MOLECULAR DEFINITION OF A NOVEL INOSITOL POLYPHOSPHATE METABOLIC PATHWAY INITIATED BY INOSITOL 1,4,5-TRISPHOSPHATE 3-KINASE ACTIVITY IN SACCHAROMYCES CEREVISIAE

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Running title: Yeast IP3 3-kinase pathway

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The production of inositol polyphosphate (IPs) and pyrophosphates (PP-IPs) from inositol 1,4,5-trisphosphate [I(1,4,5)P3] requires the 6-/3-/5-kinase activity of Ipk2 (also known as Arg82 and multikinase, IPMK). Here, we probed the distinct roles for I(1,4,5)P3 6- versus 3-kinase activities in IP metabolism and cellular functions reported for Ipk2. Expression of either I(1,4,5)P3 6- or 3-kinase activity rescued growth of ipk2 deficient yeast at high temperatures; whereas only 6-kinase activity enabled growth on ornithine as the sole nitrogen source. Analysis of IP metabolism revealed that the 3-kinase initiated the synthesis of novel pathway consisting of over eleven IPs and PP-IPs. This pathway was present in wild-type and ipk2 null cells, albeit at low levels as compared to IP6 synthesis. The primary route of synthesis was: I(1,4,5)P3 => I(1,3,4,5)P4 => I(1,2,3,4,5)P5 => PP-IP4 => PP2-IP3 and required Kcs1 (or possibly Ipk2). Ipk1, a novel inositol pyrophosphate synthase, and then Kcs1 again, respectively. Mutation of kcs1 ablated this pathway in ipk2 null cells and overexpression of Kcs1 in ipk2 mutant cells phenocopied IP3K expression, confirming it harbors a novel 3-kinase activity. Our work provides a revised genetic map of IP metabolism in yeast, and evidence for dosage-compensation between IPs and PP-IPs downstream of I(1,4,5)P3 in the regulation of nucleo-cytoplasmic processes.

Cells respond to diverse extracellular stimuli by activating phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to generate inositol 1,4,5-trisphosphate (I(1,4,5)P3) and diacylglycerol1. I(1,4,5)P3 acts through allosteric activation of the IP3 receptor that releases calcium from intracellular stores (1,2). I(1,4,5)P3 has also been shown to be a precursor for the production of other inositol polyphosphates (IPs), including inositol tetrakisphosphate (IP4), inositol pentakisphosphate (IP5), inositol hexakisphosphate (IP6) and inositol pyrophosphates (PP-IPs) (3,4). These second messengers have been implicated in the regulation of cellular functions such as mRNA export, transcription, chromatin remodeling, DNA metabolism, vesicular trafficking, chemotaxis, and environmental stress responses (5-14).

Cloning and characterization of several evolutionarily conserved inositol polyphosphate kinases (IPKs) has reinvigorated interest in “orphan” IP messengers (15-17). In yeast, activation of phospholipase C results in production of I(1,4,5)P3, and its sequential phosphorylation to IP6 via the activities of two kinases, Ipk2 and Ipk1 (5,8,18). Ipk2 has been found to sequentially phosphorylate I(1,4,5)P3 on its D-6 and D-3 positions to generate I(1,3,4,5,6)P5. Ipk1 functions as a 2-kinase to convert I(1,3,4,5,6)P5 to IP6. Additionally, both Ipk2 and Ipk1 have been shown to have promiscuous activities suggesting that each may facilitate generation of complex branches of IP metabolites. Subsequent metabolism of IP5 and IP6 by inositol pyrophosphate synthases, such as Kcs1 and a novel activity designated Ids1, generate PP-IP4 and PP-IP5 (10,13,19). It appears that the metabolic functions of IPKs have been conserved across eukaryotes as they are required for IP6 synthesis (18,20-27).

The enzymatic promiscuity Ipk2 and its action early in complex IP metabolic pathways may account for the pleiotropic biologic defects observed in ipk2 mutant yeast. To further dissect the roles of Ipk2’s kinase activity we have utilized heterologous complementation analysis in ipk2 mutant yeast cells (20,21,23). Here, in order to specifically assess the role of 3-kinase activity, we studied the effects of expression of a Drosophila...
I(1,4,5)P₃ 3-kinase beta isoform (dmIP3K), whose only reported enzymatic function was to generate inositol 1,3,4,5-tetrakisphosphate [I(1,3,4,5)P₄] (21,28). This work has led to an unexpected finding that expression of dmIP3K initiates a novel IP pathway, whose molecular basis we describe. Additionally, we provide evidence for dosage-compensation among IP species in the regulation of Ipk2-mediated nuclear and cytoplasmic processes.

MATERIALS AND METHODS

Strains and Media - Yeast were grown in either rich medium (yeast-peptone-dextrose), or complete minimal (CM) medium lacking the appropriate nutrients for maintenance of plasmids containing markers. Yeast strains used in this study were from previous studies or generated by mating strains from previous studies (5,8,10). Ornithine plates were made as previously described (5,29).

High Performance Liquid Chromatography (HPLC) Columns And Gradients – Two different Partisphere SAX columns and elution gradients were used in this study. Method 1 utilized a custom-made narrow-bore column was obtained from Capital HPLC Limited (12.5 cm x 2.1 mm). IPs were eluted with a linear gradient of ammonium phosphate (pH 3.5) (AP) from 10 mM to 1.7 M over the course of 12 min. and isocratic elution at 1.7 M AP for 23 min. (flow rate of 0.4 ml/min). Method 2 achieved higher resolution IPs by using a wider-bore Whatman column (12.5 cm x 4.6 mm) and a longer elution gradient as follows: a linear gradient from 10 mM to 85 mM AP over 5 min., then 85 mM to 1.7 M AP over 65 min. and then isocratic elution at 1.7 M AP for 30 min. all at a flow rate of 1 ml/min.

Plasmid Construction – Construction of plasmids pRS314-dmIpk2 and pRS314-dmIP3Kβ (which we will refer to as dmIP3K throughout the remainder of this manuscript) and Kcs1 expression constructs (pRS-Kcs1 and pRS-kcs1kin-) was described previously (10,21). The plasmids were then transformed into different yeast strains using standard yeast transformation techniques. The pCR®2.1 vector (Invitrogen) containing the entire scIpk1 coding region with a PCR-generated SalI site at the 5’ end was provided by Dr. Makoto Fujii (York lab, Duke University). The scIpk1 coding region was subcloned from pCR®2.1 using SalI and EcoRI and ligated into the pGEX-2T vector. The vector was transformed into Escherichia coli (DH5α) for expression of recombinant protein.

