixed with 2 ml of INSTA-GEL scintillant (Packard), and radioactivity was determined using liquid scintillation spectrometry.

Cell Culture and Extraction of Inositol Phosphates—NIH-Swiss 3T3 cells, H1299 cells, and MDA cells were all maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (Sigma) in 5% CO₂ at 37°C on 15-cm Petri dishes to about 70% confluence. Cells were treated with either vehicle or 10 mM NaF for 2 h. Subsequently, cells were rinsed with ice-cold phosphate-buffered saline and scraped into ice-cold lysis buffer (8% (w/v) trichloroacetic acid, 10 mM EDTA), followed by twice freezing and thawing in liquid nitrogen. After no more than 30 min, the precipitate was removed by centrifugation (3500 g for 10 min at 4°C), and the supernatants were extracted three times with 3 ml of water-saturated diethyl ether. After the pH had been adjusted to about 6 with Tris (0.5 M), the extract was concentrated in a SpeedVac.

RESULTS AND DISCUSSION

Phylogenetic Differences in Inositol Pyrophosphate Structure—The ability to separate and quantify inositol phosphates and inositol pyrophosphates is critically important for understanding cellular metabolism and signaling regulatory networks. The recent increased interest in the biology of inositol pyrophosphates (4) has made it especially important to determine the structures of each species within this diverse array of molecules. For example, Albert et al. (9) previously employed a MDD HPLC method for resolving different isomers of PP-IP₆. We have now further developed the MDD-HPLC protocol for routine separation of (PP)₂-IP₆ isomers.

To calibrate our chromatography system, a mixture of PP-IP₆ and (PP)₂-IP₆ isomers were prepared in “shotgun” fashion.
Structure of Inositol Pyrophosphates

from IP₆ by a nonenzymatic, dehydration-driven transphosphorylation reaction that used creatine phosphate as a phosphate donor (see “Experimental Procedures”). This mixture was resolved by MDD-HPLC into eight different peaks that eluted after IP₆ (labeled 1–8 in Fig. 1). There was some column-to-column variability in the absolute elution positions of the various peaks (compare A, B, and C in Fig. 1, which include data obtained at various times during a 4-year period). Nevertheless the elution pattern of our standard mixture was highly reproducible. Peak 1 contains 4/6-PP-IP₅ and a leading shoulder of the elution pattern of our standard mixture was highly reproducible. Peak 1 contains 4/6-PP-IP₅ and a leading shoulder of the elution pattern of our standard mixture was highly reproducible. Nevertheless the elution times of the different peaks within the standard mixture reflect column-to-column variation during the 4-year period in which these data were obtained.

It has previously (12, 15, 16, 31) been determined that the IP₆K and VIP/PPIP5K enzymes work in concert to generate (PP)₂-IP₄. Thus, when IP₆ was incubated with both human IP₆K and ScVip1, three products accumulated (labeled peaks i, ii, and iii in Fig. 1D). Peak i co-eluted with the PP-IP₅ product that was formed when IP₆ was phosphorylated by human IP₆K alone (data not shown; this material has previously been validated by NMR to be 5-PP-IP₅ (13)). Peak ii co-eluted with the (PP)₂-IP₄ product that was formed when IP₆ was incubated with yeast Vip1 alone (data not shown; this PP-IP₅ coeluted with a synthetic 1/3-PP-IP₅ standard (data not shown), and in addition it was validated by NMR to be 1/3-PP-IP₅ (see below). Peak iii contains the (PP)₂-IP₄ that was formed in these experiments, and this co-eluted with peak 7 of our standard mixture (Fig. 1D). This is also the elution point for a two-dimensional NMR-characterized 1/3,5-(PP)₂-IP₄ standard from Polysphondylium (Fig. 1A). These HPLC data provide the first evidence that ScVip1 is a 1/3-kinase (and see Fig. 2).

