The Inositol Hexakisphosphate Kinase Family

CATALYTIC FLEXIBILITY AND FUNCTION IN YEAST VACUOLE BIOGENESIS*

Saiardi et al. (Saiardi, A., Erdjument-Bromage, H., Snowman, A., Tempst, P., and Snyder, S. H. (1999) Curr. Biol. 9, 1323–1326) previously described the cloning of a kinase from yeast and two kinases from mammals (types 1 and 2), which phosphorylate inositol hexakisphosphate (InsP$_6$) to diphosphoinositol pentakisphosphate, a "high energy" candidate regulator of cellular trafficking. We have now studied the significance of InsP$_6$ kinase activity in Saccharomyces cerevisiae by disrupting the kinase gene. These ip6Δ cells grew more slowly, their levels of diphosphoinositol polyphosphates were 60–80% lower than wild-type cells, and the cells contained abnormally small and fragmented vacuoles. Novel activities of the mammalian and yeast InsP$_6$ kinases were identified; inositol pentakisphosphohydrolase (InsP$_5$) was phosphorylated to diphosphoinositol tetrakisphosphate (PP-InsP$_4$), which was further metabolized to a novel compound, tentatively identified as bis-diphosphoinositol trisphosphate. The latter is a new substrate for human diphosphoinositol polyphosphate phosphohydrolase. Kinetic parameters for the mammalian type 1 kinase indicate that InsP$_5$ (K$_m$ = 1.2 mM) and InsP$_6$ (K$_m$ = 6.7 mM) compete for phosphorylation in vivo. This is the first time a PP-InsP$_4$ synthase has been identified. The mammalian type 2 kinase and the yeast kinases are more specialized for the phosphorylation of InsP$_6$ Synthesis of the diphosphorylated inositol phosphates is thus revealed to be more complex and interdependent than previously envisaged.

The very dynamic turnover of the "high energy" diphosphorylated inositol polyphosphates (PP-InsP$_4$, PP-InsP$_5$, and [PP]$_2$-InsP$_4$)$^1$ may represent a molecular switching activity that regulates intracellular trafficking (see Ref. 1 for a review). For example, PP-InsP$_5$ represents the most potent known inhibitor of AP180-mediated assembly of clathrin cages, a key step in the endocytic retrieval of discharged synaptosomal vesicles (2). Other proteins that participate in intracellular trafficking can bind PP-InsP$_5$ very tightly, including coatamer (3, 4) and AP2 (5). The high affinity with which PP-InsP$_5$ binds to myelin proteolipid protein may be important for the vesicular delivery of the latter to the myelin sheath (6).

Prior experiments with intact cells have shown that Ins(1,3,4,5,6)P$_5$ and InsP$_6$ serve as metabolic stockpiles for the formation of the diphosphorylated inositol polyphosphates (7, 8). InsP$_6$ is the precursor for PP-InsP$_5$, which is further phosphorylated to [PP]$_2$-InsP$_4$ (5, 7–10). These reactions appear to take place within a metabolic pool that is separate from that in which Ins(1,3,4,5,6)P$_5$ and PP-InsP$_5$ are interconverted (7). Thus, there are two metabolic pools of inositol diphosphates that are turned over in parallel cycles.

There are increasing efforts to characterize the activities of the enzymes that regulate the turnover of PP-InsP$_5$, PP-InsP$_6$, and [PP]$_2$-InsP$_4$. Several phosphatases (diphosphoinositol polyphosphate phosphohydrolases) that hydrolyze these compounds have been described (11, 12). Two forms of InsP$_6$ kinase (types 1 and 2), derived from distinct genes, have been cloned from mammals (13, 14). At least in mammals, the further phosphorylation of PP-InsP$_5$ to [PP]$_2$-InsP$_4$ appears to be the function of a separate enzyme that has been purified from rat brain (9) but not yet cloned. A yeast InsP$_6$ kinase has also been cloned that shows approximately 30% sequence similarity to the two mammalian InsP$_6$ kinases (13). We also recently reported that PP-InsP$_5$ and [PP]$_2$-InsP$_4$ are present in Saccharomyces cerevisiae (15).