In Vivo Labeling Of Yeast Cultures - Yeast cultures were incubated at 30°C in minimal medium lacking the appropriate amino acids and 150 μM CuSO₄ to late logarithmic phase. [³H]-inositol (American Radiolabeled Chemicals) was added to a final concentration of 80 μCi/ml. For pulse-chase analysis: yeast strains were grown to late logarithmic phase in 50 ml of unlabeled CM medium. The cells were washed and resuspended in 500 μl of inositol-free CM medium supplemented with 1 mCi/ml [³H]-inositol. After labeling for ten minutes the cells were washed, resuspended in 50 ml inositol-replete CM medium without label and incubated at 30°C. Aliquots were taken at various time points and the cells were frozen on dry ice until they could be harvested and analyzed by Partisphere strong-anion exchange HPLC. Soluble IPs were harvested and analyzed by HPLC using a strong-anion exchange column as described previously (8). Alternatively, the IPs were harvested for enzyme treatment as described below.

Enzyme Analysis Of [³H]-Inositol Labeled Yeast Extracts - Labeled yeast strains were resuspended in 50 mM Tris pH 7.5 and lysed for 30 seconds with a bead beater (Biospec Products) using glass beads (B. Braun Biotech International). The lysate was immediately boiled for 5 min. and then the entire procedure was repeated a second time. Extracts containing soluble IPs were recovered by centrifugation. Reactions were carried out by incubating the extracts for 1 hr at 37 °C in a buffer containing 10 mM Tris pH 7.5 and 10 mM NaCl with 500 ng of human Type I 5-phosphatase and/or human diphosphoryl inositol polyphosphate phosphohydrolase (DIPP). The reactions were stopped by addition of 200 μl of 10 mM ammonium phosphate pH 3.5 and analyzed by Partisphere strong-anion exchange HPLC.
Bacterial Expression of dmIP3 and scIpkl - Transformed Escherichia coli (DH5α) were grown at 37 °C to an OD600 of 0.6 and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 30 °C. The cells were recovered by centrifugation at 4 °C, resuspended in ice-cold 50 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM dithiothreitol, Complete Mini protease inhibitor mixture (Roche Applied Science) and lysed with four passes through a cell cracker (a high shear fluid processing system for cell rupture, Microfluidics Corp.). The lysates were cleared by centrifugation at 14,000xg. The GST fusion proteins were purified over glutathione Sepharose (Amersham Biosciences) according to the manufacturer’s instructions. A buffer containing 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM dithiothreitol, and 20 mM glutathione was used to elute the proteins from the glutathione Sepharose. Proteins were quantified by modified Bradford method and SDS-PAGE analysis.

Inositol Phosphate Kinase Assay - All unlabelled IPs were purchased from Cell Signals, Inc. and [3H]-I(1,3,4,5)P4 was purchased from PerkinElmer. [32P]-I(1,3,4,5)P4 was synthesized in buffer containing 50 mM Tris pH 7.5, 50 mM NaCl, 10 mM MgCl2, 5 µM I(1,4,5)P3, trace amounts of [32P]-ATP, and 7.5 µg of dmIP3K. [3H]-I(1,3,4,5,6)P5 standard was synthesized by incubating [3H]-I(1,3,4,5)P4 with recombinant dmIpkl and ATP as described (21). Kinase assay conditions were carried out essentially as described by Stevenson-Paulik et al (20).

Kinetic Assays - The K_m and V_max of the enzymatic interaction between scIpkl and I(1,3,4,5)P4 and various substrates were determined. The following reaction mixture was prepared: 10 mM Tris pH 7.5, 10 mM NaCl, 4 mM ATP, 20 mM MgCl2, 10,000 cpm/µl [32P]-I(1,3,4,5)P4, 500ng scIpkl and various concentrations of unlabeled I(1,3,4,5)P4 in a 20 µl reaction volume. The reaction was stopped by the addition of 4 µl of 1M KH2PO4. The amount of product formed was quantified by thin layer chromatography. The K_m and V_max values were obtained from a nonlinear curve fit to the Michaelis-Menten equation using GraphPad Prism version 4.01.

RESULTS

Comparative roles of I(1,4,5)P3 6- and 3-kinase activities in yeast – Studies of yeast Ip2 have shown that its predominant in vivo catalytic function is to convert I(1,4,5)P3 => I(1,4,5,6)P3 => I(1,3,4,5,6)P5 via 6- and 3-kinase activities. Recombinant yeast Ip2 has been shown biochemically, but not in vivo, to possess a less efficient I(1,4,5)P3 3-kinase activity. Of interest, both kinase-dependent and independent roles have been described for Ip2 in regulating biological functions (5-8,18,20,30,31).

In order to further probe the kinase-dependent roles for Ip2, we compared I(1,4,5)P3 6- versus 3-kinase activities for functional complementation. To accomplish this, we genetically added back I(1,4,5,6)P4 or I(1,3,4,5)P4 production by heterologously expressing Drosophila IPKs, either dmIpkl or dmIP3K, in ipk2 deficient yeast. Both kinases have been shown to be members of a family of IP kinases bearing a signature PxxxDxKxG motif that includes: Ip2 6-/3-/5-kinases; IP6Ks which function as inositol pyrophosphate synthases and utilize IP5 and IP6 substrates; and IP3Ks which were not found in budding yeast or plants, but were present in fly, mouse and human genomes (17,32). We recently reported the cloning and biochemical characterization of dmIP3K and dmIpkl and showed they possess I(1,4,5)P3 3-kinase and I(1,4,5)P3 6-kinase activities, respectively (21). Functional analysis of ipk2 deficient cells that expressed either dmIpkl or dmIP3K revealed that 6-kinase, but not 3-kinase activity could rescue growth on ornithine as a sole nitrogen source (Fig. 1A). This data, coupled with our previous analysis of the ipk2-3 mutant (5), indicated that production of I(1,4,5,6)P4, but not I(1,3,4,5)P4, was necessary for activation of the ArgR-Mcm1 transcriptional complex. When we analyzed the transgenic strains for growth at high temperatures, we found that either 6- or 3-kinase activity was able to complement the temperature sensitive phenotype of ipk2 null yeast (Fig. 1B). In order to rule out that complementation analysis may be related to downstream metabolites, such as those required for mRNA export pathways as described for Ip1 (8), we tested the expression in ipk2 ipk1 double mutant cells. Rescue of growth on ornithine as the sole nitrogen source or
temperature sensitivity by dmIpk2 did not require the presence of Ipk1; however, the rescue of temperature sensitivity by dmIP3K did require Ipk1 (Fig. 1A and B). Additionally, to determine if 6- or 3-kinase complementation of temperature sensitivity required pyrophosphate synthesis, we analyzed ipk2 kcs1 double mutants. We report that dmipk2 and IP3K were able to partially (not fully) rescue under these conditions (Fig. 1C). Our data indicate that (1,2,3,4,5)P5 production and a downstream PP-IP were required for sustaining temperature sensitive complementation. Of interest, the ipk1 dependent temperature sensitive phenotype may be of use for forward genetic strategies aimed at identification of regulators and receptors of Ipk1 pathways. Of note, we have previously published that ipk1 mutation is not temperature sensitive by plating assays (only after 20 generations – see York et al, Science 1999).