Is the positional specificity of Vip1 from yeast conserved in mammals? To answer this question, we next characterized the nature of the (PP)₂-IP₄ produced from 5-PP-IP₅ upon its phosphorylation by full-length, recombinant human VIP1/PPIP5K1 (Fig. 3A) and, in separate assays, VIP2/PPIP5K2 (Fig. 3B). These enzymes were expressed in HEK cells with an N-terminal FLAG epitope for subsequent immunopurification. For these experiments, we incubated each of the purified kinases with [³H]labeled 5-PP-IP₅ (Fig. 3). The possible products of this reaction were 4/6,5-(PP)₂-IP₄ (equivalent to peak 3 in our standard mixture), 2,5-(PP)₂-IP₄ (peak 8), and 1/3,5-(PP)₂-IP₄ (peak 7). The only (PP)₂-IP₄ product to accumulate was 1/3,5-(PP)₂-IP₄ (Fig. 3). In further experiments, we also incubated IP₆ with IP₆K plus the catalytic domain of human VIP1/PPIP5K1 that was expressed in Escherichia coli; MDD-HPLC analysis indicated that 1/3,5-(PP)₂-IP₄ was again the only isomer of (PP)₂-IP₄ to accumulate (data not shown). These results indicate that the mammalian VIP/PPIP5K enzymes have the same positional specificity as their yeast homologue.
**Structure of Inositol Pyrophosphates**

Analysis of the Structures of IP<sub>6</sub>, PP-IP<sub>5</sub>, and (PP)<sub>2</sub>-IP<sub>4</sub> by Two-dimensional NMR—Provided that milligram quantities of pure inositol phosphates can be prepared, NMR can be used to analyze their structure (32, 33). We used ³¹P and ¹H one- and two-dimensional NMR analysis to determine the structures of the two inositol pyrophosphates (PP-IP<sub>5</sub> and (PP)<sub>2</sub>-IP<sub>4</sub>) produced by the VIP1/PIP5K family (see “Experimental Procedures” and Fig. 3). HPLC-purified IP<sub>6</sub> was also used as a standard. The ¹H-decoupled ³¹P NMR spectrum of IP<sub>6</sub> at pH 6 (Fig. 4A) reveals ³¹P signals at 2.82, 1.93, and 1.54 ppm in the ratio 1:2:3, corresponding to six phosphate signals. A 1:2:2:1 pattern was previously observed at pH 11 (21) and is expected from equivalence of the P-1/3 and P-4/6 pairs due to the symmetry of the inositol ring along an axis through positions 2 and 5. The ³¹P spectrum at pH 6 is explained by merging of the upfield single phosphate signal with the upfield double phosphate signal. The ³¹P NMR spectrum of PP-IP<sub>5</sub> (Fig. 4B), in contrast, reveals five individual phosphate singlets (2.91, 2.00, 1.66, 1.41, and 0.73 ppm) and two pyrophosphate doublets (−7.95 and −9.77 ppm, J<sub>PP</sub> = 20.4 Hz). The five distinct singlets indicate that the PP-IP<sub>5</sub> molecule no longer possesses the axis of symmetry, indicating that a pyrophosphate group is added at either position 1, 3, 4, or 6. The ³¹P NMR spectrum of (PP)<sub>2</sub>-IP<sub>4</sub> (Fig. 4C) reveals four individual phosphate singlets (1.87, 1.57, 1.20, and 0.57 ppm) and four pyrophosphate doublets (−7.90, −8.04, −9.24, and −9.62 ppm, J<sub>PP</sub> = 20.4 and 18.7 Hz). This ³¹P NMR spectrum is ambiguous for positional assignments, and we therefore turned to ¹H NMR.