A notable omission from our understanding of the intricacies of the turnover of higher inositol phosphates is any characterization of the enzyme(s) that phosphorylate Ins(1,3,4,5,6)P$_5$ to PP-InsP$_5$. Thus, an important goal of this study was to identify this "missing link" in inositide research: the PP-InsP$_5$ synthase. We now report that PP-InsP$_5$ in mammals is synthesized by the type 1 and type 2 forms of the InsP$_6$ kinase. In addition, we show that these enzymes can further phosphorylate PP-InsP$_5$, thereby forming a hitherto unknown inositol phospholipase.

We have further investigated the activity of the enzyme in yeast. We have characterized its substrate specificity in vitro. In this study, we demonstrate that disrupting the InsP$_6$ kinase gene of S. cerevisiae (ip6Δ) influences inositol polyphosphate levels in a unique fashion. We also show that ip6Δ yeast cells have fragmented vacuoles. These data support an important role for diphosphoinositol polyphosphates in regulating intracellular trafficking.

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The abbreviations used are: PP-InsP$_4$, diphosphoinositol tetraakisphosphate; PP-InsP$_5$, diphosphoinositol pentakisphosphate; [PP]$_2$-InsP$_4$, bis-diphosphoinositol tetrakisphosphate; [PP]$_2$-InsP$_5$, bis-diphosphoinositol tetrakisphosphate; carboxy-DCFDA, 5- (and 6-)carboxy-2',7'-dichlorofluorescein diacetate; carboxy-DCF, 5- (and 6-)carboxy-2',7'-dichlorofluorescein; hDPP, human diphosphoinositol polyphosphate phosphohydrolase; Ins(3,4,5,6)P$_4$, d-myo-inositol 3,4,5,6-tetrakisphosphate; InsP$_4$, inositol hexakisphosphate; InsP$_5$, inositol pentakisphosphate; Ins(1,3,4,5,6)P$_5$, d-myo-inositol 1,3,4,5,6-pentakisphosphate; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid.
EXPERIMENTAL PROCEDURES

Enzyme Assays—The various InsP6 kinases used in this study were incubated for various times at 37 °C in 25 or 50 μl of buffer containing 20 mM HEPES, pH 7.0, with KOH, 12 mM MgSO₄, 1 mM dithiothreitol, 10 mM ATP, 20 mM phosphocreatine, 1 mM EDTA, 0.02 mg/ml phosphocreatine kinase (Calbiochem 238395) and 0.5 mg/ml bovine serum albumin. The appropriate [3H]labeled inositol phosphate was added as indicated. Assays were quenched with ice-cold perchloric acid and neutralized as described previously (16).

Where specifically indicated, reactions were quenched by incubation for 3 min at 100 °C. Control experiments showed that all kinase activity was inactivated by this heat treatment, but none of the diposphorylated inositol phosphates were degraded.

HPLC Analyses—The HPLC analysis of [3H]inositol-labeled yeast cells was performed as described previously (15). Assays of the activities of recombinant enzymes employed HPLC using a Partisil SAX column (Krackeler Scientific, Durham, NC) that was eluted with a gradient generated by mixing Buffer A (1 mM Na₂EDTA) and Buffer B (Buffer A plus 1.3 m (NH₄)₂HPO₄, pH 3.85, with H₃PO₄) as follows: 0–5 min, 0% B; 5–10 min, 0–45% B; 10–60 min, 45–100% B; 60–70 min, 100% B. 1-ml fractions were collected. In some experiments, particularly when a new HPLC column was installed, the percentage of B at 10 min was increased to 50%. This change in the gradient, plus the tendency of inositol phosphates to elute earlier as the column aged, means that slightly different elution properties are seen in the different figures shown in this study.