Discovery of a novel IP metabolic pathway initiated by I(1,4,5)P3 3-kinase activity – We next analyzed the effect of heterologous expression of dmIpk2 and dmIP3K on IP metabolism in ipk2 null yeast. We previously reported that dmIpk2 expression was able to fully complement Ipk2 enzyme function in cells by converting I(1,4,5)P3 to (1,3,4,5,6)P5 (21). Remarkably, analysis of ipk2 deficient cells expressing dmIP3K revealed the synthesis of several new IP metabolites including novel IP3, IP4, IP5, and PP-IP species (Fig. 2).

In order to determine the genes required for synthesis of the new species downstream of I(1,3,4,5)P4, we examined the role of inositol pyrophosphate synthase and 2-kinase activities. Kcs1 has been shown to synthesize PP-IPα and PP-IPβ from I(1,3,4,5,6)P5 and IP6 precursors (14,33). Of note, we have implemented a symbol-based nomenclature (alpha, beta and gamma) to enable the distinction of the growing list of PP-IP isomers and species. As we describe below, the basis for assigning distinct isomers was that they had unique HPLC elution profiles and unique genetic routes of synthesis. At this time we do not have chemical structures that enable definition of the ring positions harboring pyrophosphates. Loss of Kcs1 in ipk2Δ cells expressing dmIP3K resulted in the obvious elimination of the most polar PP-IP species detected, PP2-IPβ (Fig. 2). We used the “beta” designation due to its distinct elution profile from PP3-IPα (which is synthesized downstream of I(1,3,4,5,6)P5 → PP-IPα) and fact that it is synthesized through I(1,3,4,5)P4 → I(1,2,3,4,5)P5 → PP-IPβ (also see below). These data indicate the existence of a second pyrophosphate synthase gene product, designated here as Ipsi1, required for the synthesis of PP-IPβ. It is unclear at this point whether or not Ipsi1 is similar or identical to the activity identified previously as Ids1 which is required for the synthesis of PP-IPβ (10). We next tested whether yeast lpk1 was required for the pathway. When dmIP3K was expressed in ipk2Δ ipk1Δ cells, there was a loss of IP3 and all PP-IPs, along with the accumulation of unique IP5 and IP4 species (Fig. 2). We therefore conclude that phosphorylation of I(1,4,5)P3 on the D-3 position initiates a novel IP synthesis pathway that requires Ipk1, a novel inositol pyrophosphate synthase and Kcs1.

Does this novel pathway exist in wild-type or ipk2 mutant cells? Earlier work by our lab and the Shears lab indicated that IPs with similar HPLC elution profiles to those we observed in Fig 2 exist at low levels in ipk2 deficient and wild-type cells (8,18). We therefore re-examined several combinations of kinase mutants using a high-resolution and high-sensitivity radiolabeling system (Fig. 3). Using this method, examination of ipk2 null cells revealed a similar pattern of IPs as those observed in the dmIP3K expressing cells (compare Fig 3 top and second traces). Of note, this method also exposed that the number of unique IP and PP-IP species in ipk2 deficient cells expressing dmIP3K was substantially greater than we previously thought (compare Fig. 3, top trace to Fig. 2 second trace). The presence of these IPs in ipk2 deficient cells led us to speculate that another yeast gene product harbors I(1,4,5)P3 3-kinase activity. We therefore analyzed ipk2 ipk1 and ipk2 kcs1 double mutant cells (Fig. 3, bottom two traces). Similar to results shown above, loss of lpk1 in ipk2 null cells resulted in the disappearance of IP3 and PP-IP species, and a corresponding accumulation of IP5 and IP4. Loss of Kcs1 in ipk2 null cells ablated the synthesis of nearly all IP and PP-IPs detectable using this high sensitivity method. This indicates that Kcs1 may regulate or act as a I(1,4,5)P3 3-kinase and that lpk1 functions in the conversion of IP4 to the
higher IPs of this pathway. High sensitivity labeling of wild-type cells indicated similar species were present (data not shown) consistent with results of others (18).

Ipkl harbors I(1,3,4,5)P₄ 2-kinase activity - Our data indicated that the dmiP3K-dependent IP synthesis pathway was initiated by D-3 phosphorylation of I(1,4,5)P₃ to I(1,3,4,5)P₄. Because deletion of Ipkl blocked the pathway and caused a buildup of I(1,3,4,5)P₄, we hypothesized that the second step was carried out by Ipkl phosphorylation of I(1,3,4,5)P₄ to generate I(1,2,3,4,5)P₅. To test this, we carried out in vitro kinase assays using both recombinant dmiP3K and sclIpkl (Fig. 4). Incubation of I(1,4,5)P₃ with ATP and dmiP3K resulted in the generation of IP₄ that we previously demonstrated to be I(1,3,4,5)P₁ (Fig. 4B) (21). When I(1,4,5)P₃ was incubated ATP, dmiP3K, and sclIpkl we observed the formation of an IP₃ product that did not co-elute with I(1,3,4,5,6)P₅ standard (Fig. 4C). This product was not a PP-IP based on its insensitivity to DIPP. Additionally, we observed that this IP₃ was completely hydrolyzed to IP₁ when treated with recombinant type I 5-phosphatase (Fig 4D), an enzyme previously found to utilize only I(1,4,5)P₃ and I(1,3,4,5)P₄ (3). Therefore we concluded that the structure had to be authentic I(1,2,3,4,5)P₅ based on its elution after the I(1,3,4,5,6)P₅ standard, the requirement of 2-kinase activity, sensitivity to 5-phosphatase and the fact that the only other mono-phosphorylated isomer capable of being synthesized from I(1,3,4,5)P₄ was I(1,2,3,4,5)P₅. We examined the kinetic properties of this reaction and found that scIpkl phosphorylated I(1,3,4,5)P₄ with a K_m of 148 μM and V_max of 60 nmol/min/mg (Fig. 4E).

To understand whether this novel activity is conserved in eukaryotes, we tested whether Ipkl 2-kinase orthologs were able to phosphorylate I(1,3,4,5)P₄. Recombinant rat Ipkl was not able to phosphorylate I(1,3,4,5)P₄ in vitro and co-expression of rat Ipkl and dmiP3K in ipk2Δ ipk1Δ cells did not restore I(1,2,3,4,5)P₄, PP-I₃β or PP₂-I₃β synthesis (data not shown). Conversely, Arabidopsis thaliana Ipkl was able to phosphorylate I(1,3,4,5)P₄ (Seeds, Stevenson-Paulik, and York – unpublished results). This suggests that the ability of the 2-kinase to use I(1,3,4,5)P₄ as a substrate was species-specific in eukaryotes.

