Phosphorylation of inositol hexakisphosphate and diphosphoinositol pentakisphosphate by a conserved class of kinases

Abstract

Inositol polyphosphates (IPs) comprise a major family of second messengers involved in a variety of intracellular signaling pathways. These molecules have regulatory roles in processes ranging from calcium release to transcription and mRNA export. Among these IPs, several species of highly phosphorylated inositol pyrophosphates have been identified, the functions of which are poorly understood. Recently, two classes of inositol pyrophosphate synthases have been cloned and characterized in yeast, designated Kcs1/IP6K and Vip1. Kcs1 and Vip1 are kinases capable of producing the inositol pyrophosphates diphosphoinositol pentakisphosphate (PP-IP_5 or IP_7) and bisdiphosphoinositol tetrakisphosphate (PP_2-IP_4 or IP_8). Of functional interest, Vip1 was previously identified as a regulator of the actin-related protein-2/3 (Arp2/3) complex, a vital mediator of actin branching and cytoskeleton organization. My thesis work has involved the characterization of yeast Vip1 and the cloning of its human ortholog, *hs*Vip1. I determined that yeast Vip1 possesses specific, pH-dependent IP_6 kinase activity *in vitro*, and that this kinase activity is required for genetic interactions with Arp2/3 complex members. Using biochemical and cell biological methods, I found that *hs*Vip1 has robust IP_6 and IP_7 kinase activities *in vitro* as well as in yeast and mammalian cells. The cloning and characterization of yeast and human Vip1 gene products has helped define a novel class of evolutionarily conserved inositol pyrophosphate synthases and has uncovered unanticipated roles for its IP_7 and IP_8 products in actin cytoskeleton and cellular nutrient signaling pathways.

Introduction

Inositol polyphosphates (IPs) are a diverse group of signaling molecules involved in a variety of intracellular signaling pathways. Soluble IPs are predominantly derived from inositol-1,4,5-trisphosphosphate (IP_3), which is released from lipid phosphoinositides by receptor-activated phospholipase C (PLC) (1,2). In addition to the well-studied function of $I(1,4,5)P_3$ in calcium release, signaling roles have been found for higher phosphorylated derivatives of IP_3, and numerous kinases responsible for the production of these IPs have been characterized (3-5). These inositol polyphosphate kinases, well-conserved from yeast to mammals, produce inositol tetrakisphosphate (IP_4), inositol pentakisphosphate (IP_5), and inositol hexakisphosphate (IP_6) (4). Studies have demonstrated roles for these IPs in processes ranging from transcriptional regulation, chromatin remodeling, and nuclear mRNA export, to regulation of ion channels and mouse embryogenesis (6-11).

In addition to these IPs, however, several species of inositol pyrophosphates (PP-IPs) have been identified, along with one family of inositol pyrophosphate synthase capable of producing them from less highly phosphorylated IPs. These PP-IPs were first identified and characterized in *Dictyostelium discoideum* and mammalian cells, and are distinguished by the presence of one or more pyrophosphate groups on the inositol ring (12-14). The IP_6 kinase (IP6K) family of enzymes, Kcs1 in *S. cerevisiae* and IHPK1, IHPK2, and IHPK3 in mammals, was found to convert IP_6 to the PP-IP diphosphoinositol pentakisphosphate, also known as PP-IP_5 or IP_7 (15,16). Loss of this activity in budding yeast results in defects in the response to osmotic stress, regulation of telomere length, vacuolar biogenesis, endocytosis, and other cellular processes (16-21). PP-IP_5 has also

been shown to act as a phosphate donor, capable of phosphorylating proteins directly, in a non-enzymatic process (22). Furthermore, IP6K activity is required, though not sufficient, for the synthesis of bisdiphosphoinositol tetrakisphosphate (PP_2-IP_4 or IP_8), a more highly phosphorylated PP-IP species containing two pyrophosphate groups. Studies have demonstrated that PP_2-IP_4 levels are involved in the response to osmotic and heat stress in both yeast and mammalian cells, and appear to be regulated in part by the MAP kinase pathway (23-25). PP_2-IP_4, along with PP-IP_5, also appears to have a role in certain cAMP-mediated signaling events, including chemotaxis in *Dictyostelium discoideum*, with levels of these metabolites significantly altered during cAMP signaling (23,26,27). It has not been clear, however, what enzyme produces PP_2-IP_4 from PP-IP_5 in either yeast or mammals.

In addition to the already characterized IP6K, a second IP_6 kinase activity has been detected in budding yeast, termed Vip1. In yeast mutants lacking both Kcs1/IP6K activity and the inositol pyrophosphatase activity of Ddp1 (diphosphoinositol polyphosphophosphate diphosphatase), a significant amount of PP-IP_5 production has been detected (19,28). Members of the York lab have recently cloned the gene encoding this distinct inositol pyrophosphate synthase, through a biochemical purification of the IP_6 kinase activity from a *kcs1Δddp1Δ* double knockout yeast strain (29). The gene identified, VIP1, has previously been detected through genetic interactions as a possible regulator of actin polymerization and cytoskeletal function (30). Recombinant Vip1 protein fused to glutathione S-transferase (GST) and purified from bacteria showed IP_6 kinase activity, and was also capable of phosphorylating the PP-IP_5 product of mammalian IHPK1 to PP_2-IP_4. Bioinformatic analysis revealed that Vip1 is well

conserved from yeast to mammals, and consists of two distinct domains. The first, N-terminal domain, belonging to the ATP-grasp superfamily, encodes Vip1's IP_6 kinase activity, while the C-terminal domain belongs to the histidine acid-phosphatase family of enzymes (Fig. 1A) (31,32).

My goals were first to further characterize the IP_6 kinase activity of yeast Vip1 through enzyme kinetics, supplementing the initial data of Dr. Sashi Mulugu in the York lab. Furthermore, to explore the biological relevance of this protein in yeast, I examined its genetic interactions with components of the actin polymerization complex in *S. cerevisiae*, and the dependence of these interactions on Vip1's kinase activity. I then planned to clone the human homolog of Vip1, *hs*Vip1, and determine if this enzyme also exhibited IP_6 and PP-IP_5 kinase activity, given the evolutionary conservation of the enzymes' kinase domains (Fig. 2). I looked at this first with recombinant GST-*hs*Vip1 protein, and also by transforming yeast and transfecting mammalian cells with *hs*Vip1 constructs, and examining changes in metabolic IP levels. Because of the involvement of Vip1's apparent PP-IP products in such a wide array of signal transduction pathways, characterizing the enzymatic activity of this protein in both yeast and mammals, along with the relationship of this activity to biological processes, is vital to an understanding of the increasingly evident signaling roles of inositol pyrophosphates.

Materials and Methods

Strains

Saccharomyces cerevisiae strains were typically grown in rich yeast peptone dextrose medium (YPD), while strains carrying a plasmid were grown in complete synthetic medium (CSM) lacking the nutrient corresponding to the plasmid's marker. Most strains used were from previous studies. To generate *vip1*Δ *las17*Δ double knock-outs, however, a *vip1::HIS3*/VIP1 *las17::LEU2*/LAS17 diploid strain was constructed by mating JYY915 (*MAT*α *vip1::HIS3*) and JYY916 (*MAT*a *las17::LEU2*) (Table 1) (30). This diploid was transformed with pRS426-VIP1, vip1D487A, and vip1H548A constructs, as well as with pRS426 alone, using a standard PEG/lithium acetate transformation procedure. Transformants were sporulated and dissected, then replica plated onto CSM-URA, CSM-HIS, and CSM-LEU to identify haploid *vip1::HIS3 las17::LEU2* double knockouts, as well as *vip1::HIS3* and *las17::LEU2* knockouts. These strains were then verified through PCR genotyping.