Preparations of Recombinant Proteins—Recombinant hDIPP2a was prepared as described previously (11). The cDNAs for mammalian InsP6 kinases, type 1 and 2 were recovered from the pCMV-glutathione S-transferrase vector used previously (13). The type 1 kinase cDNA was amplified using the following primers: 5′-GACGCAGGAAATGTTGTGTTGTCAACAG-3′ and 5′-GCTAAGCTTGGGCTACTGGTTCTC-3′; the polymerase chain reaction product was subcloned into the Xhol and HindIII sites of the pTrHisB expression vector (Invitrogen). The cDNA for the type 2 kinase was amplified using the following primers: 5′-CGACCTCGAGAATGTGTGTGCTC-3′ and 5′-GCTAAGCTTGGGCTACTGGTTCTC-3′; the polymerase chain reaction product was subcloned into the Xhol and BstXI restriction sites of pTrHisB. The methods used to transform Escherichia coli (strain BL21), to induce isoosopropyl-1-thio-β-D-galactopyranoside, and to isolate the poly-(His)-tagged proteins using Talon resin (CLONTECH), were all according to the manufacturer’s recommendations. The recombinant yeast InsP6 kinase was produced as described previously (13).

Creation of the ip6kΔ Strain of S. cerevisiae—The YDR017C open reading frame in S. cerevisiae comprises the kcs1 gene that encodes the yeast InsP6 kinase. Using strain P69-2A of S. cerevisiae, the portion of the kcs1 gene that encodes the C terminus of the kcs1 protein from amino acid 575 to the C-terminal stop codon was replaced by the dominant ban marker gene using the KanMX4 expression construct as described previously (17). The oligonucleotides used for the gene disruption were: 5′-GAGAGAAAGAAATCTATACAACTCAATGGGAAAACATAATGATAGCCACTGGATGAC-3′ and 5′-TAAAAGCCGGCTAAAGAATATGCAATTTTCTGTTTCACTGGA-3′.

Northern Analysis of Wild-type and ip6kΔ yeast—10 μg of total RNA prepared from exponentially growing yeast was fractionated on a 1% agarose/MOPS-formaldehyde gel and transferred to Hybond N+ membranes, according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The blot was hybridized with a 1.2-kilobase BamHI-HindIII fragment corresponding to the 3′-region of the gene. This fragment was obtained by the digestion of the CDNA for the yeast InsP6 kinase (13).

Vacuole Analyses of Wild-type and ip6kΔ yeast—Wild-type and ip6kΔ yeast were grown at 30 °C in YPD medium (2 g/liter peptone, 10 g/liter yeast extract, 2% glucose). Yasts of 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose). Yeasts from 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose). Yeasts from 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose). Yeasts from 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose). Yeasts from 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose). Yeasts from 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose). Yeasts from 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose). Yeasts from 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose). Yeasts from 5 ml of early logarithmic phase yeast were grown at 30 °C in YPD medium (20 g/liter peptone, 10 g/liter glucose).

RESULTS

Creation and Analysis of a ip6kΔ Strain of S. cerevisiae—We recently showed that PP-InsP₆ and [PP]₂-InsP₆ were present in S. cerevisiae (15). One goal of this study was to determine the importance of the yeast InsP₆ kinase to the synthesis of the diposphorylated inositol phosphates in vivo. The yeast InsP₆ kinase protein is about three times larger in mass than either of the type 1 and type 2 forms of the mammalian InsP₆ kinases (13). The domain of the yeast InsP₆ kinase that is homologous to the smaller, mammalian enzymes resides in a region close to the C terminus. The latter portion of the yeast protein was deleted by disrupting the appropriate 3′-region of the kinase gene (Fig. 1 and see “Experimental Procedures”). To confirm the correct integration of the marker gene, two diagnostic Southern blots were performed. Yeast genomic DNA was digested by SnaBI, and a band of approximately 5.5 kilobases was detected only in the gene-disrupted strain upon hybridization with the ban′ gene (data not shown). Coincidentally, the ban′ cassette used for the gene disruption is almost identical in size to the InsP₆ kinase gene it replaces (Fig. 1). Thus, a band of approximately 5.5 kilobases was detected only in wild-type
levels of PP-InsP₅ in ratus (23, 24). Thus, we studied the effect of the deletion of the cell surface as well as biosynthetic traffic from the Golgi appa-
vacuole (23). This organelle receives endocytic traffic from the InsP₅ and Ins(1,3,4,5,6)P₅.