Characterization of the IP species produced by the I(1,4,5)P₃ 3-kinase pathway – To understand the molecular structure of the species produced by this novel pathway, we used enzymatic digestion of the novel IPs derived from mutant cells. Our typical method for preparing IPs from radiolabeled extracts utilized 0.5 M HCl, which impaired enzymatic analysis. To circumvent this problem, we rapid boiled radiolabeled extracts isolated under neutral pH conditions to ensure the inactivation of endogenous enzymes. Extracts prepared from ipk2 null cells expressing dmiP3K in this manner appeared similar to those prepared by the acid-chloroform extraction method used in previous analysis (compare Fig. 2, second trace with Fig. 5, top trace). To determine if any of the species were I(1,4,5)P₃, I(1,3,4,5)P₄ or I(1,2,3,4,5)P₅ we treated extracts with recombinant human Type I 5-phosphatase, which dephosphorylates the D-5 these substrates. Surprisingly, none of the IPs or PP-IPs in this extract were hydrolyzed by the 5-phosphatase (Fig. 5, second trace). To confirm that the 5-phosphatase was active under these conditions, we spiked these extracts with [3H]-I(1,3,4,5)P₄ standard, and observed that it was completely hydrolyzed by I(1,3,4,5)P₃ by the 5-phosphatase (not shown). We next treated extracts prepared from ipk2 ipk1 double mutant cells expressing dmiP3K and found that while the IP₄ species was sensitive to 5-phosphatase, consistent with it being I(1,3,4,5)P₄, the IP₃ species was not (data not shown). Thus, the IP₃ species found in either extract expressing dmiP3K, was likely I(3,4,5)P₃ based on its elution profile. Additionally, we have partially purified an IP 1-phosphatase activity from yeast, which we have designated Inp1, encoded by an unknown gene (York lab, unpublished results). Given that he IP₄ species in ipk2 null cells expressing dmiP3K was not 5Pτase sensitive, the elution position relative to other IP₄ standards, and the inability to be phosphorylated by either recombinant Ipkl or Ipk2 (not shown) suggested that it was likely I(2,3,4,5)P₄. This species may arise from either Inp1 cleavage of I(1,2,3,4,5)P₅ or by phosphorylation of I(3,4,5)P₃ by a 2-kinase. Of note, we were unsuccessful in our attempts to phosphorylate I(3,4,5)P₃ with
recombinant yeast Ipkl (not shown); however, it was possible that the specific activity of the kinase towards this substrate was low or that we did not have proper conditions that mimicked those in cells.

We next examined the identities of PP-IP species in ipk2Δ cells expressing dmIP3K. DIPP specifically removes the beta-phosphate from PP-IPs and has no reported activity against the IP monophosphates (34). Recombinant human DIPP completely dephosphorylated the two major species that eluted at 20 and 30 min. with a concomitant increase in the levels of I(1,2,3,4,5)P5 (Fig. 5, third trace). The observed increase in IP4 levels in DIPP treated extracts confirmed that a PP-IP4β and PP2-IP3β were present as shown in Fig 3, top trace (note under low resolution HPLC this species co-eluted with IP5). We did not observe evidence of IP6 after treatment. Additionally, we simultaneously treated extracts with both DIPP and 5-phosphatase. We observed a disappearance of PP-IP4β and PP2-IP3β, the reduction of a major peak of I(1,2,3,4,5)P5, and the formation of I(1,3,4)P3 and I(1,2,3,4)P4 (Fig 5, bottom trace). We conclude that the major IP3 released by DIPP treatment was (1,2,3,4,5)P5 based on: 1) this IP3 species co-eluted exactly with authentic I(1,2,3,4,5)P5; 2) it was fully susceptible to 5-phosphatase treatment generating a product that co-eluted with the I(1,2,3,4)P4 standard and 3) that its synthesis in cells required Ipkl. Furthermore, recombinant type I 5-phosphatase was unable to hydrolyze any additional IP3 species tested, and we are not aware of any literature demonstrating that 5-phosphatases have ring-position promiscuity (ie 4- or 3-phosphatase activities). Collectively, these data corroborated our genetic evidence that PP-IP4β and PP2-IP3β arose through sequential phosphorylation of I(1,2,3,4,5)P5, via the action of two pyrophosphate synthase steps, likely through Ips1 or Ids1 and then Kcs1. Additionally they suggested that I(1,3,4,5)P4 was the likely precursor for PP-IP3α and PP2-IP2α. A second IP3 (IP3α) species was identified through this experiment, based on the observation of an IP3 species that was not susceptible to 5-phosphatase treatment (compare Fig. 5, second and bottom traces).

Pulse chase analysis of the IP3K-dependent synthesis pathway – To further examine the order of cellular IP synthesis we used pulse-chase analysis of ipk2 null cells expressing dmIP3K. Overnight cultures were grown to late logarithmic phase, pulse labeled with medium labeled with [3H]-inositol for ten min., washed, and then chased with medium supplemented with excess cold inositol. Examination of IP profiles revealed that IP3 and IP4 pools accumulated first, and were followed by the synthesis of IP5, PP-IP4β and PP2-IP3β (Fig. 6A). These data were consistent with the kinetics we observed for scIpkl phosphorylation of I(1,3,4,5)P4. The relatively high Km that scIpkl exhibits for I(1,3,4,5)P4 may help explain our observation that the 2-kinase’s substrate (I(1,3,4,5)P4) accumulated before a significant amount of I(1,2,3,4,5)P5 was synthesized.

Treatment of the pulse-labeled extracts with 5-phosphatase revealed that at early time points, I(1,4,5)P3 and I(1,3,4,5)P4 were the predominant species (Table I). At progressively later time points I(3,4,5)P4 (90 min. or more) and I(2,3,4,5) (after 240 min.) became the predominant species, as the majority of IP3 and IP4 were no longer 5-phosphatase susceptible. Presumably, I(3,4,5)P3 accumulates early from the high levels of I(1,3,4,5)P4, whereas I(2,3,4,5)P4 was not synthesized until later because it was generated from I(1,2,3,4,5)P5. I(1,2,3,4,5)P5 was the only IP5 species detected at any of the tested time points, further supporting its role as the substrate for PP-IP3 and PP2-IP3 synthesis (Table I).