The ¹H NMR spectrum of our IP<sub>6</sub> standard at pH 6.0 (Fig. 4D) reveals a distinct double-triplet near 5.03 ppm, a two-proton quartet near 4.53 ppm, and three proton resonances that overlap near 4.28 ppm. The 5.03 ppm double-triplet is assigned to H-2, since this is the sole equatorial proton on the inositol ring, and will be shifted downfield relative to the five other protons that are in axial positions (33). The 9.5 Hz splitting is identified as a proton-phosphorus coupling (J<sub>HP</sub>) from ³¹P decoupling experiments (see below and Fig. 6). The 2.4 Hz splitting is recognized as the equatorial-axial coupling from H-2 to H-1/3 (<J<sub>HP</sub>, and J<sub>HP</sub>). Two of the three resonances at 4.27 ppm show triple-triplet line shape with 2 Hz axial-equatorial coupling, indicating that these arise from H-1 and H-3. These resonances are chemically equivalent due to the axis of symmetry through positions 2 and 5. The two-proton quartet with large proton-phosphorus coupling (~9 Hz) and axial-axial proton-proton coupling (~9 Hz) likewise must be chemically equivalent H-4 and H-6 resonances due to the symmetry axis. The remaining one-proton quartet at 4.28 ppm with large proton-phosphorus and axial-axial couplings is therefore assigned as H-5. Comparison with the reported IP<sub>6</sub> spectrum at pH 5 (33) confirmed assignment of the 5.03 ppm double-triplet to H-2. In the previously described IP<sub>6</sub> spectrum at pH 5 (33), the chemically equivalent H-1 and H-3 triple-triplets appear at a chemical shift of ~4.21 ppm (scale corrected from HOD solvent signal at 4.67 ppm to HOD at 4.784 ppm) and overlap almost completely with the H-5 quartet. This accords with the spectrum of our IP<sub>6</sub> standard at pH 6, in which the H-1, H-3, and H-5 resonances overlap at 4.27 ppm. This leaves the remaining two-proton quartet signal near 4.53 ppm assigned to H-4 and H-6.

Subsequent comparison of the ¹H NMR spectra of the kinase products with the IP<sub>6</sub> spectrum allows deductions on the ring positions of the pyrophosphate groups. The ¹H NMR spectrum of PP-IP<sub>5</sub> (Fig. 4E) shows the double-triplet near 5.08 ppm (H-2), two overlapped multiplets near 4.55 ppm (H-4/6), a resolved triple-triplet near 4.37 ppm (H-1/3), and an overlapped quartet at 4.31 ppm (H-5) and a hidden triple-triplet at 4.29 ppm (H-1/3) (Fig. 4). The 4.37 ppm triple-triplet features the small (~2 Hz) equatorial-axial coupling and is assigned as either H-1 or H-3, whereas the 4.31 ppm quartet features only large proton-phosphorus and axial-axial couplings and is assigned as H-5. Since the resolved triple-triplet (4.37 ppm) is well shifted from the hidden triple-triplet (4.28 ppm), the PP-IP<sub>5</sub> very likely has its pyrophosphate group at the 1- or 3-position. NMR cannot further distinguish between these two alternatives, due to the symmetry axis of the inositol ring. The ¹H NMR spectrum of (PP)<sub>2</sub>-IP<sub>4</sub> (Fig. 4F) reveals the H-2 double-triplet near 5.07 ppm, two overlapped multiplets near 4.63 ppm (H-4/6), a distinct quartet near 4.48 ppm, and two resolved triple-triplets at 4.43 and 4.33 ppm. The two triple-triplets with the ~2 Hz couplings are clearly the H-1/3 pair,
leaving the 4.48 ppm quartet to be assigned to H-5. Thus, the 

\[(PP)_2\text{IP}_4\] probably has its pyrophosphate groups at the 1/3- and 5-positions.