There is an unexpected link between the metabolism of PP-
lower than those of wild-type cells (Table I). Thus, in yeast,
urrence of the gene disrupted strain is hereafter designated ip6kΔ.

We investigated the consequences of the gene disruption upon the insositol phosphophosphate profile of wild-type and ip6kΔ cells. Levels of PP-InsP₅ in ip6kΔ cells were >80% lower than those of wild-type cells (Table I). This result demonstrates that this kinase is quantitatively important for the expression of PP-
InsP₅ synthase activity in vivo. Nevertheless, our results indi-
cate that there must be an alternative, hitherto unrecognized,
pathway for PP-InsP₅ synthesis in yeast. Levels of [PP₃]-InsP₄ were 60% lower in the ip6kΔ cells compared with the wild type (Table I). This result indicates that the InsP₆ kinase plays only a partial role in the pathway of [PP₃]-InsP₄ synthesis. Note also that the levels of Ins(1,3,4,5,6)P₅ in the ip6kΔ cells were 80% lower than those of wild-type cells (Table I). Thus, in yeast,
there is an unexpected link between the metabolism of PP-
InsP₅ and Ins(1,3,4,5,6)P₅.

The ip6kΔ cells grew more slowly than the wild-type cells at 23 and 30 °C (Fig. 2). At 37 °C, the ip6kΔ cells did not grow at all (Fig. 2). Prior to the identification of the yeast InsP₆ kinase (13), the gene that encodes this protein was known as kcs1 (21). In that earlier study, the deletion of the kcs1/InsP₆ kinase gene from S. cerevisiae yielded no apparent growth phenotype (21), possibly because that strain of yeast had a different genetic background from the strain that we have used.

Nuclear mRNA Export Is Not Impaired in ip6kΔ Cells—We (15) and others (22) have previously shown that drastic impair-
ment of the pathway of InsP₆ synthesis in S. cerevisiae is accompanied by a decreased efficiency in the rate of export of mRNAs from the nucleus. We (15) did point out, however, that mRNA export may be regulated by metabolites of InsP₆ (such as PP-InsP₅ and [PP₃]-InsP₄) rather than by InsP₆ itself. Indeed, we previously showed that one particular gene-disrupted strain of yeast (ipmkΔ), which displayed an impaired mRNA export phenotype and decreased synthesis of InsP₆, also had 60–80% lower levels of PP-InsP₅ and [PP₃]-InsP₄ (15). The ip6kΔ cells gave us the first opportunity to study whether mRNA export was affected by quantitatively similar changes in levels of PP-InsP₅ and [PP₃]-InsP₄ under conditions where InsP₆ levels were not significantly affected (Table I). We measured mRNA export as described previously (15) but found no significant difference between wild-type and ip6kΔ cells (data not shown).

This negative result redirected our efforts to the hypothesis (see the Introduction) that diphosphoinositol polyphosphates regulate protein trafficking.