In ipk2Δipk1Δ cells expressing dmIP3K I(1,3,4,5)P4 was the only IP3 species detected at any of the tested time points. At later time points the IP3 species was not susceptible to 5-phosphatase hydrolysis indicating it was I(3,4,5)P3. Based on these findings, together with the genetic and pulse labeling data presented above, we propose the primary synthesis pathway to be I(1,4,5)P3 → I(1,3,4,5)P4 → I(1,2,3,4,5)P5 → PP-IP4β → PP2-IP3β. Given the complexity of additional species, it also appears that there were several branches to the pathway, which may arise from phosphatase and promiscuity of IPK kinase activities.
Kcs1 functions as an I(1,4,5)P_3 3-kinase in ipk2Δ cells – Two lines of evidence support that Kcs1 may function as an I(1,4,5)P_3 3-kinase: the work of Dubois and colleagues that reported that Kcs1 had an undetermined I(1,4,5)P_3 kinase activity in vitro (13); and our observation that deletion of KCS1 ablated synthesis of higher IPs in an ipk2 null. Dubois and co-workers also reported that Kcs1 overexpression in ipk2Δ cells resulted in the production of several higher phosphorylated IPs, which resembled profiles we reported here for ipk2 null cells expressing dmIP3K. We directly compared the IP profiles generated by either expression of dmIP3K or Kcs1 in ipk2Δ cells. This analysis revealed that I(1,3,4,5)P_4, I(2,3,4,5)P_4, I(1,2,3,4,5)P_5, and PP-IP_4β were synthesized in both extracts; however, there were significant differences in the relative levels of the different PP-IP_4 species (Fig. 7A, top and middle traces). When Kcs1 was expressed in ipk2Δ ipk1Δ cells, the synthesis of I(1,2,3,4,5)P_5, PP-IP_4β and PP2-IP3β was abolished; however we observed the production of PP-IP_3α and PP2-IP2α species (Fig. 7A, bottom trace). Treatment of extracts from ipk2Δ ipk1Δ cells overexpressing Kcs1 with 5-phosphatase and/or DIPP confirmed that I(1,3,4,5)P_4 was a precursor to PP-IP synthesis demonstrating that Kcs1 functioned in cells as an I(1,4,5)P_3 3-kinase (Fig. 7B). Of note, treatment with 5-phosphatase caused almost complete hydrolysis of IP3 to I(1,4)P_2 indicating that it was I(1,4,5)P_3 and not I(3,4,5)P_4 (Fig. 7B).

Our results indicate that Kcs1 is both a pyrophosphate synthase and a I(1,4,5)P_3 3-kinase. Previous experiments examining the pyrophosphate synthase activity of Kcs1 demonstrated that its activity requires a highly conserved PxxxDxKxG motif that is required for ATP binding (10,35). To determine if this site is also important for the 3-kinase activity of Kcs1, we generated a double point mutant (D786A; K788A) that lacks its diphosphoryl synthase activity. When this mutant was expressed in ipk2Δ cells, we did not observe the Kcs1-dependent synthesis pathway, indicating that the 3-kinase activity uses the same catalytic domain as the pyrophosphate synthase activity (data not shown).

DISCUSSION

Our results may be summarized into four main findings: 1) we distinguish between the roles of 6- versus 3-kinase activities associated with Ipk2 function in cells; 2) we serendipitously have discovered and defined a molecular basis for a new IP/PP-IP pathway in yeast that is initiated by I(1,4,5)P_3 3-kinase activity; 3) we demonstrate novel in vivo activities for Kcs1 and Ipk1 which have ramifications for re-interpreting previously published work; and 4) we provide evidence for new phosphatase and inositol pyrophosphate synthase activities in yeast: designated Inp1 and Ips1, respectively.

Our initial hypothesis for the heterologous expression of Drosophila IPKs in ipk2 mutant yeast was that such experiments would allow us to distinguish between 6- and 3-kinase activities. These experiments also provide a means to “add-back” I(1,4,5,6)P_4 and I(1,3,4,5)P_4 production in cells without complications due to protein components, as the Drosophila enzymes have less than 50 residues, out of over 350, in common with yeast Ipk2 (note: the concept of “add-back” was first proposed in the York lab by Drs. Audrey Odom and Jill Stevenson-Paulik while studying the Arabidopsis thaliana Ipk2). While dmIpk2, and inferred 6-kinase activity, was able to complement growth on ornithine as a sole nitrogen source and growth at high temperatures, dmIP3K I(1,4,5)P_3 3-kinase activity was only able to complement temperature growth. Thus, providing the first evidence that synthesis of I(1,3,4,5)P_4 was unable to restore regulation of gene expression as judged by growth on ornithine. These data further support our initial evidence that I(1,4,5,6)P_4 production plays a role in gene expression through studies of the ipk2-3 mutant which appears to be a 6-kinase selective enzyme in cells (5). This work also demonstrates that production of I(1,4,5,6)P_4, but not I(1,3,4,5)P_4, was able to bypass a kinase-independent function of Ipk2 in transcriptional control as proposed by Messenguy and colleagues (30). Subsequent work of this group (31), and work of the O’Shea and Wu labs (6,7), further support our initial claim that Ipk2’s kinase activity was and still is required for its role in gene expression and biological processes (for example, ArgR-Mcm1 transcription, chromatin remodeling, ...
growth on arginine or ornithine as a sole nitrogen source; and growth at high-temperatures).

Perhaps the most surprising aspect of this work arose from the metabolic analysis of ipk2 deficient cells expressing dmIP3K. In these cells, we expected to observe conversion of I(1,4,5)P$_3$ and stoichiometric accumulation of I(1,3,4,5)P$_4$. However as Figs. 2 and 3 illustrate, we instead found over 11 new species of IPs and PP-IPs that were downstream metabolites of I(1,3,4,5)P$_4$ production. Of interest, when we expressed dmIP3K in wild-type yeast, we did not observe stimulation of this pathway and the metabolic profiles of these cells were identical to wild-type, having a signature major peak of IP$_6$ (not shown). These data indicate that when Ipk2 was present the 6-kinase pathway was the major route of metabolism for I(1,4,5)P$_3$. Having said this, the 3-kinase pathway was present in wild-type and ipk2 deficient cells, albeit at low levels as compared to IP$_6$ synthesis.

Our data helps interpret the observations that we and others made previously that several other low abundance IPs are present in ipk2 null and wild-type cells (5,8,18). In contrast to the report of Saiardi et al (18), we found that ipk2Δ cells do not generate I(1,3,4,5,6)P$_5$, IP$_6$, PP-IP$_5$ and PP$_2$IP$_5$. Rather, they synthesize I(1,2,3,4,5)P$_5$, PP-IP$_2$, PP-IP$_4$, PP$_2$IP$_3$, and PP$_2$IP$_4$ (see Fig 3). Of note, when using a Partisphere SAX HPLC column, the PP$_2$IP$_2$ species that is generated co-elutes with IP$_6$, which may have led to its misidentification. We therefore, confirmed our results through treatment of the ipk2 null IP extracts with DIPP and found that the PP$_2$IP$_2$ peak was completely hydrolyzed (not shown). Additionally, DIPP treatment of the extracts did not result in the formation of IP$_6$, providing evidence that the PP-IPs were only synthesized from IP$_4$ and/or I(1,2,3,4,5)P$_5$. We note that the data presented in this study do not reveal the chemical structures of the PP-IP species produced in the pathway, specifically which ring-positions harbor the pyrophosphate. However their unique elution profiles and their synthesis via different precursors provided evidence that they appear structurally distinct (see Fig 8).