Cloning, recombinant expression and purification of human Vip1

The coding sequence (CDS) of the *S. cerevisiae* VIP1 gene, along with 1-535 and 538-1047 truncation mutants, were PCR amplified from a wild-type yeast strain by Dr. Sashi Mulugu, and cloned into the pGEX-KG gluthathione S-transferase (GST) fusion vector. Kinase-dead (D487A) and kinase-only (H548A) point mutants were made by Dr. James Otto through site-directed mutagenesis (29).

The human VIP1 CDS (*hs*Vip1) was PCR amplified from a cDNA clone (accession number BC050263) obtained from Open Biosystems (Huntsville, AL). Sal I

sites were installed at the 5' and 3' ends, using hsVIP1-1-Sal sense primer 5'-CG TCC AGT CGA CTC ATG TGG TCA TTG ACG GCC AGT GAG GGC-3' and hsVIP1-1433-Sal anti-sense primer 5'-GCT CCA GTC GAC CTA ATT TAT CTC CTC AGG GAC CTC CTG GGC-3' (Sal I sites are underlined). A truncation mutant of the kinase domain, residues 1-387, was also cloned using the hsVIP1-1-Sal sense primer, and the hsVIP1-387-Sal anti-sense primer 5'-GCT CCA GTC GAC CTA CAT AGT GCC AGA TGT GGT GGG AAC AAT GG-3'. A mutant containing the putative acid-phosphatase domain (residues 390-1433) was cloned using the hsVIP1-390-Sal sense primer 5'-CG TCC AGT CGA CTC GAA CTT CGT TGT GTC ATT GCA ATT ATT CGT CAT GG-3' and the hsVIP1-1433-Sal anti-sense primer. All cloned constructs were confirmed by sequencing at the Duke University DNA Analysis Facility.

Yeast GST-Vip1 constructs were expressed and purified by Dr. Sashi Mulugu as described previously (29,33). Human Vip1 constructs were transformed into BL21 DE3 Star *E. Coli* (Invitrogen, Carlsbad, CA), and protein was expressed by inducing at 18°C with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours. Cells were resuspended in lysis buffer (25 mM Tris, pH 8.0, 350 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and lysed by passing twice through a Microfluidics M110L homogenizer. The lysate was cleared by centrifugation, and the supernatant was applied to a column of gluthathione-sepharose (Sigma, St. Louis, MO) equilibrated in lysis buffer. After washing, protein was eluted in buffer containing 25 mM Tris, pH 8.0, 350 mM NaCl, 1mM DTT, and 10 mM glutathione. Eluted protein was stored at -80°C.

Assays of Vip1 IP_6 kinase activity

Enzyme assays were typically run in 10 μL reactions, containing 50 mM HEPES, pH 6.2, 1 mM ATP, 5 mM $MgCl_2$, and approximately 80,000 cpm of D-2-[^{32}P]-IP_6. For kinetic analyses, IP_6 concentrations ranged from approximately 0.1 μM to 100 μM. Reactions were generally incubated 20-40 minutes at 37°C, and stopped by adding 1 μL of 2.5 N HCl and placing on ice. Reactions were spotted onto PEI-cellulose TLC plates in 2.5 μL increments, with samples dried in between spotting, and developed in a TLC tank equilibrated with a buffer consisting of 1.09 M KH_2PO_4, 0.72 M K_2HPO_4, and 2.07 M HCl. Plates were exposed to phosphor storage screens, which were read on a 4500 SI PhosphorImager (Amersham Biosciences/Molecular Dynamics, Piscataway, NJ). [^{32}P]-IP_6 was produced enzymatically in 50uL reactions, with 1.7 pmol γ-[^{32}P]-ATP (specific activity 6,000 Ci/mmol, Perkin Elmer, Waltham, MA), 50 pmol I(1,3,4,5,6)P_5, and 1μg of the IP_5 2-kinase GST-*at*IPK1. This reaction was run in a buffer of 50 mM HEPES, pH 7.5, 1 mM ethylene glycol tetraacetic acid (EGTA), 100 mM KCl, and 3mM $MgCl_2$, and was incubated at 37°C for 30 minutes, with the enzyme subsequently heat-inactivated at 95°C for 10 minutes. Kinetic parameters were obtained using a Lineweaver-Burke plot.

Complementation analysis of yeast Vip1 mutants

For plasmids used in Vip1 complementation analysis, a genomic fragment containing the VIP1 promoter and coding sequence was PCR amplified from a wild-type yeast strain and cloned into pRS315 plasmid by Robert Bastidas. Kinase-dead (D487A) and kinase-only (H548A) point mutants were also produced by Robert Bastidas by subcloning mutant fragments from the appropriate pGEX-KG GST fusion construct. The

wild-type, kinase-dead, and kinase-only genomic fragments were further subcloned into pRS426 using BamHI and Sal I restriction sites. Constructs were confirmed by DNA sequencing. The human Vip1 full length sequence, as well as its kinase domain (residues 1-387) and acid phosphatase domain (residues 390-1433) were PCR amplified from the above cDNA clone, and Nde I and Sal I restriction sites were added to the 5' and 3' ends, respectively. For the full-length coding sequence, the primers used were the hsVIP1-1-Nde sense primer 5'-CGT CCA CAT ATG TGG TCA TTG ACG GCC AGT GAG GGC-3' (Nde I site is underlined) and hsVIP1-1433-Sal anti-sense primer. For the kinase domain, the primers used were the hsVIP1-1-Nde sense primer, and the hsVIP1-390-Sal anti-sense primer. For the putative acid phosphatase domain, the hsVIP1-390-Nde sense primer 5'-CGT CCA CAT ATG GAA CTT CGT TGT GTC ATT GCA ATT ATT CGT CAT GG-3' and the hsVIP1-1433-Sal anti-sense primer were used. These PCR products were then ligated into the pUNI10 univector plasmid (34). Full-length and truncation mutants were then cloned into the pRS426-loxP-GFP-myc3 vectors through loxP recombination in a GST-Cre recombinase reaction, as previously described (34).

Spot assays were performed first by resuspending a similar number of cells in 50 μL of water and bath sonicating to eliminate clumping. Cells were then serially diluted 1:10 five times, and 5 μL of each dilution was spotted onto plates of the appropriate medium.

Yeast steady state inositol labeling for HPLC analysis

1 mL volumes of CSM lacking appropriate nutrients and containing 20 μCi/mL [^{3}H]-*myo*-inositol (American Radiolabel Corp., St. Louis, MO) were inoculated with

single colonies of a *kcs1*Δ *ddp1*Δ *vip1*Δ triple mutant transformed with appropriate plasmids. After incubating until saturation (about 2 days), soluble inositols were harvested as previously described (7). Samples were diluted 1:5 in 10 mM ammonium phosphate (AP), and run by Dr. Shean-Tai Chiou on a 4.6 mm x 125 mm Partisphere SAX-HPLC strong anion exchange column (Whatman, Clifton, NJ).

Cell culture

To clone human Vip1 constructs into a mammalian vector, *hs*VIP1 full-length, kinase domain, and acid phosphatase domain sequences were PCR amplified from cDNA as with GST constructs, with Kpn I and Not I sites added at the 5' and 3' ends. For the full-length protein, the primers hsVIP1-1-5kpn, GGTA GGT ACC ATG TGG TCA TTG ACG GCC AGT GAG GGC, and hsVIP1-1433-3not, GGAT GCGGCCGC CTA ATT TAT CTC CTC AGG GAC CTC CTG GGC, were used. For the kinase domain, primers hsVIP1-1-5kpn and hsVIP1-387-3not, GGAT GCGGCCGC CTA CAT AGT GCC AGA TGT GGT GGG AAC AAT GG, were used. For the phosphatase domain, the hsVIP1-1433-3not primer was used along with hsVIP1-390-5kpn, GGTA GGT ACC ATG GAA CTT CGT TGT GTC ATT GCA ATT ATT CGT CAT GG. These fragments were subcloned into pCFP-N, a CFP fusion vector containing a human cytomegalovirus (CMV) promoter. A 293T line of cells was transfected by Dr. James Otto with these constructs using the FuGENE 6 transfection reagent (Roche, Indianapolis, IN), and radiolabeled for 2 days in inositol-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 25 μCi/mL [^{3}H]-*myo*-inositol (American Radiolabel Corp., St. Louis,

MO). Cells were washed in PBS, soluble inositols were released by resuspending in 0.5 N HCl, and samples were diluted in 10 mM AP for HPLC analysis as described (35).