Altered Vacuolar Morphology in ip6kΔ Cells—In yeast cells,
several different vesicle transport pathways converge upon the vacuole (23). This organelle receives endocytic traffic from the cell surface as well as biosynthetic traffic from the Golgi apparatus (23, 24). Thus, we studied the effect of the deletion of the

| Inositol phosphate | WT (1[^H] dpm) | ip6kΔ (1[^H] dpm) | ip6kΔ/WT |
|--------------------|--------------|-----------------|----------|
| Ins(1,3,4,5,6)P₅   | 3238 ± 1274  | 718 ± 102       | 0.22     |
| Ins(1,2,4,5,6)P₅   | 10,624 ± 3066| 10,287 ± 3249   | 0.97     |
| InsP₆             | 169,333 ± 18,883| 133,720 ± 24,931| 0.79     |
| PP-InsP₃          | 951 ± 120    | 161 ± 30        | 0.17     |
| [PP₃]-InsP₄       | 1505 ± 199   | 613 ± 92        | 0.41     |

We also found that the yeast InsP₆ kinase phosphorylated Ins(1,3,4,5,6)P₅ (Fig. 4B). The Vₘₐₓ values for InsP₆ and InsP₅ were each approximately 2 μmol/mg/min. The affinity of the enzyme for InsP₆ (mean Kₘ = 3.3 μM) was about 3-fold less than the affinity for InsP₅ (mean Kₘ = 1.2 μM). This is the first time an enzyme with PP-InsP₄ synthase activity has been identified. In the case of S. cerevisiae, cellular levels of InsP₆ are 50-fold higher than those of InsP₅ (Table I), so we would not anticipate substantial phosphorylation of InsP₅ by this kinase in intact yeast cells. However, in mammalian cells, levels of Ins(1,3,4,5,6)P₅ and InsP₆ are very similar to each other (27). We therefore next investigated whether either of the two mam-

![Fig. 2. Growth phenotype of wild-type and ip6kΔ strains of S. cerevisiae.](image-url)
malian InsP6 kinases (named types 1 and 2; see Ref. 13) could also phosphorylate Ins(1,3,4,5,6)P5.

Phosphorylation of InsP 5 by the Mammalian Type 1 InsP 6 Kinase—The type 1 InsP6 kinase was expressed in E. coli as a His-tagged protein and purified using Talon resin (Fig. 5). The enzyme was incubated with trace amounts of [3H]InsP6 and an ATP regeneration system (see “Experimental Procedures”). The InsP6 was found to be completely phosphorylated to PP-[3H]InsP5 (Fig. 6 and Ref. 13). No [PP]2-InsP4 was formed (data not shown and see Ref. 13). Several other inositol polyphosphates, namely inositol 1,4-bisphosphate, inositol 1,4,5-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate, have also previously been found not to be significant substrates for this enzyme (13). However, Ins(1,3,4,5,6)P5 was not previously tested as a substrate. We now studied this issue, in view of the observation that the yeast kinase phosphorylated both InsP6 and Ins(1,3,4,5,6)P5 (Fig. 4).

The type 1 InsP6 kinase phosphorylated Ins(1,3,4,5,6)P5 (Fig. 6B). The Vmax values for Ins(1,3,4,5,6)P5 and InsP6 were very similar (Table II). The affinity of this enzyme for InsP6 was only 5-fold higher than that for Ins(1,3,4,5,6)P5 (Table II). Because levels of both InsP6 and Ins(1,3,4,5,6)P5 in mammalian cells each range from 15 to 50 μM (27), we can anticipate that these two substrates will compete for phosphorylation by this enzyme in vivo.

InsP5 Is Converted to a Novel Inositol Polyphosphate by Mammalian Type 1 InsP6 Kinase—Another unexpected result to emerge from the studies of InsP5 phosphorylation by the type 1 mammalian kinase was the accumulation of a novel product (Fig. 6B, peak X), at a rate that was approximately 10% of the rate of accumulation of PP-InsP5 (Fig. 6B, inset). A comparison of panels A and B in Fig. 6 also shows that the elution position of peak X was between those of InsP6 and PP-InsP5.