The molecular and biochemical analysis of the 3-kinase pathway has allowed us to define most species and the gene products required for their synthesis. We now provide a revised genetic and metabolic map of IP/PP-IP metabolism in budding yeast (Fig. 8). Unlike the Ipk2/Ipk1-dependent pathway that synthesized IP$_6$, the IP3K-dependent pathway required Kcs1 (possibly Ipk2), Ipk1, and Ips1 (possibly Ipd1). Interestingly, despite that role of these kinases in both yeast pathways, their sequential order, substrates and, in at least one case, activities were unique to each pathway.

Through our studies we have assigned a novel activities and specificities to Kcs1 and Ipk1. We assign an I(1,4,5)P$_3$ 3-kinase activity to Kcs1 that was functional in cells. This extends previous work of Dubois and co-workers that ascribed an undetermined I(1,4,5)P$_3$ kinase activity to Kcs1 (13). The metabolic phenocopy of dmIP3K and Kcs1 expression in ipk2 null cells, and the loss of I(1,3,4,5)P$_4$ production observed in a ipk2 kcs1 double mutant demonstrate this new activity is present in cells. It is not entirely surprising that Kcs1 can function as an I(1,4,5)P$_3$ kinase given that it is evolutionarily related to two I(1,4,5)P$_3$ kinases, Ipk2/IPMKs and IP3Ks (17,35), thus it may have retained I(1,4,5)P$_3$ kinase activity, in addition to acquiring pyrophosphate synthase activities. Also, we note in the context of wild-type cells, our data do not exclude a role for Ipk2 in initiating the 3-kinase pathway. We show for the first time, that Ipk1 from yeast acts as a 2-kinase on I(1,3,4,5)P$_4$. Analysis of the kinetic properties of this reaction indicated that this is not as efficient as those described for other Ipk1 substrates, but clearly under conditions of I(1,3,4,5)P$_4$ accumulations in cells, this activity is relevant. Thus, it is possible that cellular alterations in Ipk2, Ipk1 and/or Kcs1 activity or specificity would regulate flux to either the 6- or 3-kinase pathways.

The discovery of the 3-kinase pathway has also enabled the identification of new IP phosphatase and inositol pyrophosphate synthase activities. Recently, we reported the existence of Ipd1, an inositol pyrophosphate synthase present in cells lacking Kcs1 and DIPP whose activity appeared to convert IP$_6$ to PP-IP$_5$. Here we provide evidence of an inositol pyrophosphate synthase, designated Ips1, capable of phosphorylating I(1,2,3,4,5)P$_5$ to generate PP-IP$_4$. At this point we have not determined if
these are two distinct enzymes or a single gene product, nor have we determined which phosphate position serves as an acceptor for the synthesis of the pyrophosphate. Lastly, we find evidence for an IP 1-phosphatase activity, designated Inp1, which does not appear to be related to INPP1, a lithium-inhibited inositol polyphosphate 1-phosphatase (36,37). Our previous studies of ipk2 mutant cells showed evidence for this activity towards I(1,4,5)P$_3$ substrates (5,8) and here we find evidence that such an activity also utilizes I(1,3,4,5)P$_4$ to generate I(3,4,5)P$_3$. The molecular identity of Inp1 has yet to be established. However, we have ruled out that this activity was encoded by Inp5’s and SAC1-like inositol phosphatases (5,8,38-40) based on genetic and biochemical analysis (Bryan Spiegelberg and John York, unpublished results).

The discovery of the 3-kinase pathway may have important ramifications for interpreting genetic evidence. The 6-kinase and IP$_6$ synthesis pathway has been implicated in the regulation of cellular functions including transcription, chromatin remodeling, RNA export, vacuole function, DNA metabolism and telomere maintenance (10,15,17). Clearly, the 3-kinase pathway that remains in ipk2 deficient cells does not appear to compensate for loss of 6-kinase, thereby supporting the parsimonious explanation that the 6-kinase pathway is most relevant to these functions. However, upregulation of the 3-kinase activity by expression of dmIP3K or Kcs1 indicated that some of the IP/PP-IPs generated by this pathway were able to dosage-compensate for at least some, but not all, of the functions attributed to 6-kinase metabolites. It is intriguing to speculate the alterations in Ipk2 specificity (i.e. 6- versus 3-kinase), and/or Kcs1/IPk1/lis1/lps1 activity may provide the yeast cell with a complex repertoire of signaling molecules to enable adaptation to changes in cellular environment.

