Biochemical and Functional Characterization of Inositol 1,3,4,5,6-Pentakisphosphate 2-Kinases*

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Synthesis of inositol 1,2,3,4,5,6-hexakisphosphate (IP₆), also known as phytate, is integral to cellular function in all eukaryotes. Production of IP₆ predominately occurs through phosphorylation of inositol 1,3,4,5,6-pentakisphosphate (IP₅) by a 2-kinase. Recent cloning of the gene encoding this kinase from Saccharomyces cerevisiae, designated scpIpk1, has identified a cellular role for IP₆ production in the regulation of mRNA export from the nucleus. In this report, we characterize the biochemical and functional parameters of recombinant scpIpk1. Purified recombinant scpIpk1 kinase activity is highly selective for IP₅ substrate and exhibits apparent Km values of 644 nm and 62.8 μM for IP₅ and ATP, respectively. The observed apparent catalytic efficiency (kcat/Km) of scpIpk1 is 31,610 s⁻¹ M⁻¹. A sequence similarity search was used to identify an IP₅ 2-kinase from the fission yeast Schizosaccharomyces pombe. Recombinant scpIpk1 has similar substrate selectivity and catalytic efficiency to its budding yeast counterpart, despite sharing only 24% sequence identity. Cells lacking sc-Ipk1 are deficient in IP₆ production and exhibit lethality in combination with a gle1 mutant allele. Both of these phenotypes are complemented by expression of the spIpk1 gene in the sc-ipk1 cells. Analysis of several inactive mutants and multiple sequence alignment of scpIpk1, spIpk1, and a putative Candida albicans Ipk1 have identified residues involved in catalysis. This includes two conserved motifs: E(i/l/m)KPKWL(t/y) and LXMTLRLDV(t/y)UC(t/y)I. Our data suggest that the mechanism for IP₆ production is conserved across species.

Inositol polyphosphates (IPs)¹ in eukaryotic cells are key regulatory molecules whose levels transiently fluctuate in response to diverse cellular stimuli (1, 2). A major route for synthesis of IPs is through activation of phosphatidylinositol-specific phospholipase C. Phospholipase C cleaves lipids such as phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate, a regulator of calcium efflux from the endoplasmic reticulum. The release of a soluble inositol head group from its anchoring lipid also represents the first step in the pathway for generation of more highly phosphorylated inositols (3). The most abundant of these is inositol 1,2,3,4,5,6-hexakisphosphate (IP₆), also known as phytate. IP₆ can represent up to 1% of the mass of a plant seed, where it may serve as an antioxidant and a phosphate storage source (4, 5). The role of IP₆ is less clear in mammalian cells, although there is evidence suggesting that it may regulate inflammation, neurotransmission, and cell growth (reviewed in Ref. 3).

Recently, a metabolic pathway converting inositol 1,4,5-trisphosphate to IP₆ was delineated in budding yeast Saccharomyces cerevisiae cells (6–9). It has been shown that IP₆ also serves as a precursor for diphosphorylated inositols, such as diphosphoryl inositol 1,3,4,5,6-pentakisphosphate (PP-IP₅), in both yeast and vertebrate cells (3, 10). Combined in vivo and in vitro studies show that three genes (PLC1, IPK1, and IPK2/ARG82) account for the pathway converting phosphatidylinositol 4,5-bisphosphate to IP₆. Ipk2 is a dual-specificity inositol-1,4,5-trisphosphate 6-kinase and inositol-1,4,5,6-tetrakisphosphate (IP₄) 3-kinase (7). Ipk1 is an IP₆ 2-kinase (6). Physiological roles for soluble IPs in at least two nuclear functions have been established: mRNA export and transcription. Mutant alleles of plc1, ipk1, and ipk2 were all identified in a synthetic lethal genetic screen with a temperature-sensitive gle1-2 mutant defective for an essential mRNA export factor (6, 11). Moreover, the plc1, ipk1, and ipk2 mutants each individually have mRNA export defects at a restrictive growth temperature (6). These mutants do not show defects in nuclear envelope morphology, nuclear protein import, or protein export (6). Overexpression of PLC1 in cells suppresses the temperature sensitivity of the gle1-4 mutant in a manner that depends on a functional IP₆ 2-kinase (6).² Taken together, these results suggest a direct role for this IP₆ pathway, specifically IP₆ production, in either stimulating or regulating mRNA export. Interestingly, Ipk2 is identical to Arg82, a regulator of the ArgR/Mcm1 transcription complex (7, 12, 13). Ipk2 may control transcriptional responses by at least two mechanisms. First, the Ipk2 protein itself, but not IP generation, is necessary for assembly of ArgR/Mcm1 complexes on DNA promoter elements (7). Second, the production of IP₆/PP₂ through both phospholipase C and Ipk2 kinase activities is required for proper execution of ArgR/Mcm1-mediated gene expression (7). In this way, complexes may be poised on DNA awaiting activation of inositol signaling for