We considered the possibility that X represents one of two different PP-InsP4 isomers, each formed by direct phosphorylation of InsP5. This option seemed unlikely, because in our HPLC experiments, peak X eluted 10 min later than did PP-InsP4 (Fig. 6B); this HPLC system can only resolve isomers of diphosphoinositol polyphosphates by 1–2 min even when a considerably more shallow elution gradient is employed (5). Furthermore, when the kinase was separately incubated with PP-[3H]InsP5, approximately 50% of this substrate was phosphorylated to peak X (Fig. 7). In other words PP-InsP4, and not InsP5, is the immediate precursor of peak X.
We next considered whether peak X might be another isomer of PP-InsP₅. This explanation also seems an unlikely possibility, because the synthesis of PP-InsP₅ from PP-InsP₄ would not involve the formation of a diphosphate group. Nevertheless, we checked the nature of peak X by using, as a diagnostic tool, one of the several hDIPP isoforms that we have cloned (hDIPP₂α) (11). The hDIPP₂α enzyme specifically removes β-phosphates from diphosphorylated inositol polyphosphates; hDIPP₂α does not hydrolyze monoester phosphates (11). When hDIPP₂α was incubated with a mixture of PP-InsP₄ and peak X, both were completely dephosphorylated back to InsP₅ (Fig. 8). Thus, peak X cannot be PP-InsP₅, because the latter is dephosphorylated to InsP₆ by hDIPP₂α (11).

We therefore propose that peak X may be a hitherto unknown, doubly diphosphorylated derivative of InsP₅, namely, [PP]₂-InsP₃. The [PP]₂-InsP₃ would be analogous to [PP]₂-InsP₄ (Fig. 9).

Although PP-InsP₄ is a substrate for the type-1 kinase, this enzyme was very ineffective at further phosphorylating the PP-InsP₅ that was formed from InsP₆ (Fig. 4, A and C). The difference between PP-InsP₄ and PP-InsP₅ lies in the latter having a 2-OH instead of a 2-phosphate group, and this clearly has a substantial impact upon substrate specificity.

Comparisons between the Type 1 and Type 2 Mammalian InsP₆ Kinases—The amino acid sequences of the mammalian type 1 and type 2 InsP₆ kinases are about 60% similar (13). We therefore compared the activities of these two enzymes in more detail. We found that the purified, recombinant type 2 kinase (Fig. 5) was able to phosphorylate both InsP₅ and InsP₆ (Table II).
II). In kinetic experiments, we established that $V_{\text{max}}$ values for both Ins(1,3,4,5,6)P$_5$ and InsP$_6$ were very similar (Table II). The affinity of the type 2 kinase for Ins(1,3,4,5,6)P$_5$ ($K_m = 8.4 \mu M$, Table II) was approximately 20-fold lower than the affinity for InsP$_6$ ($K_m = 0.43 \mu M$, Table II). These data therefore identify an important difference between the two kinases, namely, that Ins(1,3,4,5,6)P$_5$ will compete with InsP$_6$ much less effectively for phosphorylation by the type 2 kinase, compared with the type 1 enzyme.

In view of the ability of the mammalian InsP$_6$ kinases to phosphorylate Ins(1,3,4,5,6)P$_5$, we examined whether Ins(3,4,5,6)P$_4$ was a substrate. Ins(3,4,5,6)P$_4$ is a cellular signal that regulates the conductance of Ca$^{2+}$-activated chloride channels (28, 29), so the understanding of the metabolism of this inositol phosphate is a topic of some importance. We found that for both the type 1 and type 2 kinases, Ins(3,4,5,6)P$_4$ was a 40–50-fold weaker substrate compared with Ins(1,3,4,5,6)P$_5$ (Table III). This observation and our knowledge that cellular levels of Ins(1,3,4,5,6)P$_5$ are 5–10-fold higher than those of Ins(3,4,5,6)P$_4$ (27) lead us to conclude that there will not be significant phosphorylation of Ins(3,4,5,6)P$_4$ by this route in vivo. Nevertheless, these data usefully indicate that the 1-phosphate is important in determining substrate specificity of the InsP$_6$ kinases (Table III).