The \(^1\)H NMR assignments of the chemically distinct protons on the inositol ring of \(\text{IP}_6\), \(\text{PP-IP}_5\), and \(\text{(PP)}_2\text{IP}_4\) were all further confirmed by two-dimensional COSY (21, 22, 33) and are discussed here only for \(\text{(PP)}_2\text{IP}_4\). COSY cross-peaks from the H-2 double-triplet (5.07 ppm) assign the 4.33 and 4.43 ppm proton multiplets as the H-1/3 peak pairs. The latter each show cross-peaks to two proton multiplets at 4.64 ppm, thus assigning the 4.64 ppm resonances to H-4 and H-6. The H-4/6 multiplets in turn show a cross-peak with the quartet at 4.47 ppm, which is confirmed as H-5 (22, 33).

With \(^1\)H NMR assignments at hand, two-dimensional \(^1\)H-\(^{31}\)P HMQC spectra were pursued to obtain \(^{31}\)P NMR assignments. HMQC for IP\(_6\) (Fig. 5A) revealed cross-peaks from the 4.28 ppm quartet (H-5) to the single phosphorus signal at 2.82 ppm (P-5), from the 2-proton quartet at 4.53 ppm (H-4/6) to the two-phosphorus signal at 1.93 ppm (P-4/6), and from the 5.03 ppm double-triplet (H-2) and equivalent 4.27 ppm triple-triplets (H-1/3) to the three-phosphorus signal at 1.54 ppm. HMQC for \(\text{PP-IP}_5\) (Fig. 5B) reveals the \(\alpha\)-pyrophosphate peak at \(-9.77\) ppm to show a prominent cross-peak with the previously assigned H-1/3 \(^1\)H peak (4.37 ppm), confirming that the pyrophosphate group on \(\text{PP-IP}_5\) is at the 1- or 3-position. The H-2 \(^1\)H double-triplet (5.07 ppm) shows a distinct HMQC cross-peak to the 0.73 ppm \(^{31}\)P signal, assigning it to P-2. The H-4/6 multiplets (4.55 ppm) show connectivities to the 2.00 and 1.66 ppm \(^{31}\)P signals, assigning these as the P-4/6 pairs. The H-5 quartet (4.30 ppm) shows a cross-peak to the 2.92 ppm \(^{31}\)P signal, assigning it to P-5. The remaining H-1/3 peak (4.29 ppm) correlates to the remaining \(^{31}\)P signal at 1.41 ppm as a P-1/3 peak. HMQC for \(\text{(PP)}_2\text{IP}_4\) (Fig. 5C) shows the \(\alpha\)-pyrophosphate peaks at \(-9.24\) and \(-9.62\) ppm with strong cross-peaks to the previously assigned H-1/3 and H-5 \(^1\)H peaks, confirming that the pyrophosphate groups on \(\text{(PP)}_2\text{IP}_4\) are at the 5- and 1- or 3-positions. The H-2 \(^1\)H double-triplet (5.07 ppm) shows a distinct HMQC cross-peak to the 0.57 ppm \(^{31}\)P signal, assigning it to P-2. The H-4/6 multiplets (4.64 ppm) show connectivities to the 1.57 and 1.20 ppm \(^{31}\)P signals,
assigning these as the P-4/6 pairs. The remaining H-1/3 peak (4.33 ppm) correlates to the remaining 31P signal at 1.81 ppm as a P-1/3 peak.