REFERENCES

1. Berridge, M. J. (1993) Nature 361, 315-325
2. Mikoshiba, K. (1997) Curr Opin Neurobiol 7(3), 339-345
3. Majerus, P. W. (1992) Annu Rev Biochem 61, 225-250
4. Irvine, R. V., Schell, M.J. (2001) Nature Reviews 2, 327-338
5. Odom, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000) Science 287(5460), 2026-2029.
6. Shen, X., Xiao, H., Ranallo, R., Wu, W. H., and Wu, C. (2003) Science 299(5603), 112-114
7. Steger, D. J., Haswell, E. S., Miller, A. L., Wente, S. R., and O'Shea, E. K. (2003) Science 299(5603), 114-116
8. York, J. D., Odom, A. R., Murphy, R., Ives, E. B., and Wente, S. R. (1999) Science 285(5424), 96-100.
9. Luo, H. R., Saiardi, A., Yu, H., Nagata, E., Ye, K., and Snyder, S. H. (2002) Biochemistry 41(8), 2509-2515
10. York, S. J., Armbruster, B. N., Greenwell, P., Petes, T. D., and York, J. D. (2005) J Biol Chem 280(6), 4264-4269
11. Luo, H. R., Huang, Y. E., Chen, J. C., Saiardi, A., Iijima, M., Ye, K., Huang, Y., Nagata, E., Devreotes, P., and Snyder, S. H. (2003) Cell 114(5), 559-572
12. Hanakahi, L. A., Bartlet-Jones, M., Chappell, C., Pappin, D., and West, S. C. (2000) Cell 102(6), 721-729.
13. Dubois, E., Scherens, B., Vierendeels, F., Ho, M. M., Messenguy, F., and Shears, S. B. (2002) J Biol Chem 277(26), 23755-23763
14. Saiardi, A., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (2000) J Biol Chem 275(32), 24686-24692.
15. Shears, S. B. (2004) *Biochem J* 377(Pt 2), 265-280
16. York, J. D., Guo, S., Odom, A. R., Spiegelberg, B. D., and Stolz, L. E. (2001) *Adv Enzyme Regul* 41, 57-71
17. York, J. D. (2003) *Inositol Polyphosphate Regulation of Nuclear Function*, Academic Press, Incorporated
18. Saiardi, A., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (2000) *FEBS Lett* 468(1), 28-32.
19. Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., and Snyder, S. H. (1999) *Curr Biol* 9(22), 1323-1326
20. Stevenson-Paulik, J., Odom, A. R., and York, J. D. (2002) *J Biol Chem* 277(45), 42711-42718
21. Seeds, A. M., Sandquist, J. C., Spana, E. P., and York, J. D. (2004) *J Biol Chem* 279(45), 47222-47232
22. Saiardi, A., Nagata, E., Luo, H.R., Sawa, A., Luo, X., Snowman, A.M., Snyder, S.H. (2001) *Proc. Natl. Acad. Sci. U S A* 98(5), 2306-2311
23. Fujii, M., and York, J. D. (2005) *J Biol Chem* 280(2), 1156-1164
24. Chang, S. C., Miller, A. L., Feng, Y., Wente, S. R., and Majerus, P. W. (2002) *J Biol Chem* 277(46), 43836-43843
25. Nalaskowski, M. M., Deschermeier, C., Fanick, W., and Mayr, G. W. (2002) *Biochem J* 366(Pt 2), 549-556
26. Verbsky, J. W., Wilson, M. P., Kisseleva, M. V., Majerus, P. W., and Wente, S. R. (2002) *J Biol Chem* 277(35), 31857-31862
27. Verbsky, J. W., Chang, S. C., Wilson, M. P., Mochizuki, Y., and Majerus, P. W. (2005) *J Biol Chem* 280(3), 1911-1920
28. Monnier, V., Girardot, F., Audin, W., and Tricoire, H. (2002) *Free Radic Biol Med* 33(9), 1250-1259
29. Gimeno, C. J., Ljungdahl, P. O., Styles, C. A., and Fink, G. R. (1992) *Cell* 68(6), 1077-1090
30. Dubois, E., Dewaste, V., Erneux, C., and Messenguy, F. (2000) *FEBS Lett* 486(3), 300-304
31. El Alami, M., Messenguy, F., Scherens, B., and Dubois, E. (2003) *Mol Microbiol* 49(2), 457-468
32. Irvine, R. F., and Schell, M. J. (2001) *Nat Rev Mol Cell Biol* 2(5), 327-338
33. Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., and Snyder, S. H. (1999) *Curr Biol* 9(22), 1323-1326.
34. Safrany, S. T., Caffrey, J. J., Yang, X., Bembenek, M. E., Moyer, M. B., Burkhart, W. A., and Shears, S. B. (1998) *Embo J* 17(22), 6599-6607
35. Miller, G. J., and Hurley, J. H. (2004) *Mol Cell* 15(5), 703-711
36. York, J. D., Ponder, J. W., and Majerus, P. W. (1995) *Proc Natl Acad Sci U S A* 92(11), 5149-5153
37. York, J. D., and Majerus, P. W. (1999) *Proc Natl Acad Sci U S A* 87(24), 9548-9552
38. Guo, S., Stolz, L.E., Lemrow, S.M., York, J.D. (1999) *J Biol Chem* 274, 12990-12995
39. Stolz, L. E., Kuo, W. J., Longchamps, J., Sekhon, M. K., and York, J. D. (1998) *J Biol Chem* 273(19), 11852-11861.
40. Stolz, L. E., Huynh, C. V., Thorner, J., and York, J. D. (1998) *Genetics* 148(4), 1715-1729.
FOOTNOTES

We thank members of the York laboratory for helpful discussions, especially Drs. Odom and Stevenson-Paulik for studies related to inositol polyphosphate “add-back” experiments. This work was supported by grants from the Howard Hughes Medical Institute (J.D.Y.), and from the National Institutes of Health – R01 HL-55672 (J.D.Y.) and minority supplement grant (R.J.B.), and R33 DK070272 (J.D.Y).

The abbreviations used are: PLC, phospholipase C; I(1,4,5)P₃, inositol 1,4,5-trisphosphate HPLC, high pressure liquid chromatography; Vₘₓ, maximal velocity attained with excess substrate; Kₘ, substrate concentration permitting a half-maximal velocity, PP-IP₄, diphosphoinositol tetrakisphosphate; IP, inositol polyphosphate; IP₃, inositol trisphosphate; IP₄, inositol tetakisphosphate; IP₅, inositol pentakisphosphate; IP₆, inositol hexakisphosphate; Ipk2, inositol polyphosphate kinase 2; dmIP3K, Drosophila I(1,4,5)P₃ 3-kinase beta isoform; DIPP, diphosphoryl inositol polyphosphate phosphohydrolase.

Fig. 1. Comparison of I(1,4,5)P₃ 6- versus 3-kinase function in the rescue of ipk2Δ-specific growth defects. Strains of ipk2Δ, ipk2Δ ipk1Δ or ipk2Δ kcs1Δ (panel B only) were transformed with empty pRS314 vector (Vec) or pRS314 containing dmlpk2 or dmIP3K. dmlpk2 has I(1,4,5)P₃ 6-kinase activity; whereas dmIP3K has 3-kinase activity. The strains were then serially diluted (1/10) and spotted onto (A) plates containing complete minimal (CM), ornithine as the sole nitrogen source (ORN), or (B) YPD medium and grown for two days at indicated temperatures.

Fig. 2. Molecular dissection of a new IP pathway initiated by I(1,4,5)P₃ 3-kinase activity. Kinase mutant yeast strains: ipk2Δ, ipk2Δ kcs1Δ, or ipk2Δ ipk1Δ were transformed with either empty pRS314 vector (Vec) or pRS314 vector containing dmIP3K (dmIP3K) as indicated. Strains were radiolabeled to isotopic equilibrium with metabolic precursor [³H]-inositol, harvested at late logarithmic phase and soluble extracts containing inositol polyphosphates were separated by strong-anion exchange HPLC. Elution positions of various IP and PP-IP species are indicated. The PP-IPs were identified as PP-IP₄ (pyrophosphate species generated through phosphorylation of IP₅) and PP₂-IP₃ (pyrophosphate species generated through phosphorylation of PP-IP₄). Symbols are assigned to the PP-IPs to distinguish between the different isomers identified in this study and in previous studies. Black bars indicate multiple species.

Fig. 3. Kcs1 and Ipk1 are required for ipk2Δ-independent IP synthesis. High resolution and sensitivity isotopic equilibrium radiolabeling was performed on ipk2Δ + dmIP3K (Upper trace), ipk2Δ (second trace), ipk2Δipk1Δ (third trace), or ipk2Δkcs1Δ (bottom trace) strains as described in Materials and Methods. Soluble extracts containing [³H]-IP and PP-IP molecules were separated by HPLC using higher resolution column and protracted elution gradient as compared to Fig 2. Elution positions of various IP and PP-IP species are indicated.