Results

Biochemical activity of recombinant *S. cerevisiae* Vip1

Dr. Sashi Mulugu's identification of Vip1 as an inositol pyrophosphate synthase through biochemical purification from yeast demonstrated the kinase activity of the endogenous yeast protein (29). To confirm that this activity was intrinsic to Vip1, and to further characterize it, Dr. Mulugu cloned and purified recombinant GST-*sc*Vip1 from *E. Coli*. Together with Dr. Mulugu, I helped determine the kinetics of the IP_6 kinase activity using *in vitro* IP_6 kinase assays (Fig. 3A). Vip1 showed strong specificity for IP_6 over other inositol polyphosphates, with a K_M of 17.63 μM. The maximum velocity V_{max} determined for the production of PP-IP_5 was 22.63 nmol/min/mg. To distinguish the activities of Vip1's two domains, truncation mutants of the kinase domain (residues 1-535) and the putative acid phosphatase domain (residues 538-1047) were also cloned and purified by Dr. Mulugu (Fig. 1B). Similar values for K_M (20.66 μM) and V_{max} (99 nmol/min/mg) were found for the yeast kinase domain alone (residues 1-535), while no IP_6 kinase activity was seen for the acid phosphatase domain (Fig. 3A and 3C). Kinase activity was also ablated by a point mutation of a conserved catalytic glutamic acid residue at position 487 to alanine (Fig. 1A). Activity remained, however, after a similar point mutation in the acid phosphatase domain of a catalytic histidine at position 548 to alanine (29). The pH-dependence of Vip1's IP_6 kinase activity was also jointly examined, with buffer conditions ranging from pH 4.0 to 8.8 (Fig. 3D) (29). Optimal

activity was observed at pH 6.2, though greater than 80% of activity was observed from pH 6 to pH 6.8.

Dependence of *sc*Vip1's genetic interactions on kinase activity

While Vip1's kinase activity has been detected both *in vitro* and through *in vivo* [^{3}H]-inositol labeled yeast studies, I also examined the biological relevance of this activity in yeast (29). A previous report identified Vip1 as a possible regulator of the Arp2/3-mediated actin polymerization pathway (30). *sc*Vip1 showed a severe synthetic growth defect and temperature sensitivity with Las17, a yeast ortholog of Wiscott-Aldrich Syndrome Protein (WASP), a key regulator of the Arp2/3 complex (36). To examine the involvement of Vip1's kinase activity in this synthetic interaction, a complementation analysis was done with various *sc*Vip1 constructs (Fig. 4). The severe growth defect was rescued by overexpression of both full-length *sc*Vip1 and a kinase-only mutant with a point mutation in the acid-phosphatase domain. However, a kinase-dead mutant with a deactivating mutation in the kinase domain did not complement this interaction.

Kinase activity of recombinant human Vip1

To explore the evolutionary conservation of Vip1's IP_6 kinase activity, I examined the biochemical activity of the human ortholog, *hs*Vip1. I therefore cloned the human Vip1 gene (*hs*Vip1) and expressed and purified recombinant GST constructs of the full-length protein, as well as of kinase (residues 1-387) and acid-phosphatase (residues 390-1433) domains, as determined by homology. Preliminary enzymological

studies have demonstrated that full-length *hs*Vip1, as well as the kinase domain alone, exhibit robust dose-dependent IP_6 kinase activity (Fig. 5A). This activity shows similar specificities and maximum velocities as *sc*Vip1, with a K_M of 17.98 μM and a V_{max} of 24.69 nmol/min/mg (Fig. 3B and 3C). From these *in vitro* experiments, mammalian Vip1 appears to retain the inositol pyrophosphate synthase activity observed in the yeast enzyme. Additionally, PP-IP_5 from the mammalian IP6K IHPK1 was converted by recombinant *hs*Vip1 to PP_2-IP_4, an activity also seen with the yeast protein. The relative kinetic parameters of *hs*Vip1's two kinase activities have not yet been resolved.

_hs_Vip1 kinase activity in yeast and mammalian cells

In addition to these biochemical results, *hs*Vip1's kinase activity was also observed through *in vivo* studies. First, yeast mutants overexpressing *hs*Vip1 were radiolabeled with [^{3}H]-*myo*-inositol and extracts were analyzed through HPLC. In yeast mutants lacking both Kcs1 and *sc*Vip1 genes, no PP-IP_5 was detected in radiolabeled extracts. However, when either full-length or kinase domain *hs*Vip1 constructs were overexpressed in these mutants, a significant amount of PP-IP_5 was detected (Fig. 5B). While the acid phosphatase domain was also overexpressed in yeast, no change in soluble inositol levels was observed, with either yeast or human Vip1 constructs.

*hs*Vip1 constructs were also overexpressed in [^{3}H]-*myo*-inositol labeled mammalian 293T cells, with transfections performed by James Otto. There was little change in IP levels in wild-type cells, however, with no noticeable accumulation of PP-IP_5. To increase the flux of IP_6 in transfected cells, *hs*Vip1 was coexpressed with the G protein Gαq, a strong activator of PLC that significantly increases IP concentrations,

along with Ipk1, an I(1,3,4,5,6)P_5 2-kinase that synthesizes IP_6 (Fig. 6) (37,38). Under these conditions, a small peak of PP-IP_5 was detectable, as well as a small peak of PP_2-IP_4. More significantly, however, when *hs*Vip1 was coexpressed with IHPK1, a member of the IP_6 kinase enzyme family, a much larger peak of PP_2-IP_4 was observed. As expression of IHPK1 substantially increases levels of its PP-IP_5 product, this suggests that *hs*Vip1's phosphorylation of PP-IP_5 to PP_2-IP_4 is a major activity in mammalian cells.

Discussion

Enzyme kinetics of *S. cerevisiae* Vip1 demonstrate, as previously reported, significant IP_6 kinase activity. It has a high, specific affinity for IP_6, with a K_M within the normal range of intracellular IP_6 levels in yeast and mammalian cells (39). The specific activity of the enzyme, while relatively weak, is in the same range as other IP kinases, and is biologically significant (28). While this activity is dependent on pH, peak activity occurs in the range of 6-7, indicating that *sc*Vip1 should be capable of normal activity when localized to the cytoplasm. Further, genetics studies suggest that this kinase activity is biologically relevant in yeast. *sc*Vip1 has previously been found to have synthetic interactions with elements of Arp2/3 actin polymerization, specifically the Las17 protein (30). Las17 regulates the Arp2/3 complex, which catalyzes the nucleation of actin filaments, and is required for the actin branching necessary for actin's cytoskeletal functions (36,40). This synthetic interaction appears to depend on the presence of kinase activity, as only constructs with an intact kinase domain can rescue the *vip1*Δ*las17*Δ synthetic growth defect. This suggests that *sc*Vip1's kinase activity, and

likely its PP-IP_5 product, are required for whatever involvement *sc*Vip1 might have in actin polymerization. This is consistent with work by other members of the York lab observing defects in cell growth, morphology, and Arp2/3 synthetic interactions in *Schizosaccharomyces pombe* yeast overexpressing kinase-dead mutants of Asp1, the *S. pombe* Vip1 ortholog (29).