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‡ The abbreviations used are: IPs, inositol polyphosphates; IP₆, inositol 1,2,3,4,5,6-hexakisphosphate; IP₅, inositol 1,3,4,5,6-pentakisphosphate; IP₄, inositol 1,4,5,6-tetrakisphosphate; PP-IP₅, diphosphoryl IP₅; PP-IP₄, diphosphorylated IP₄; PP-IP₂, bis(diphospho)-IP₂; sc, S. cerevisiae; sp, S. pombe; ca, C. albicans; 5-FOA, 5-fluoroorotic acid; GST, glutathione S-transferase; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.

² A. R. Odom and J. D. York, unpublished data.
TABLE I  
**S. cerevisiae strains used in this study**

| Strain             | Genotype                                      | Derivation     |
|--------------------|-----------------------------------------------|----------------|
| W303a              | Mata ade1-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 | Ref. 6         |
| SWY1659            | Mata ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ipk1::kan<sup>+</sup> | Ref. 6         |
| SWY1793            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-1-4 g1e1-2 | Ref. 6         |
| SWY1834            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-1-4 g1e1-2 | Ref. 6         |
| SWY1853            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-1-4 g1e1-2 | Ref. 6         |
| SWY1836            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-3 g1e1-2 | Ref. 6         |
| SWY1837            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-5 g1e1-2 | Ref. 6         |
| SWY2227            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-4 g1e1-2 | SWY1793 transformed with pRS315 |
| SWY2228            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-4 g1e1-2 | SWY1281        |
| SWY2229            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-4 g1e1-2 | SWY1793 transformed with pSW406 |
| SWY2230            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-4 g1e1-2 | SWY1793 transformed with pSW989 |
| SWY2231            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-4 g1e1-2 | SWY1793 transformed with pSW989 |
| SWY2232            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-4 g1e1-2 | SWY1793 transformed with pSW989 |
| SWY2233            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-4 g1e1-2 | SWY1793 transformed with pSW989 |
| SWY2234            | Mata ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 1-100 ipk1-4 g1e1-2 | W303a transformed with pRS315 |

**Materials and Methods**

Strains and Media—Yeast strains were grown either in 1% yeast extract and 2% peptone or in synthetic minimal medium plus appropriate amino acids supplemented with 2% glucose. Yeast transformations were completed by the lithium acetate method (15), and general genetic manipulations of yeast cells were conducted as described (16). 5-Fluoroorotic acid (5-FOA) was obtained from United States Biologicals and used at a working concentration of 0.5 mg/mL. The *S. cerevisiae* strains used in this study are described in Table I. DH1α was used as the bacterial host for all plasmids. Bacterial strains were cultured in LB medium and transformed by standard methods (17).

**Bacterial Glutathione S-Transferase (GST) Expression Plasmids—** The plasmid for expression of GST-sclp1 fusion protein in bacteria was described previously (6). For the GST-sclp1-C plasmid, sequence encoding *sclp1* residues 557–640 was made by PCR amplification of purified *S. pombe* genomic DNA (the generous gift of Dr. Paul R. Russell, Scripps Research Institute, La Jolla, CA) using 4 ng/μl sense and antisense primers (5′-atgcatgcaggttatactgaattcgtgccgcttttttaactcc-3′ and 5′-atgcatgccacccttactttatgctgctcagcggcatg3′, respectively; uppercase letters denote *sclp1* coding sequence) with Taq polymerase and manufacturer’s buffer (Roche Molecular Biochemicals). The reactions were cycled 30 times under the following conditions: denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min. The resulting product was digested with EcoRI and SalI, gel-purified, and inserted into the EcoRI and SalI sites of pGEX-4T. The regions generated by PCR were sequenced to confirm the product. The sequences of the oligonucleotides for cloning *sclp1*-C were based on a candidate *S. pombe* IP2-kinase (283-residue partial-length gene product; GenBankTM/EBI Data Bank accession number D899240). The candidate was identified by sequence comparison of the primary amino acid sequence of *sclp1* with the GenBankTM/EBI non-redundant data base (November 1998 release) using the grouped BLASTP program (18).