Two additional decoupling strategies employing highly digitized one-dimensional spectra were then pursued. These experiments remove interpretative ambiguities from limited resolution in the two-dimensional data and allowed direct measurement of the relevant coupling constants. In the first strategy, one-dimensional 1H NMR spectra incorporating selective 31P decoupling were collected and are illustrated first for IP6 and second for (PP)2-IP4 (Fig. 6). Selective decoupling of the 1.54 ppm three-phosphate 31P signal of IP6 (P-2; P-1 and P-3) (Fig. 6A) removes the phosphorus-proton coupling (9.9 Hz) and simplifies the double-triplet (5.03 ppm; H-2) to a simple triplet with axial-equatorial coupling (2.4 Hz) and the triple-triplet to a double-triplet (4.27 ppm; H-1 and H-3) with axial-axial and axial-equatorial couplings. Selective decoupling of the 1.93 ppm two-phosphate 31P signal (P-4/6) (Fig. 6B) removes the phosphorus-proton coupling (9.5 Hz) and leaves a simplified triplet at 4.53 ppm (H-4/6) with axial-axial proton-proton couplings. Similarly, selective decoupling of the 2.82 single 31P signal (P-5) (Fig. 6C) pinpoints the location of a 1H triplet (H-5) at 4.27 ppm with axial-axial proton-proton couplings (9.5 Hz). Selective decoupling of the 0.57 ppm 31P signal (P-2) of (PP)2-IP4 (Fig. 6D) removes the phosphorus-proton coupling (9.9 Hz) and simplifies the double-triplet (H-2) to a simple triplet, with small axial-equatorial coupling (2.4 Hz). The splittings of the other proton peaks are not affected, and subtraction of a control spectrum gives complete cancellation in the 4.3–4.7 ppm region of the difference spectrum (data not shown). Selective decoupling of the 1.87 ppm 31P signal (P-1/3) removes the phosphorus-proton coupling (9.5 Hz) and leaves a simplified double-triplet at 4.33 ppm (H-1/3) with proton-proton couplings of 9.5 and 2.4 Hz. Similarly, selective decoupling of the 1.57 and 1.20 ppm 31P signals (P-6 and P-4) pinpoints the location of 1H triplets at 4.629 and 4.641 ppm, respectively, with large axial-axial proton-proton couplings (9.5 Hz). Selective decoupling of the 0.57 ppm 31P signal of (PP)2-IP4 (Fig. 6E) collapses the 4.473 ppm quartet (H-5) to a triplet with 10.5 Hz proton-proton coupling. Selective decoupling of the 4.43 ppm complex triplet (H-3/H-1) into a “doublet” with one large proton-proton coupling (9.4 Hz) and an unresolved proton-proton coupling (2.4 Hz).

In the second strategy, one-dimensional 31P NMR spectra with selective 1H decoupling were collected and are only discussed for (PP)2-IP4 (spectra not shown). The 1H-coupled 31P NMR spectrum reveals doublets for each phosphomonoester signal and double-doublets for each pyrophosphate signal. Selective 1H decoupling of the 5.07 ppm 31H signal (H-2) removes the large phosphorus-proton coupling (9.87 Hz) and simplifies the 0.57 ppm 31P doublet (P-2) to a singlet with no other changes in the 31P NMR spectrum. The splittings of the other phosphorus peaks are unaffected, and subtraction of a control spectrum gives complete cancellation in all other

![FIGURE 5. Two-dimensional 1H-31P HMQC spectra at pH 6 of IP6, PP-IP5, and (PP)2-IP4. A, IP6; B, PP-IP5; C, (PP)2-IP4. Singlet monophosphate peaks that could not be unequivocally assigned from the two-dimensional spectra were assigned by selective 31P-decoupled 1H spectra.](image-url)
regions of the difference spectrum (data not shown). Selective $^1$H decoupling of the two overlapped 4.64 ppm $^1$H multiplets (H-6 and H-4) similarly simplifies the 1.20 and 1.57 $^{31}$P doublets (P-6 and P-4) to singlets. Selective decoupling of the 4.33 ppm complex triplet (H-3/H-1) collapses the 1.87 ppm $^{31}$P double-doublet to a singlet (P-3/P-1). Selective decoupling of the 4.47 ppm quartet (H-5) collapses the −9.24 ppm $^{31}$P double-doublet to a doublet (P-5α). Selective decoupling of the 4.43 ppm triple-triplet (H-1/3) collapses the −9.62 ppm $^{31}$P double-doublet to a

\[ \text{Table 1} \]

| Position | $\delta H$ | $^{3}J_{H-H}$ | $\delta P$ | $^{3}J_{P-H}$ | $\delta P$ |
|----------|----------|-------------|---------|------------|----------|
| 1/3      | 1.536    | 9.5         | 1.536   | 9.5        |
| 2        | 1.536    | 9.5         | 1.536   | 9.5        |
| 3/1      | 1.536    | 9.5         | 1.536   | 9.5        |
| 4/6      | 1.536    | 9.5         | 1.536   | 9.5        |
| 5        | 1.536    | 9.5         | 1.536   | 9.5        |
| 6/4      | 1.536    | 9.5         | 1.536   | 9.5        |

a Error in coupling constant from one-dimensional $^1$H NMR data is 0.16 Hz.
b Error in coupling constant from one-dimensional $^{31}$P NMR data is 0.25 Hz.