Sashi Mulugu's cloning of the *S. cerevisiae* Vip1 gene immediately allowed a bioinformatic analysis of the structure and evolutionary conservation of the enzyme. In addition to the dual domain structure revealed by this analysis, a sequence alignment of Vip1 genes from yeast, mammals, and other model species revealed a conservation of these domains throughout evolutionary history, with known catalytic residues universally conserved among species (Fig. 2) (29). This suggested that Vip1 orthologs in other species might have a similar role as an IP_6 kinase. Consistent with this, the human ortholog, *hs*Vip1, possessed *in vitro* IP_6 and PP-IP_5 kinase activities comparable to those of yeast Vip1. Additionally, yeast overexpressing *hs*Vip1 constructs showed conversion of IP_6 to PP-IP_5. These data are consistent with studies by other members of the York lab examining yeast mutants overexpressing the yeast Vip1 protein, and suggest that the human enzyme exhibits IP_6 kinase activity in the cytosolic environment of a eukaryotic cell, and is capable of producing a physiologically relevant amount of PP-IP_5 in cells (29). Vip1 IP_6 kinase activity does therefore appear to be evolutionary well-conserved between yeast and mammals.

Significantly, this conserved IP_6 kinase activity appears to have a biological signaling function in budding yeast. A recent report has identified the PP-IP_5 product of *sc*Vip1 as a regulator of the cyclin/CDK complex Pho80/Pho85, a transcriptional

regulator involved in phosphate homeostasis (41). PP-IP_5 from *sc*Vip1, and not from Kcs1, is required for inhibition of Pho80/Pho85 by the CDK inhibitor Pho81, through direct binding of this complex. Additionally, PP-IP_5 levels appear to rise upon phosphate starvation, when Pho80/Pho85 is normally inhibited. *vip1Δ* null yeast strains also appear deficient for Pho80/Pho85 inhibition, leading to a defective phosphate starvation response. This biological function of *sc*Vip1 reveals a specific role for its kinase activity, mediated by direct interaction between its PP-IP_5 product and its regulatory target. This vital, specific signaling role of Vip1 in yeast reveals a clear biological function for the protein. It is not inconsistent with a possible role in Arp2/3 regulation, however, as other soluble IPs have been found to have numerous independent signaling functions. The integration of these single molecules' diverse signaling roles continues to be an important question in inositol signal transduction, and further elucidation of mechanisms regulating Vip1's activity would be useful in exploring the problem.

In addition to studies in yeast, *hs*Vip1 was overexpressed in inositol radiolabeled mammalian cells. Only a small amount of PP-IP_5 production was detected, however. Considering the considerable IP_6 kinase activity detectable in yeast and with recombinant protein, this failure to detect significant IP_6 kinase activity could be a result of some mechanism in mammalian cells regulating or inhibiting this activity under normal conditions. Alternatively, it could be a result of sequestration of intracellular IP_6 from *hs*Vip1, possible through either protein binding or compartmentalization. It is also possible, however, that IP_6 kinase activity is not a primary activity of Vip1 in mammalian cells. Given the many biological differences between yeast and mammalian phosphate

regulation and actin polymerization mechanisms, it is not inconceivable that Vip1's kinase domain, despite its conservation, has different signaling functions across species.

Despite the lack of robust IP_6 kinase activity seen in mammalian cells, *hs*Vip1 did exhibit a high level of PP-IP_5 kinase activity, producing PP_2-IP_4 when coexpressed with the IHPK1 IP_6 kinase. Vip1's apparent ability to produce PP_2-IP_4 from PP-IP_5 indicates possible involvement with several previously reported examples of inositol pyrophosphate signaling. PP_2-IP_4 appears to be involved in certain stress responses, as well as in some cAMP-mediated signaling events (23-27). Some of these responses appear to be mediated by MAP kinase pathways, suggesting one possible regulatory mechanism for this activity. An inositol pyrophosphate synthase with a robust PP-IP_5 kinase activity has not yet been reported, and it appears that *hs*Vip1 is a major producer of PP_2-IP_4 in cells. Given these results, and the biochemical kinase activities observed for both human and yeast proteins, production of PP-IP_5 and PP_2-IP_4 can be tentatively assigned to the IP6K and Vip1 enzymes (Fig. 7) (29). However, further studies examining the importance of *hs*Vip1 to the regulation of PP_2-IP_4 levels are needed to explore Vip1's precise involvement in this pathway.

While *hs*Vip1 appears to possess both IP_6 and PP-IP_5 kinase activities, the relative strength and importance of these is not yet clear. Further enzymological studies are necessary to determine the relative affinities and specific activities for these two substrates. This would help determine whether the apparent lack of IP_6 kinase activity in mammalian cells, and the much stronger PP-IP_5 kinase activity observed, is a result of PP-IP_5 being a significantly better substrate. It would also be helpful to overexpress both *hs*Vip1 and Kcs1/IP6K in yeast, to determine whether this PP-IP_5 kinase activity can be

detected outside of mammalian cells. Additionally, it would be valuable to examine the availability of intracellular IP_6 to soluble enzymes, which might indicate whether substrate sequestration plays a role in regulating Vip1's IP_6 kinase activity. Performing these experiments with the yeast Vip1 protein would also be valuable, allowing comparison of activities between orthologs. As different organisms appear to utilize IP_6 and inositol pyrophosphates in different manners, it would be interesting to determine if the function of this enzyme varied between species, or if its relative activities as an IP_6 or PP-IP_5 kinase were evolutionarily conserved.

References

1. Majerus, P. W., Ross, T. S., Cunningham, T. W., Caldwell, K. K., Jefferson, A. B., and Bansal, V. S. (1990) *Cell* **63**(3), 459-465
2. Berridge, M. J., and Irvine, R. F. (1984) *Nature* **312**(5992), 315-321
3. Berridge, M. J., and Irvine, R. F. (1989) *Nature* **341**(6239), 197-205
4. Shears, S. B. (2004) *Biochem J* **377**(Pt 2), 265-280
5. York, J. D. (2006) *Biochim Biophys Acta* **1761**(5-6), 552-559
6. El Alami, M., Messenguy, F., Scherens, B., and Dubois, E. (2003) *Mol Microbiol* **49**(2), 457-468
7. Odom, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000) *Science* **287**(5460), 2026-2029
8. Steger, D. J., Haswell, E. S., Miller, A. L., Wente, S. R., and O'Shea, E. K. (2003) *Science* **299**(5603), 114-116
9. York, J. D., Odom, A. R., Murphy, R., Ives, E. B., and Wente, S. R. (1999) *Science* **285**(5424), 96-100
10. Ho, M. W., Yang, X., Carew, M. A., Zhang, T., Hua, L., Kwon, Y. U., Chung, S. K., Adelt, S., Vogel, G., Riley, A. M., Potter, B. V., and Shears, S. B. (2002) *Curr Biol* **12**(6), 477-482
11. Frederick, J. P., Mattiske, D., Wofford, J. A., Megosh, L. C., Drake, L. Y., Chiou, S. T., Hogan, B. L., and York, J. D. (2005) *Proc Natl Acad Sci U S A* **102**(24), 8454-8459