The plasmids harboring GST fusion proteins of the five *sc-ipk1* mutant alleles were generated as follows. The *sc-ipk1* mutant strains were isolated from a synthetic lethal screen with a g1e1-2 mutant (Table I) (6). Genomic DNA was prepared from wild-type and mutant cells for use as a PCR template using 4 ng/μl sense and antisense oligonucleotides: 5′-ggtcagtgaatcgcatatgcatacgcatcgatcagctgctcgaggg- GCA-3′ and 5′-gcacattgatcgcacctgccactgctcagcaaatggtg- GAAAC-3′. To generate *IPK1* fragments, KlenTaq or Taq polymerase was used with the respective manufacturer’s buffer (Sigma and Roche Molecular Biochemicals). The reactions were conducted using a decremental annealing temperature program for 25 total cycles with denaturing at 96 °C for 1 min, annealing over a range from 59 to 49 °C (decreasing by 0.4 °C/cycle) for 1 min, and extension at 72 °C for 2 min, followed by 10 additional cycles at 48 °C annealing. The resulting product was purified and digested with EcoRI and SalI and inserted into the EcoRI and SalI sites of pGEX-4T. The integrity of this and all other constructs was verified by DNA sequencing using the ABI Prism dGTP BigDye Terminator Ready Reaction kit (PE BioSystems). The GST expression plasmids for each of the *sc-ipk1* alleles were as follows: *pSW1285*, sc-ipk1-1; *pSW1284*, sc-ipk1-3; *pSW1282*, sc- ipk1-4, and *pSW1285*, sc-ipk1-5, *pSW1287*. In the course of constructing the pGST-sclp1 plasmid, a construct with a PCR mutation was isolated and further characterized (*pSW1288*). The mutation results in a change of the codon AAT for Asn10 to GAT for Asp, designated *sclp1*-C. Protein Purification of GST Fusion Proteins—All proteins were expressed in DH5α. For large-scale purifications, 1-liter cultures of bacteria in LB medium were allowed to grow at 37 °C to 0.6. Protein expression protein was induced by addition of 0.3 ml isopropyl-b-D-thiogalactopyranoside (final concentration), and growth was continued for 4 h at 37 °C. Cells were pelleted and resuspended in 20 ml of lysis buffer (50 mM Hepes pH 7.5, 50 mM KCl, 8 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 15 min, the cells were disrupted by sonication. The lysate was clarified by a 10,000 × g spin for 30 min, and the resulting supernatant was inclu...
bated with a 1-ml bed volume of glutathione-Sepharose beads at 4 °C for 1 h (with agitation). The beads were washed with 20 ml of lysis buffer and eluted in glutathione buffer (10 mM glutathione, 5 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0), and 50 mM NaCl). For small-scale purifications, 50-ml cultures of cells were grown in LB medium and induced for 3 h as described above. The cells were collected, resuspended in 0.5 ml of lysis buffer, incubated on ice for 15 min, and disrupted by sonication. Lysates were clarified by a 13,000 × g spin for 5 min at 4 °C. The resulting supernatant was incubated with 50 μl of glutathione-Sepharose for 1 h at 4 °C. The beads were washed with 3 ml of lysis buffer and eluted in glutathione buffer. 

**[^32P]IP₅ Production**—The radiolabeled [^32P]IP₅ substrate was made by incubating 125 pmol of unlabeled pure IP₅ (Matreya, Inc., Pleasant Gap, PA) with 3 pmol of [γ-[^32P]]ATP and 9 ng of GST-Ipk2 (7) in a total volume of 25 μl of buffer containing 40 mM Hepes (pH 7.5), 100 mM KCl, 3 mM MgCl₂, and 1 mM EGTA for 1 h at 37 °C. The [^32P]IP₅ product was purified by HPLC (as described below) and dialyzed overnight against 10 mM Hepes (pH 7.5).