\[ \text{Table 2} \]

| Position | $\delta H$ | $^{3}J_{H-H}$ | $\delta P$ | $^{3}J_{P-H}$ | $\delta P$ |
|----------|----------|-------------|---------|------------|----------|
| 1/3      | 1.536    | 9.5         | 1.536   | 9.5        |
| 2        | 1.536    | 9.5         | 1.536   | 9.5        |
| 3/1      | 1.536    | 9.5         | 1.536   | 9.5        |
| 4/6      | 1.536    | 9.5         | 1.536   | 9.5        |
| 5        | 1.536    | 9.5         | 1.536   | 9.5        |
| 6/4      | 1.536    | 9.5         | 1.536   | 9.5        |

a Error in coupling constant from one-dimensional $^1$H NMR data is 0.16 Hz.
b Error in coupling constant from one-dimensional $^{31}$P NMR data is 0.25 Hz.

\[ \text{Table 3} \]

| Position | $\delta H$ | $^{3}J_{H-H}$ | $\delta P$ | $^{3}J_{P-H}$ | $\delta P$ |
|----------|----------|-------------|---------|------------|----------|
| 1/3      | 1.536    | 9.5         | 1.536   | 9.5        |
| 2        | 1.536    | 9.5         | 1.536   | 9.5        |
| 3/1      | 1.536    | 9.5         | 1.536   | 9.5        |
| 4/6      | 1.536    | 9.5         | 1.536   | 9.5        |
| 5        | 1.536    | 9.5         | 1.536   | 9.5        |
| 6/4      | 1.536    | 9.5         | 1.536   | 9.5        |

a Error in coupling constant from one-dimensional $^1$H NMR data is 0.16 Hz.
b Error in coupling constant from one-dimensional $^{31}$P NMR data is 0.25 Hz.
MDD-HPLC to investigate if the spectrum of (PP)2-IP4 iso-and axial-axial proton-proton interactions (3 of the large couplings observed from the proton-phosphorus figures and “Results”).

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The complex splitting patterns of the peaks seen in the one-dimensional 1H spectra can now be understood. Consideration of the large couplings observed from the proton-phosphorus and axial-axial proton-proton interactions (3) about 9 Hz leads to predictable doublet, triplet, and quartet peaks at the H-2, H-1/3, and H-4/H-5/H-6 positions, respectively. The presence of the smaller coupling (2.4 Hz) adds fine splittings at the H-2 and H-1/3 positions, resulting in the “small triplets,” as expected from previous studies (33). The results of the two decoupling strategies corroborate and validate the HMQC and COSY NMR data, reinforcing our conclusion that the (PP)2-IP4 has its pyrophosphate groups at the 1/3- and 5-positions.