We also compared the rates at which the two kinases phosphorylated PP-InsP$_4$ to [PP]$_2$-InsP$_3$. We could not prepare sufficient mass amounts of PP-InsP$_4$ to ascertain $K_m$ and $V_{\text{max}}$ values. Instead we compared rates of PP-InsP$_4$ phosphorylation under first order conditions. The activity of the type 2 kinase toward PP-InsP$_4$ was approximately 7-fold lower than the activity of the type 1 kinase (Table III). The two enzymes showed only a 2-fold difference in activity toward Ins(1,3,4,5,6)P$_5$ (Table III).

DISCUSSION

In this study we have obtained important new information concerning the physiological significance of the InsP$_6$ kinase family. We have discovered that Ins(1,3,4,5,6)P$_5$ can also be phosphorylated by these enzymes (Fig. 9), and we have obtained data indicating that the yeast InsP$_6$ kinase plays an important role in vacuole biogenesis (Fig. 3).

Ins(1,3,4,5,6)P$_5$, and PP-InsP$_4$ have previously been allocated to a metabolic pool that is different from the pool in which InsP$_6$, PP-InsP$_6$, and [PP]$_2$-InsP$_3$ are contained (7). It would, therefore, not have been surprising if the turnover of these two metabolically distinct groups of compounds had turned out to be independently regulated and also functionally discrete. As a result of the work described in the current study, we can now appreciate for the first time just how interdependent are these metabolic cycles, in mammalian cells at least (Fig. 9). Levels of InsP$_6$ and Ins(1,3,4,5,6)P$_5$ in mammalian cells range from 15 to 50 $\mu M$ (27), and in some cells Ins(1,3,4,5,6)P$_5$ levels are 4-fold higher than those of InsP$_6$ (7). This information, together with the new data on the kinetic parameters for Ins(1,3,4,5,6)P$_5$ and InsP$_6$ phosphorylation by mammalian type 1 InsP$_6$ kinase (Table II), indicates that both of these substrates will compete for phosphorylation by this enzyme in vivo. If the size of the metabolic reservoir of either substrate is modified, as happens for example during passage through the cell cycle (30, 31), the metabolism of all of the diphosphorylated compounds will be affected. From this intertwined metabolism, we can further conclude that the physiological activities of all of the diphosphorylated inositol phosphates are likely to be closely linked.

Our studies also provide new insight into the structural determinants of InsP$_6$ kinase specificity. With regards to the mammalian type 1 InsP$_6$ kinase, the $V_{\text{max}}$ and $K_m$ values were similar for phosphorylation of both Ins(1,3,4,5,6)P$_5$ and InsP$_6$ (Table II). Thus, for these two substrates, there is only a minor impact on the catalytic activity of this enzyme when a phosphate group substitutes for a hydroxyl group at the 2-position on the inositol ring. On the other hand, PP-InsP$_4$ can be further phosphorylated much more readily than can PP-InsP$_5$ (Figs. 6 and 7). The difference between PP-InsP$_4$ and PP-InsP$_5$ is that the latter has a 2-phosphate, which in this context imposes an important constraint upon catalytic activity. Thus, the impact of the 2-phosphate group on enzyme activity is critically dependent upon the nature of the substrate. Note also that two very weak substrates for the kinases are Ins(3,4,5,6)P$_4$ (Table III) and inositol 1,3,4,5-tetrakisphosphate (13), so phosphate groups at both the 1- and 6-positions are also very important to substrate recognition.

Our identification of a novel diphosphorylated inositol phosphate (Fig. 6, peak X) may also provide an unexpected new direction for future research into this area. This compound, tentatively identified as [PP]$_2$-InsP$_3$, was synthesized by both mammalian InsP$_6$ kinases (Table III), and by the yeast InsP$_6$ kinase (data not shown), suggesting that the catalytic site of these enzymes has not had some flexibility in the positions on the inositol ring at which the diphosphate groups are added. The mammalian type 1 kinase had the most active [PP]$_2$-InsP$_3$ synthase activity (Table III). It would therefore be useful to ascertain whether [PP]$_2$-InsP$_3$ can be detected in vivo.