**Enzymatic Analysis of IP₅ 2-Kinase Activity**—Enzymatic activity was measured using [^32P]IP₅. For kinetic analysis, standard reactions were conducted in a total volume of 10 μl. Purified GST fusion proteins were diluted in buffer containing 50 mM Hepes (pH 7.5), 2 mM MgCl₂, 20 mM KC1, and 0.2 mg/ml bovine serum albumin and added in a 2-μl volume to an 8-μl reaction mixture of 100 mM Hepes (pH 7.5), 10 mM MgCl₂, ATP, and IP₅. Individual kinetic parameters for scIpk1 were determined by varying the concentration of ATP (0.02–5 mM) while holding IP₅ constant (20 μM). Alternatively, IP₅ was varied (0.125–32 μM) while holding ATP constant (5 mM). Parameters for spIpK1-C were determined using a range of IP₅ concentrations from 0.5 to 32 μM and a range of ATP concentrations from 0 to 400 μM. The amounts of recombinant protein added to individual assays and the time of incubation at 30 °C were varied to maintain substrate conversion within a linear range (5–50% conversion). The reactions were stopped by addition of 2 μl of TLC running buffer (1.08 mM KH₂PO₄, 0.64 mM K₂HPO₄, and 1.84 mM HCl). The samples were spotted onto Baker-flex polyethyleneimine cellulose thin-layer chromatography sheets (J. T. Baker Inc.) and allowed to dry. Separation of IP₅ and IP₆ by TLC was achieved by incubation of the neat sample with solvent (chloroform/methanol (1:2, v/v) and 100 mM NaCl). For high-performance liquid chromatography analysis, the sample was injected onto a BioRad Aminex HPX-87H column and eluted with a 25 mM H₂SO₄ gradient at 0.6 ml/min at 90 °C. The reaction products were analyzed by polyethyleneimine cellulose thin-layer chromatography, which readily resolved IP₅ and IP₆ isomers (Fig. 1A). The results shown are typical of an assay with 10 ng of protein incubated with 20 μM IP₅, 4.5 mM ATP, and 22.5 mM MgCl₂ for the times indicated. The dependence of IP₅ 2-kinase activity on the concentration of IP₅ (B) and ATP (C) was determined. Results shown are the averages of three independent assays presented as saturation curves with specific activity (micromoles of IP₆ formed per min/mg of protein) versus IP₅ (B) or ATP (C) concentration. For the experiments in B, the ATP concentration was constant at 5 mM; in C, the IP₅ concentration was constant at 20 μM. The insets in B and C are Lineweaver-Burk plots of the average values for each data set, with 1/ν as (mmol/min/mg)⁻¹ and 1/S as μM⁻¹ IP₅ in B and mM⁻¹ ATP in C.

**RESULTS**

**Biochemical Characterization of Recombinant scIpK1**—To characterize the biosynthesis of IP₆ by scIpk1, kinetic experiments were performed with bacterially expressed and purified GST-scIpK1. The ability of the enzyme to synthesize IP₆ was assayed by incubation with [^32P]IP₅ and ATP in a buffered system. The reaction products were analyzed by polyethyleneimine cellulose thin-layer chromatography, which readily resolved IP₅ and IP₆ isomers (Fig. 1A). No IP₆ was formed at time 0 incubation, in the absence of ATP, in the absence of GST-scIpK1, or in the presence of the stopping buffer. Reactions were determined to be dose- and time-dependent (Fig. 1A; data not shown). The optimal pH range, temperature, and MgCl₂ concentrations were determined under zero-order conditions to be pH 6.7–7.5, 30 °C (89% maximal activity was observed at 36577
Michaelis-Menten kinetic parameters for GST-scIp1 were determined by monitoring the conversion of IP₅ to IP₆ under a variety of conditions. Data from three experiments are shown as saturation curves with specific activity (micromoles of IP₆ formed per min/mg of protein) plotted versus the IP₅ substrate concentration (Fig. 1B). Apparent kinetic parameters were calculated from the best fit line derived from double-reciprocal plots of 1/v versus 1/S (shown in the inset). The average apparent Kₘ and Vₘₐₓ for IP₅ were found to be 644 nM and 0.020 μmol/min/mg, respectively, using 40 ng of recombinant protein and 5 mM ATP. This corresponds to an apparent catalytic efficiency (Kₘ/Vₘₐₓ) of 31,610 s⁻¹ μM⁻¹. Similar experiments were performed with constant GST-scIp1 and IP₅ levels (80 ng and 20 μM, respectively) and varying concentrations of ATP (0.020 to 5 mM). Data from the experiments are shown in Fig. 1C. The apparent Kₘ for ATP was determined to be 62.8 μM.