12. Europe-Finner, G. N., Gammon, B., and Newell, P. C. (1991) *Biochem Biophys Res Commun* **181**, 191-196
13. Menniti, F. S., Miller, R.N., Putney, J.W. Jr., Shears, S.B. (1993) *J Biol Chem* **268**, 3850-3856
14. Stephens, L., Radenberg, T., Thiel, U., Vogel, G., Khoo, K.H., Dell, A., Jackson, T.R., Hawkins, P.T., Mayr, G.W. (1993) *J Biol Chem* **268**, 4009-4015
15. Saiardi, A., Erdjument-Bromage, H., Snowman, A. M., Tempst, P., and Snyder, S. H. (1999) *Curr Biol* **9**(22), 1323-1326
16. Saiardi, A., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (2000) *J Biol Chem* **275**(32), 24686-24692
17. Dubois, E., Scherens, B., Vierendeels, F., Ho, M. M., Messenguy, F., and Shears, S. B. (2002) *J Biol Chem* **277**(26), 23755-23763
18. Saiardi, A., Resnick, A. C., Snowman, A. M., Wendland, B., and Snyder, S. H. (2005) *Proc Natl Acad Sci U S A* **102**(6), 1911-1914
19. York, S. J., Armbruster, B. N., Greenwell, P., Petes, T. D., and York, J. D. (2005) *J Biol Chem* **280**(6), 4264-4269
20. Saiardi, A., Sciambi, C., McCaffery, J. M., Wendland, B., and Snyder, S. H. (2002) *Proc Natl Acad Sci U S A* **99**(22), 14206-14211
21. Bennett, M., Onnebo, S. M., Azevedo, C., and Saiardi, A. (2006) *Cell Mol Life Sci* **63**(5), 552-564
22. Saiardi, A., Bhandari, R., Resnick, A. C., Snowman, A. M., and Snyder, S. H. (2004) *Science* **306**(5704), 2101-2105
23. 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
24. Pesesse, X., Choi, K., Zhang, T., and Shears, S. B. (2004) *J Biol Chem* **279**(42), 43378-43381
25. Choi, K., Mollapour, E., and Shears, S. B. (2005) *Cell Signal* **17**(12), 1533-1541
26. Safrany, S. T., and Shears, S. B. (1998) *Embo J* **17**(6), 1710-1716
27. Laussmann, T., Pikzack, C., Thiel, U., Mayr, G. W., and Vogel, G. (2000) *Eur J Biochem* **267**(8), 2447-2451
28. Seeds, A. M., Bastidas, R. J., and York, J. D. (2005) *J Biol Chem* **280**(30), 27654-27661
29. Mulugu, S., Bai, W., Fridy, P.C., Bastidas, R.J., Otto, J.C., Dollins, D.E., Haystead, T.A., Ribeiro, A.A., York, J.D. (2007) *Science* **IN PRESS**
30. Feoktistova, A., McCollum, D., Ohi, R., and Gould, K. L. (1999) *Genetics* **152**(3), 895-908
31. Van Etten, R. L., Davidson, R., Stevis, P. E., MacArthur, H., and Moore, D. L. (1991) *J Biol Chem* **266**(4), 2313-2319
32. Mullaney, E. J., and Ullah, A. H. (2003) *Biochem Biophys Res Commun* **312**(1), 179-184
33. Stevenson-Paulik, J., Bastidas, R. J., Chiou, S. T., Frye, R. A., and York, J. D. (2005) *Proc Natl Acad Sci U S A* **102**(35), 12612-12617
34. Liu, Q., Li, M. Z., Leibham, D., Cortez, D., and Elledge, S. J. (1998) *Curr Biol* **8**(24), 1300-1309
35. Stevenson-Paulik, J., Chiou, S. T., Frederick, J. P., dela Cruz, J., Seeds, A. M., Otto, J. C., and York, J. D. (2006) *Methods* **39**(2), 112-121

36. Li, R. (1997) *J Cell Biol* **136**(3), 649-658
37. Smrcka, A. V., Hepler, J. R., Brown, K. O., and Sternweis, P. C. (1991) *Science* **251**(4995), 804-807
38. Ives, E. B., Nichols, J., Wente, S. R., and York, J. D. (2000) *J Biol Chem* **275**(47), 36575-36583
39. Ingram, S. W., Safrany, S. T., and Barnes, L. D. (2003) *Biochem J* **369**(Pt 3), 519-528
40. Higgs, H. N., and Pollard, T. D. (2001) *Annu Rev Biochem* **70**, 649-676
41. Lee, Y. S., Mulugu, S., York, J. D., and O'Shea, E. K. (2007) *Science* **316**(5821), 109-112
42. Stolz, L. E., Huynh, C. V., Thorner, J., and York, J. D. (1998) *Genetics* **148**(4), 1715-1729

Figures and Tables

Strain	Relevant Genotype	Reference
W303	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	(42)
JYY911	W303 *MATα kcs1::HIS3 ddp1::HIS3 vip1::kanMX4*	This work
JYY915	KGY1350 *MATα vip1::HIS3 ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52*	Gift of K. Gould (30)
JYY916	KGY2142 *MATa las17::LEU2 his3 leu2 trp1 ura3*	Gift of K. Gould (30)
JYY917	*MATα/a vip1::HIS3/VIP1 las17::LEU2/LAS17*	This work
JYY918	*MATα vip1::HIS3 las17::LEU2*	This work
JYY919	JYY918 + pRS426	This work
JYY920	JYY918 + pRS426-VIP1	This work
JYY922	JYY918 + pRS426-Vip1D487A	This work
JYY923	JYY918 + pRS426-Vip1H548A	This work

Table 1. *S. cerevisiae* strains used in this report.

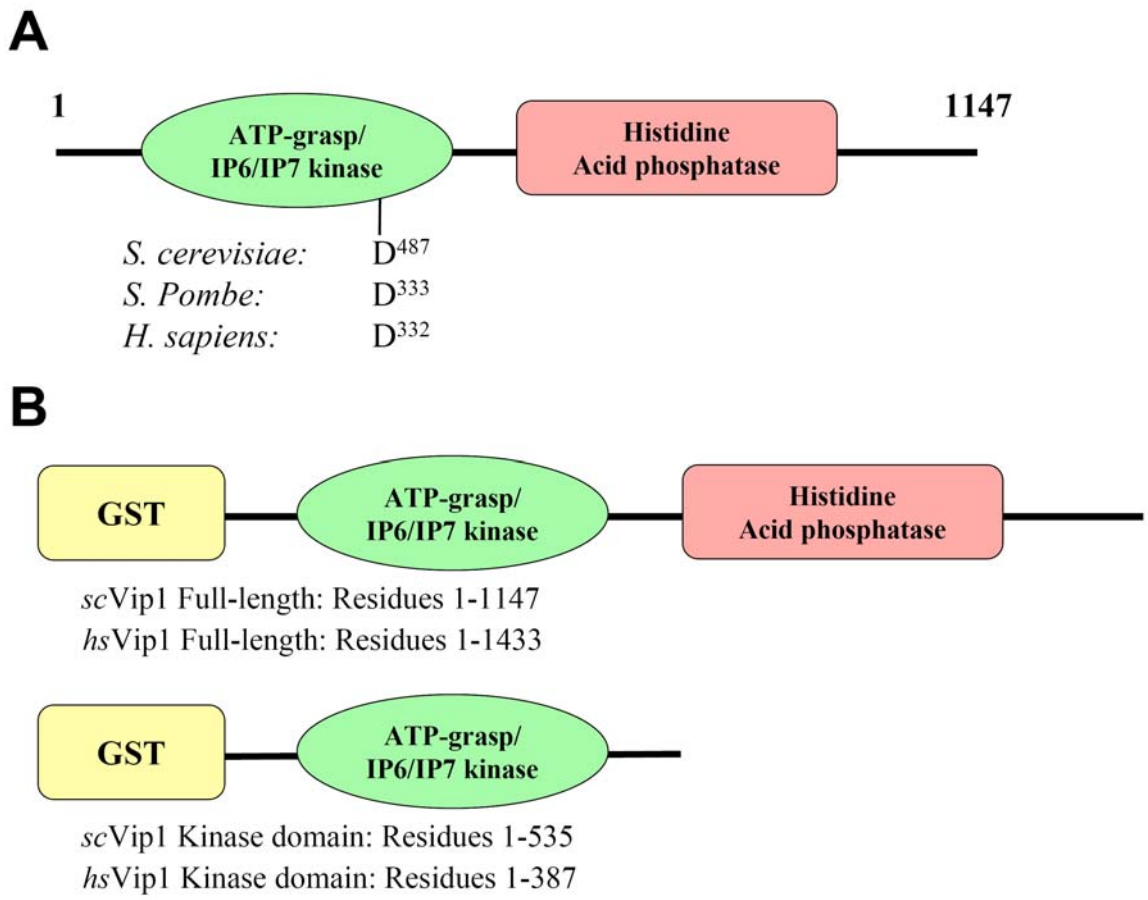


Fig. 1. Schematics of Vip1 structure and GST constructs used. **(A)** In the *S. cerevisiae* protein, conserved ATP-grasp and histidine acid phosphatase domains are located at residues 200-525 and 530-1025, respectively. The ATP-grasp domain was found to exhibit kinase activity specific for IP_6 and IP_7 ($PP\text{-}IP_5$) substrates. In yeast, this activity depended on the presence of a highly conserved catalytic aspartic acid residue, shown here. **(B)** Several constructs were used in the purification of recombinant Vip1 enzymes from bacteria. All enzymes were fused at the amino terminus to glutathione S-transferase (GST), and full-length and kinase domain-only constructs were used, for both yeast and human proteins.