One final point to emerge from these NMR experiments was the absence of any signals indicating that a triphosphate was formed (the estimated limit of detection is 2% of total products). This finding indicates another difference between the VIP/PPIP5K family and the IP6K family, in that the latter can convert IP6 to a limited quantity of the triphosphate, 5-PPP-IP5 (22) also differs from that in mammalian cells (5-PP-IP5 and 6-PP-IP5) (22) also differs from that in mammalian cells (5-PP-IP5 and 1/3-PP-IP5, see above). In fact, our work represents the first identification of the presence of 1/3-PP-IP5 in any organism. These phylogenetic differences are especially intriguing when considering that many of the mammalian inositol lipids and inositol phosphates are conserved in slime molds. Indeed, phylogenetic analysis has placed the Dictyostelids in the so-called “crown taxa” that includes green plants, fungi, and animals (34). Nevertheless, our data now suggest that previous (35) use of NaNF has therefore unmasked an ongoing flux through this metabolite in vivo. In three different cell types, the NaNF treatment also caused a single (PP)2-IP4 peak to accumulate (Fig. 7). Its co-elution with peak 7 from our standard mixture is consistent with this material being 1/3,5-(PP)2-IP4 (e.g. see Fig. 1A). These experiments verify the significance in vivo of the (PP)2-IP4 product that we have defined to be produced in vitro by the VIP/PPIP5K class of inositol phosphate kinases.

General Conclusions — The universality and versatility of inositol-based signaling owes much to the wealth of second messengers that are synthesized by combinatorial phosphorylation of the six hydroxyl groups that are situated around the inositol ring. In the current study, we characterize a new aspect of this diversity by determining the spatially specific placement of pyrophosphate groups by the VIP/PPIP5K family. We report that the yeast and mammalian VIP/PPIP5K proteins are 1/3-kinases, thereby contributing to the synthesis of 1/3-PP-IP5 and 1/3,5-(PP)2-IP4 in vivo. This finding is significant for a number of reasons.

First, we find that yeast and mammals do not synthesize 5,6-(PP)2-IP4, which is the exclusive isomer of (PP)2-IP4 in several species of slime molds (21, 22) and the major isomer (>80% of total (PP)2-IP4) in others (two species of Polyphondylium). The pattern of PP-IP5 isomers in Dictyostelium (5-PP-IP5 and 6-PP-IP5) (22) also differs from that in mammalian cells (5-PP-IP5 and 1/3-PP-IP5; see above). In fact, our work represents the first identification of the presence of 1/3-PP-IP5 in any organism. These phylogenetic differences are especially intriguing when considering that many of the mammalian inositol lipids and inositol phosphates are conserved in slime molds. Indeed, phylogenetic analysis has placed the Dictyostelids in the so-called “crown taxa” that includes green plants, fungi, and animals (34). Nevertheless, our data now suggest that previous (35) use of Dictyostelium as a model system for probing mechanisms of actions of inositol pyrophosphates may not actually be applicable to mammalian cells.

Second, the vicinal diphosphate groups in 5,6-(PP)2-IP4 give this molecule a significantly higher standard free energy of hydrolysis than the 1/3,5-PP-IP5 in yeast and mammalian cells (8). This may provide slime molds with a more energetically efficient donor molecule for transphosphorylation reactions,
such as those that directly phosphorylate certain proteins, in vitro at least (36, 37).

Third, the availability of the structure of both (PP)$_2$-IP$_4$ (this study) and DIPP1 (Protein Data Bank entry 2FVV) provides new opportunities to understand some enigmatic aspects of this hydrolase activity. For example, modeling of substrate into the active site may help us understand why the positional specificity of DIPP varies depending upon whether (PP)$_2$-IP$_4$ or PP-IP$_5$ is the substrate (17). This modeling might also help explain why F84Y and H91L mutations in DIPP specifically compromise (PP)$_2$-IP$_4$ hydrolysis but leave PP-IP$_5$ hydrolysis largely unaffected (38). The increased understanding of the structures of inositol pyrophosphates described in the current study will also help us understand the structural basis for their synthesis and the development of isomers of inositol pyrophosphates and the development of the transcriptional activity of a cyclin-cyclin-dependent kinase complex that controls cellular phosphate accumulation (6, 7).

Finally, our studies also represent a significant step forward to the goal of the chemical synthesis of physiologically relevant isomers of inositol pyrophosphates and the development of structurally based inhibitors of the metabolism and action of this class of molecules. This is likely to be a field of growing interest, since evidence that the inositol pyrophosphates are important cellular regulators continues to accumulate (4).

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