Identification of S. pombe and C. albicans Ipk1 Homologues—To determine whether homologues of scIp1 were present in the available sequence data bases, we conducted a series of searches with the gapped BLASTP program (18). Computer analysis identified putative proteins from S. pombe (GenBank™/EBI accession number CAB60684.1) and C. albicans (Stanford DNA Sequencing and Technology Center accession number Contig5-2849) with high probability scores (Fig. 2). The C-terminal region of spIp1 shows high similarity to full-length scIp1 and putative calp1 (dark-gray boxes), including two conserved amino acid spans designated Boxes A and B (hatched boxes). The N-terminal region of spIp1 has a region (residues 114–253; light-gray boxes) with homology to scRvs167 (19% identity; residues 128–272) and scRvs161 (20% identity; residues 116–214). A span within the Rvs homology region of spIp1 is predicted to form a coiled-coil domain (residues 159–188; black box). B, an alignment of the related sequences from scIp1, spIp1-C, and putative calp1 was generated using multiple sequence alignment analysis (22). Black boxes indicate residues that are identical between at least two of the proteins, and gray boxes highlight similar residues. The spans of amino acid sequences for Boxes A and B are shown. The asterisks designate residues that were changed in the sc-ipk1-6 (N10D) and sc-ipk1-2 (C139Y) mutants. The carets note the points of truncation for the sc-ipk1-4 (Trp128 to a stop codon), sc-ipk1-5 (Trp237 to a stop codon), and sc-ipk1-1/3 (Gln268 to a stop codon) mutants.
of splpk1, and residues 250–261 of putative calpk1. The consensus sequence for each motif was deduced as E(i/l/m)KPK-WL(t/y) for Box A and as LXMTLRDLV(t/g/u)le(t/y)f for Box B.

Interestingly, the N-terminal region of splpk1 is not present in sclpk1 or putative calpk1. The open reading frame for splpk1 encodes a predicted 640-amino acid protein with a molecular mass of ~73 kDa. This compares with 281 residues and ~33 kDa for sclpk1 and 361 residues and ~43 kDa for putative calpk1. A search of the *S. cerevisiae* data base by BLASTP Version 2.0 with the splpk1 sequence revealed that a span in the N-terminal region of splpk1 is related to scRvs161 and the N-terminal region of scRvs167 (Fig. 2A). The scRvs161 and scRs167 proteins may play roles in endocytosis, actin function, and cell signaling (23–27). These regions of the yeast Rvs167 and Rvs161 proteins as well as the related region in the mammalian amphiphysins are predicted to form coiled-coil domains that mediate the formation of hetero- and homodimers (28–32). Using the SMART program (33, 34) to identify modular domains in splpk1, residues 102–131 were designated as having that mediate the formation of hetero- and homodimers (28–32).

The C-terminal Region of splpk1 is an *IP$_5$* 2-Kinase That Functionally Complements *S. cerevisiae* ipk1 Mutants in Vivo—To directly test if the C-terminal region of splpk1 (splpk1-C) is an intrinsic *IP$_5$* 2-kinase, recombinant splpk1-C was purified as a GST fusion protein and analyzed for biochemical activity. Incubation of GST-slpk1-C with pure IP$_5$ and [$\gamma$-32P]ATP resulted in the specific conversion of IP$_5$ to IP$_6$ (data not shown). For the kinetic characterization, the experimental conditions were as described for GST-sclpk1, except that purified GST-slpk1-C at 6 ng/reaction was used with substrate concentration ranges appropriate for the given $K_m$. Varying the IP$_5$ concentration by 2-fold dilutions between a range of 0.5 and 32 $\mu$M resulted in the determination of an average apparent $K_m$ for IP$_5$ of 5.89 $\mu$M and $V_{max}$ of 0.240 $\mu$mol/min/mg (n = 3) (Table II). The apparent $K_m$ for ATP was 9.3 $\mu$M (determined with 30 ng of protein/reaction). Under these conditions, the *S. pombe* enzyme had a lower affinity for IP$_5$ substrate, but a higher maximal velocity compared with the *S. cerevisiae* enzyme; however, the apparent catalytic efficiencies of the enzymes are similar (31,610 versus 42,105 s$^{-1}$ $\mu$mol$^{-1}$) (Table II).