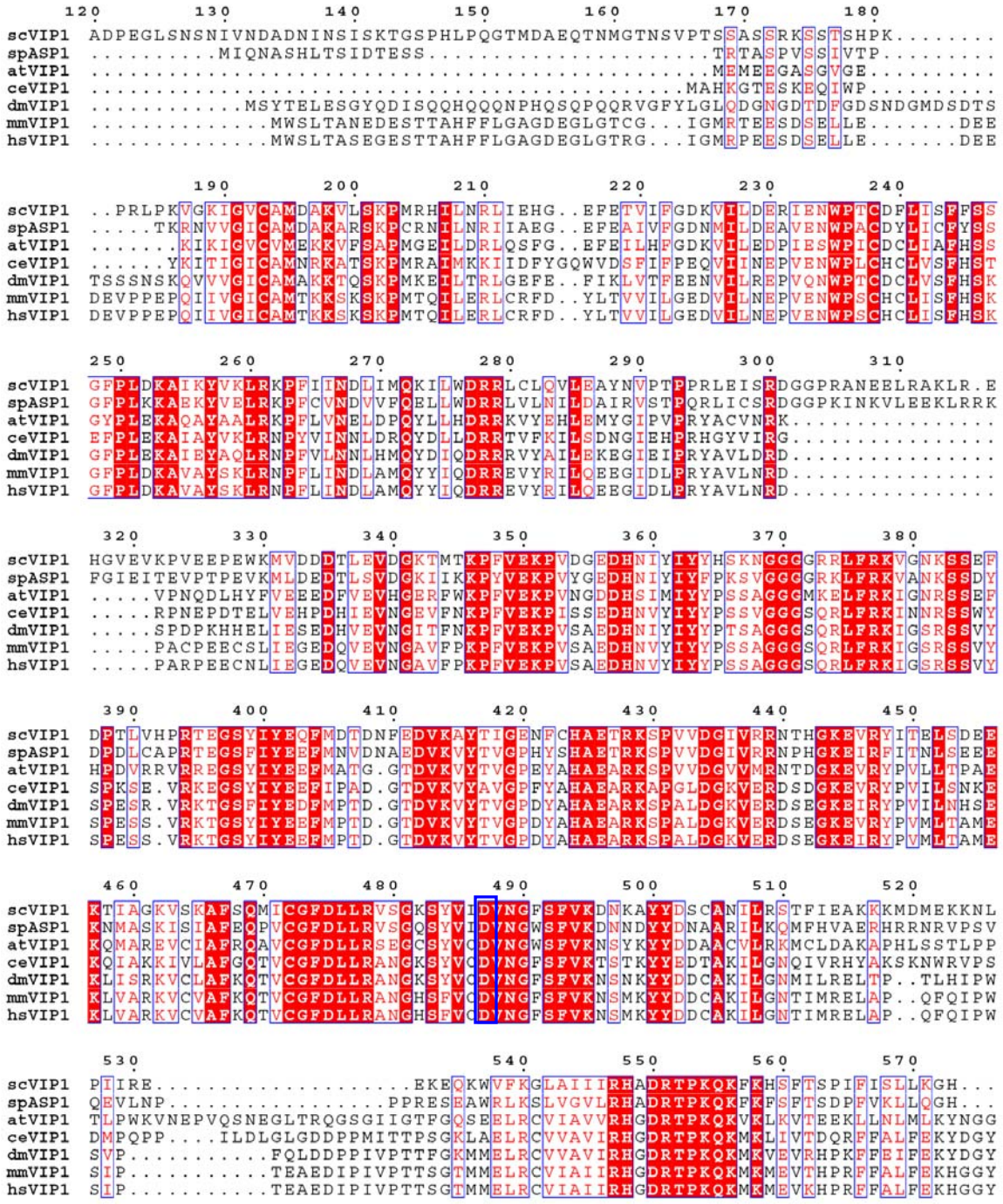
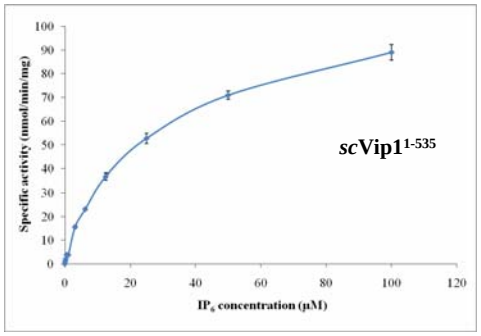
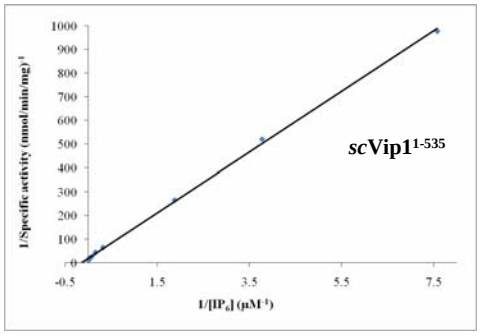


Fig. 2. Evolutionary conservation of Vip1 kinase domain across species. A multi-sequence alignment was performed with VIP1 homologs from *Saccharomyces cerevisiae* (*sc*, accession NP_013514), *Schizosaccharomyces pombe* (*sp*, SPCC1672.06c), *Arabidopsis thaliana* (*at*, NP_001030614), *Caenorhabditis elegans* (*ce*, NP_740855), *Drosophila melanogaster* (*dm*, CG14616-PE), *Mus musculus* (*mm*, NP_848910), and *Homo sapiens* (*hs*, AAH57395). Residues 200-525 in *S. cerevisiae* were identified as having homology to the ATP-grasp domain superfamily, and have been found to encode IP_6 kinase activity in both yeast and humans. A catalytic aspartic acid residue required

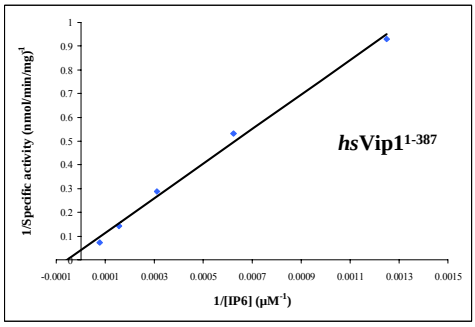
for this activity is boxed in blue. Identical residues are shown in solid red boxes, while similar residues are shown as red text in blue boxes. Alignment was printed using the ENDScript/ESPript 2.2 tool, accessed at <http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>. Alignment was generated with the EMBL-EBI ClustalW tool, accessed at <http://www.ebi.ac.uk/clustalw/>.