Fig. 4. Budding yeast Ipk1 functioned as an I(1,3,4,5)P₄ 2-kinase. 100 nM I(1,4,5)P₃ was incubated at 37 °C with no kinase (A), 100ng recombinant dmIP3K (B), or both dmIP3K and sclpk1 (C) in a buffer containing 50 mM HEPES (pH 7.5), 50 mM NaCl, 20 mM MgCl₂, and 4 mM ATP. A duplicate reaction to C was treated with human Type I 5-phosphatase at 37 °C (D). In panels A-D, reaction products were resolved by Partisphere strong anion exchange HPLC and elution profile of a I(1,3,4,5,6)P₅ standard was superimposed in panel C – gray line. (E) The kinetic parameters of sclpk1 phosphorylation of I(1,3,4,5)P₃ were determined using the above reaction conditions while the I(1,3,4,5)P₃ concentration was varied. Kₘ and Vₘₓ values were obtained from a nonlinear curve fit to the Michaelis-Menten equation using GraphPad Prism version 4.01. The R² value was 0.9697.
Fig. 5. Enzymatic identification of IP and PP-IP species present in the I(1,4,5)P_3 3-kinase pathway. DIPP and Type I 5-phosphatase treatment of labeled yeast extracts. Radiolabeled IP extracts were prepared from *ipk2* deficient cells expressing *dmIP3K* and digested with control (no enz), 500 ng recombinant human type I inositol polyphosphate 5-phosphatase (5Ptase), 500 ng recombinant human diphosphoryl inositol polyphosphate phosphatase (DIPP) or both (DIPP/5Ptase). The resulting reactants were then separated by HPLC as described in figure 2. Elution positions of various IP and PP-IP species are indicated.

Fig. 6. Pulse-chase analysis of IP metabolism in yeast mutants expressing *dmIP3K*. *ipk2Δ* (A) or *ipk2Δ ipk1Δ* (B) expressing pRS314 vector containing *dmIP3Kβ* were grown overnight to logarithmic phase, pulse labeled with 1 mCi/ml [³H]-inositol for 10 min., washed and chased for indicated times with medium containing excess cold-inositol. Radiolabeled extracts were harvested and analyzed by Partisphere strong-anion exchange HPLC as described for Fig. 2. Elution positions of various IP and PP-IP species are indicated.

Fig. 7. Identification of a novel I(1,4,5)P_3 3-kinase activity for Kcs1. (Panel A) *ipk2Δ* was transformed with vector containing pRS314-*dmIP3K* (top trace) or pRS426-Kcs1 under control of the Gal4 promoter (middle trace). Additionally, *ipk2Δ ipk1Δ* was transformed with pRS426-Kcs1 (bottom trace). Strains were radiolabeled to isotopic equilibrium and soluble extracts were harvested and separated by HPLC as described for Fig. 2. (Panel B) Enzymatic treatment of radiolabeled prepared from *ipk2 ipk1* double mutant cells overexpressing Kcs1. The extracts were then incubated at 37 °C with no enzyme (top trace), 500 ng of human Type I 5-phosphatase (second trace) or 500 ng human DIPP (third trace), or both (bottom trace). The products were then analyzed as described for Fig. 2. Elution positions of various IP and PP-IP species are indicated.

Fig. 8. Revised molecular map of IP and PP-IP pathways in budding yeast. The pathway designated by the thick bold arrows represents the originally described I(1,4,5)P_3 6-kinase pathway. The novel 3-kinase dependent IP/PP-IP pathway in *S. cerevisiae* is shown with thin arrows and may be initiated artificially by expression of *dmIP3K* or endogenously by a 3-kinase activity of Kcs1 and/or possibly *ipk2*. The gene products involved in the synthesis of each reaction are designated. Symbols were assigned to the PP-IPs to distinguish between the different isomers. The PP-IP chemical structures and ring-positions harboring the pyrophosphate have yet to be determined. *Gray font* illustrates portions of the model that are postulated from the data in this and/or previous studies.
Table I. 5-phosphatase susceptibility of extracts from pulse labeled strains

| Time after pulse | IP$_3$ | IP$_4$ | IP$_5$ | IP$_3$ | IP$_4$ |
|------------------|--------|--------|--------|--------|--------|
| 30 min           | ND     | ND     | ND     | +      | +      |
| 90 min           | ND     | ND     | ND     | -      | +      |
| 180 min          | -      | +      | +      | -      | +      |
| 360 min          | -      | +      | +      | -      | +      |
| 540 min          | -      | -      | +      | -      | +      |

+, ≥50% of the total IP$_3$, IP$_4$, or IP$_5$ content is 5-phosphatase susceptible; -, ≥50% of total IP$_3$, IP$_4$, or IP$_5$ content is 5-phosphatase resistant; ND, not determined. 5-phosphatase susceptibility was determined by comparing HPLC traces of extracts incubated with or without 5-phosphatase.
Figure 1

A  
CM (30°C)  ORN (30°C)

B  
YPD (30°C)  YPD (37°C)

- ipk2Δ + Vec
- ipk2Δ + dmlpk2
- ipk2Δ + dmlIP3K
- ipk2Δ ipk1Δ + Vec
- ipk2Δ ipk1Δ + dmlpk2
- ipk2Δ ipk1Δ + dmlIP3K
- ipk2Δ kcs1Δ + Vec
- ipk2Δ kcs1Δ + dmlpk2
- ipk2Δ kcs1Δ + dmlIP3K
Figure 2

Relative Counts

Ins/IP₁  IP₂  IP₃  IP₄  IP₅  PP-IP₃β  PP₂-IP₃β

ipk2Δ + Vec

ipk2Δ + dmIP3K

ipk2Δ kcs1Δ + dmIP3K

ipk2Δ ipk1Δ + dmIP3K

time (min)
Figure 3

Relative Counts vs. time (min)

- *ipk2Δ*
- *ipk2Δ ipk1Δ*
- *ipk2Δ kcs1Δ*

Species:
- IP₂
- I(1,4,5)P₃
- I(3,4,5)P₃
- I(1,3,4,5)P₄/IP₄ₜ
- I(1,2,3,4,5)P₅
- PP-IP₃
- PP₂-IP₃
- PP₂-IP₃

Legend:
- ΔdmIP3K
- Δipk2
- Δipk1
- Δkcs1
Figure 4

A

I(1,4,5)P₃

B

I(1,3,4,5)P₄

C

I(1,3,4,5,6)P₅ Std.

I(1,2,3,4,5)P₅

I(1,3,4,5)P₅

D

I(1,3,4)P₃

I(1,2,3,4)P₄

E

V_{MAX} 60.3 nmol/min/mg

Kₐ 148.3 µM

nmol/min/mg

time (min)

[I(1,3,4,5)P₄] µM
Figure 6

A

B

Relative Counts

IP₃, IP₄, IP₅, PP-IP, PP₂-IP

30min

60min

90min

120min

150min

180min

240min

330min

time (min)

2 5 10 15 20 25 30

2 5 10 15 20 25 30

2 5 10 15 20 25 30

2 5 10 15 20 25 30

2 5 10 15 20 25 30

2 5 10 15 20 25 30

2 5 10 15 20 25 30

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2 5 10 15 20 25 30
Figure 7

A

B
Revised Yeast IP and PP-IP Pathways

Figure 8