In conclusion, we now have a new understanding of the metabolic reactions catalyzed by the mammalian type 1 and type 2 InsP$_6$ kinases, and we have implicated a novel role for the inositol hexakisphosphate kinases in vacuole biogenesis.
cells (7), primary cultured rat hepatocytes (34), or hamster DDT1, MF-2 smooth muscle cells (35). However, this search has so far only involved a small number of cell types. Additionally, the metabolism of the diphosphorylated inositol phosphates is carefully regulated (1), and so we may not have incubated cells under the appropriate conditions. By analogy, it is worth noting that it was some years after the identification of PtdIns(4,5)P2 before the more minor lipids such as PtdIns(3,4,5)P3 and PtdIns(3,5)P2 were detected and found to be significant (36).

Our studies have also provided new insights into the activities of the yeast InsP6 kinase in vivo. The persistence of significant levels of diphosphorylated inositol phosphates in ip6kΔ cells (Table I) indicates that the InsP6 kinase we have studied plays a major, but not an exclusive, role in the synthesis of this group of compounds in vivo. Thus, we need to search for an independent (albeit more minor) metabolic pathway that yeast can use to synthesize PP-InsP6. Moreover, this alternative pathway can sustain a certain amount of [PP]2-InsP4 synthesis in vivo (2–5). Thus, the altered vacuolar morphology in the yeast cells (Table I) indicates that the InsP6 kinase we have studied is some years after the identification of ip6k2-InsP4 activity was deleted (22). Because the elimination of the Ins(1,3,4,5,6)P5 2-kinase activity was deleted (22). Because the elimination of the Ins(1,3,4,5,6)P5 2-kinase was accompanied by loss of cellular InsP6 (22), we can now appreciate that this would have removed an inhibitory constraint upon the conversion of Ins(1,3,4,5,6)P5 to PP-InsP6. Another interesting phenomenon in the ip6kΔ cells was their dramatically (approximately 80%) reduced levels of Ins(1,3,4,5,6)P5 compared with wild-type cells (Table I). Understanding the molecular mechanisms that link Ins(1,3,4,5,6)P5 metabolism to InsP6 kinase activity will be an interesting topic for future studies. At the very least, we should be cognizant that the deletion of the InsP6 kinase has unexpected repercussions that may contribute to the growth-impaired phenotype of ip6kΔ cells.

In addition to the decrease in PP-InsP6 levels (Table I), the ip6kΔ yeast contain abnormally small and fragmented vesicles (Fig. 3). Yeast vacuoles are homologues of mammalian lysosomes, possessing acidic interiors where protein degradation takes place (23). The vacuoles derive from fusion of cytoplasm-derived vesicles as well as clathrin-coated vesicles associated with the endocytic apparatus or the trans-Golgi network (23, 24). PP-InsP6 binds tightly to adaptor proteins that help assemble both clathrin-coated and non-clathrin-coated vesicles (2–5). Thus, the altered vacuolar morphology in the ip6kΔ yeast may reflect some abnormality in the inositol polyphosphate-dependent assembly of those vesicles that normally form vacuoles.

By determining the structural and functional relationships that exist between proteins from different organisms, we can gain insight into their evolutionary origins and physiological roles. Clearly, the catalytic activities of the yeast and mammalian InsP6 kinases are all rather similar, suggesting that there has been evolutionary conservation of their active site domains. The ability of Ins(1,3,4,5,6)P5 to compete with InsP6 for phosphorylation by these kinases does differ between the type 1 and type 2 mammalian kinases. This is suggestive of some divergence in the expression of these two proteins. This conclusion is pertinent to understanding the physiological consequences that might arise from tissue-dependent differences in the expression of the type 1 and 2 isoforms, as has been described in the rat, for example (13). Overall, the dual specificity of the type-1 kinase toward Ins(1,3,4,5,6)P5 and InsP6 has uncovered an unexpectedly close metabolic and functional linkage between the turnover of all of the diphosphorylated inositol phosphates.