A

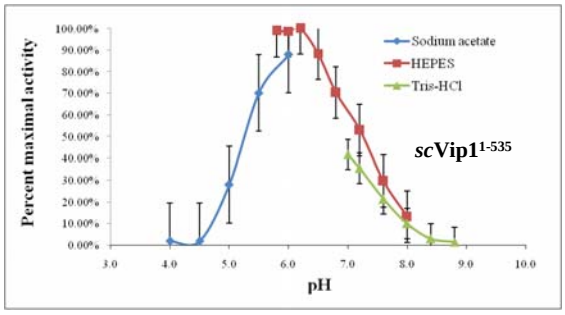




B



D



C

	K_M (μM)	V_{max} (nmol/min/mg)
*sc*Vip1	17.63	22.63
*sc*Vip1 1-535	20.66	98.79
*hs*Vip1 1-387	17.98	24.69

Fig. 3. Biochemical analysis of IP_6 kinase activity of Vip1's kinase domain. **(A)** In enzyme kinetics studies of the *sc*Vip1 kinase domain truncation mutant (residues 1-535), the dependence of initial velocity on substrate concentration was examined in triplicate. K_M and V_{max} were also determined from a Lineweaver-Burke plot. **(B)** Lineweaver-Burke plot of IP_6 kinase activity of the *hs*Vip1 kinase domain. **(C)** Kinetic parameters of *sc*Vip1 and full-length and kinase domain (residues 1-535), as well as the *hs*Vip1 kinase domain (residues 1-387). **(D)** The pH dependence of *sc*Vip1 kinase domain activity was determined in triplicate in buffers ranging from pH 4.0 to pH 8.8. Maximal activity was observed at pH 6.2.

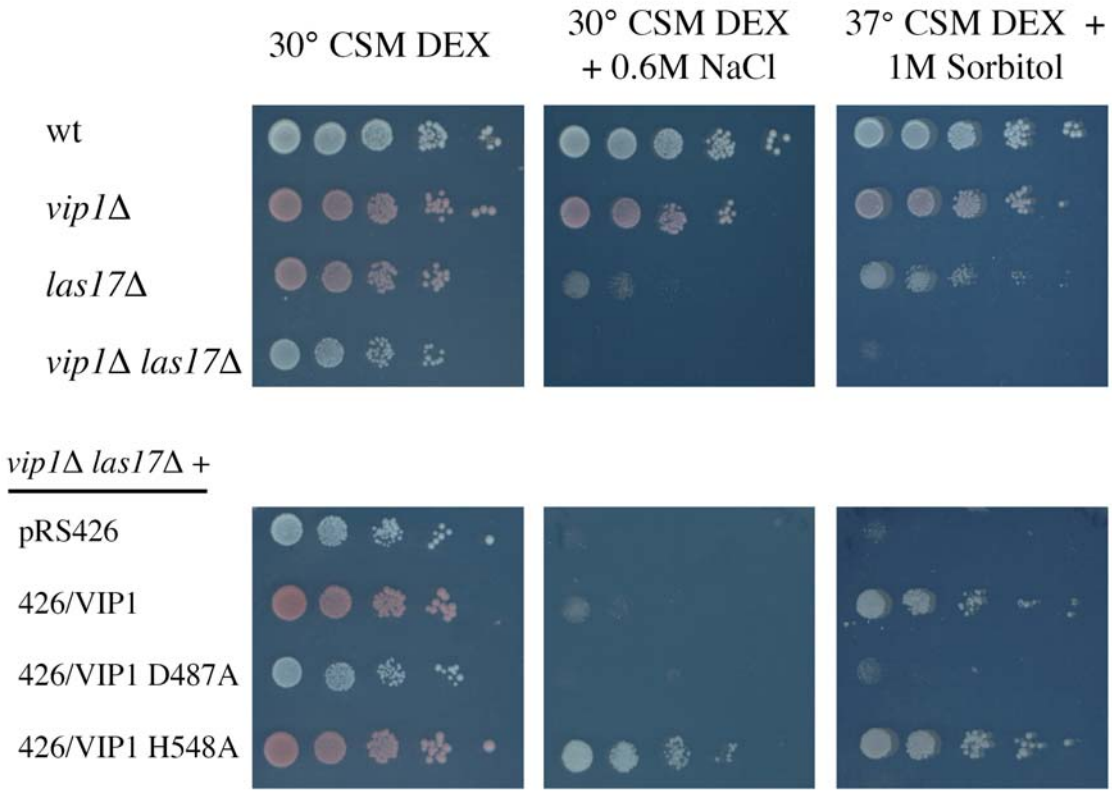


Fig. 4. Complementation analysis of synthetic interaction between *sc*Vip1 and Las17 genes. v*ip1*Δ *las17*Δ double mutants show a severe sensitivity to osmotic stress and temperature that is not seen in single mutants (top panel). This synthetic growth defect is substantially rescued by overexpression of full-length *sc*Vip1, driven by an endogenous promoter in a high-copy vector (bottom panel). Expression of a Vip1 D487A point mutant, which has no IP_6 kinase activity, does not complement the defect, while an H548A acid phosphatase mutant can.

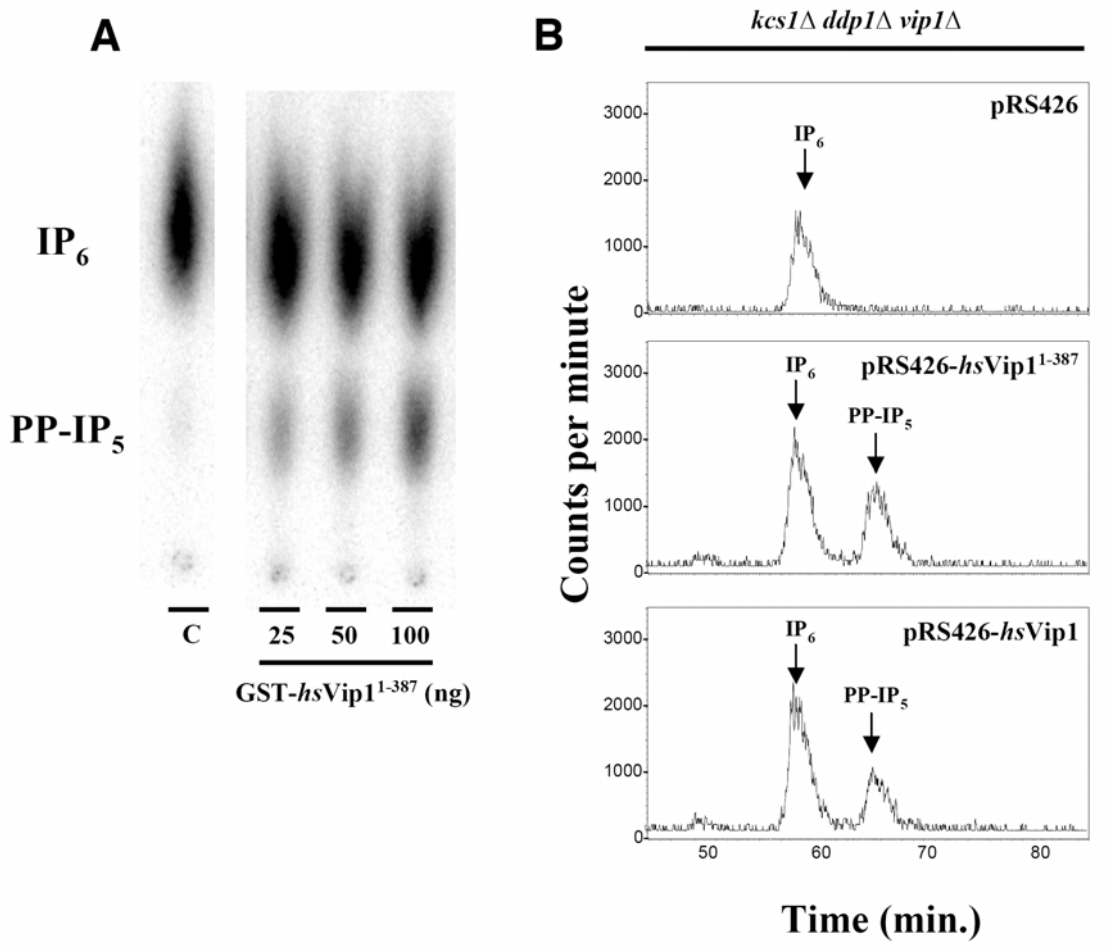


Fig. 5. IP_6 kinase activity of human Vip1 enzyme. **(A)** IP_6 kinase assays were run i*n vitro* with ATP and human GST-Vip1 kinase domain protein (residues 1-387) and resolved on a PEI-cellulose TLC plate. [^{32}P]-IP_6 is converted to [^{32}P]-PP-IP_5 in a dose-dependent manner. (B) Yeast cells deficient for the IP_6 kinases *kcs1* and *vip1* and the inositol pyrophosphatase *ddp1* do not show a PP-IP_5 peak in [^{3}H]-inositol radiolabeled extracts resolved by HPLC. However, when full-length human Vip1 or its kinase domain are overexpressed, a relatively large peak of PP-IP_5 is detected, demonstrating *hs*Vip1's IP_6 kinase activity in yeast.

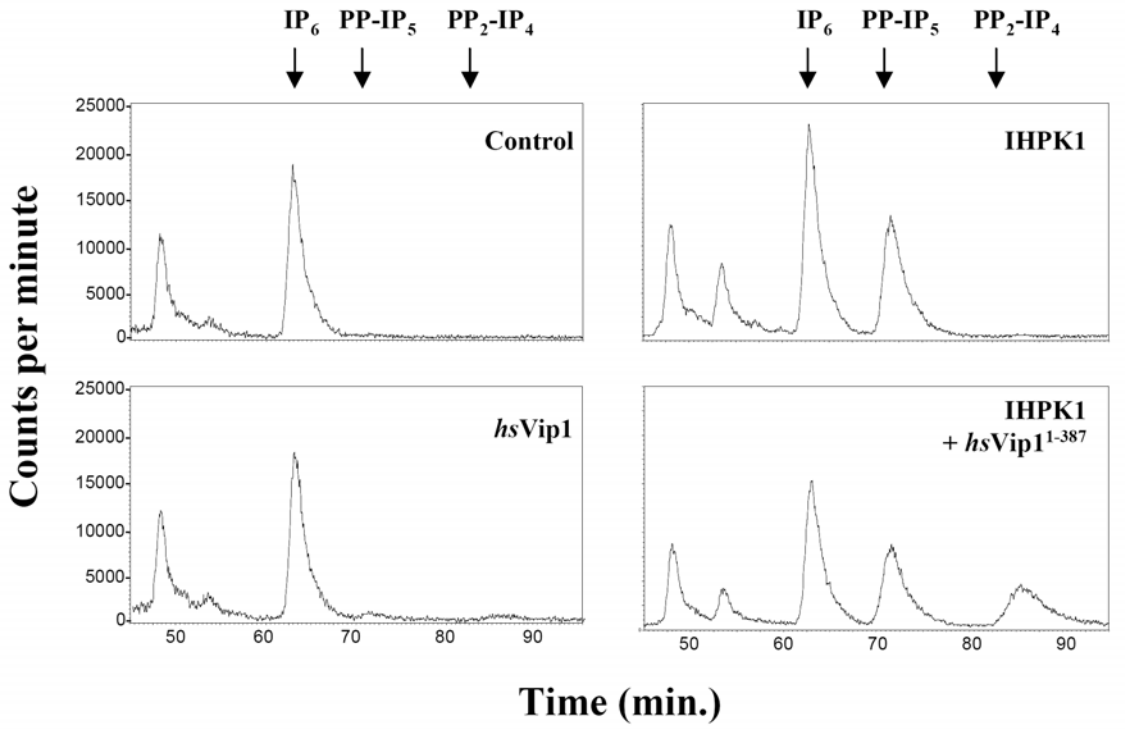


Fig. 6. IP_6 and PP-IP_5 kinase activity of human Vip1 in a mammalian 293T cell line. Control cells overexpressing the PLC activator Gαq and the IP_5 kinase Ipk1 produce a large IP_6 peak in HPLC-analyzed [^{3}H]-*myo*-inositol radiolabeled extracts, but no detectable pyrophosphates. Overexpression of human Vip1 kinase domain (residues 1-387) leads to the appearance of very small PP-IP_5 and PP_2-IP_4 peaks, while expression of a human IP6K, IHPK1, produces a much stronger PP-IP_5 peak. Coexpression of these two IP_6 kinases leads to the appearance of a relatively large PP_2-IP_4 peak, indicating that one or both enzymes act as a PP-IP_5 kinase.

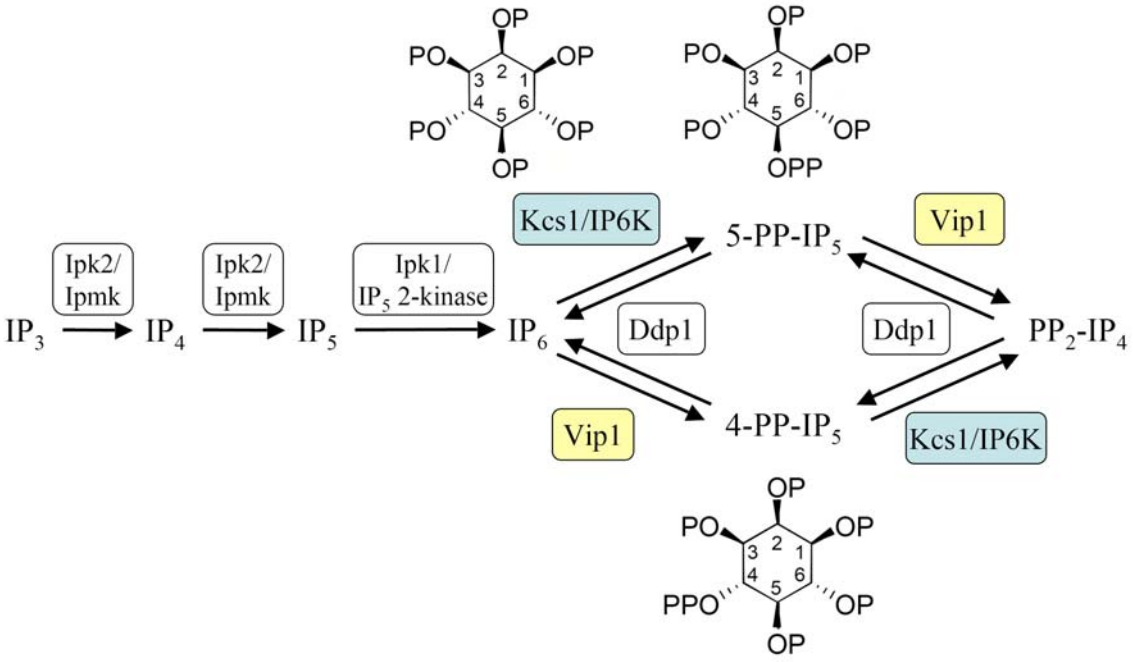


Fig. 7. Outline of the inositol pyrophosphate synthesis pathway. With the identification of Vip1 as an IP_6 and PP-IP_5 kinase, this is a simplified outline of the currently understood pathway of inositol pyrophosphate synthesis in yeast and humans. The PP-IP_5 products of IP6K and Vip1 have been identified as structurally distinct through NMR studies, with the pyrophosphate group on the 5 or 4 position, respectively (29). This report and previous studies have also shown that IP6K and Vip1 can act on each other's PP-IP_5 products, together producing PP_2-IP_4.