To test if the *IP$_5$* 2-kinase region of splpk1 is functional in budding yeast, a *LEU2/CEN* plasmid expressing the carboxy-terminal region of splpk1 under the control of the *S. cerevisiae* GLE1 promoter was constructed (splpk1-C). The splpk1-C plasmid, an *scIPK1* expression plasmid constructed in an identical manner, and the empty control vector were each transformed into the *S. cerevisiae* strain SWY1659, a null strain. A search of the *S. cerevisiae* genome database by BLASTP revealed that the splpk1-C construct also did not restore full IP$_6$ production. This suggests that the plasmid-based expression by the GLE1 promoter was not sufficient. The subtle differences between the splpk1-C and *scIPK1* profiles in terms of the levels of IP$_5$ and PP-IP$_4$ may also reflect differences in the kinetic properties of the proteins or subcellular localization.

To further examine potential functional overlap between *splpk1-C* and *scIPK1*, we tested if expression of *splpk1-C* could complement the gle1-1 ipk1-4 synthetic lethal phenotype. The *LEU2/CEN* expression plasmids harboring splpk1-C, scIPK1, or scGLE1 were transformed into the gle1-1 ipk1-4 tester strain containing plasmid-borne (URA3/CEN) wild-type GLE1. The assay for complementation of synthetic lethality was based on the toxicity of the drug 5-FOA in a URA3 background. Only strains that can lose the URA3 plasmid and survive without the expressed GLE1 gene will be viable on 5-FOA medium. As expected, the tester strain carrying either the GLE1/LEU2 or scIPK1/LEU2 plasmid was viable, and the strain with the empty LEU2 vector was dead (Fig. 4). Strikingly, the strain with the splpk1-C plasmid was viable at 23 °C. Thus, splpk1-C rescued the synthetic lethality and fulfilled the functional role of the *IP$_5$* 2-kinase required for Gle1 and mRNA export. Overall, these results indicate that the

| Protein       | $K_m$(IP$_5$) | $K_m$(ATP) | $V_{max}$ | $V_{max}$/[$K_m$] |
|---------------|---------------|-------------|-----------|------------------|
| scIPK1        | 5.89 $\mu$M   | 9.3 $\mu$M  | 0.240     | 42,105           |

**TABLE II**

Enzymatic properties of the recombinant *IP$_5$* 2-kinases

![Graphs](image309x368to553x729)

**Fig. 3.** Expression of *splpk1-C* rescues IP$_6$ production in an *sc-ipk1* null strain. The *S. cerevisiae* ipk1 null (Δ) strain (SWY1659) was transformed with empty vector (A) (SWY2230), *splpk1-C* under the control of the GLE1 promoter (B) (SWY2231), or *scIPK1* under the control of the GLE1 promoter (C) (SWY2232) and grown in minimal medium lacking leucine and containing 40 $\mu$Ci/ml [3H]inositol. Soluble cell extracts were prepared and separated by Partisephrone strong-anion exchange HPLC. Individual IP isomers were assigned on the basis of coelution with known IP standards.
enzymes for IP₆ biosynthesis are conserved between S. pombe and S. cerevisiae.

Characterization of sc-iplk1 Mutant Strains—Five mutant alleles of sc-iplk1 were identified in the gle1-2 synthetic lethal screen (6) and designated sc-iplk1-1 to sc-iplk1-5. To investigate potential phenotypic differences between the isolated mutant strains, cells were assayed for their ability to generate IP₆ (Fig. 5). Strains were grown in synthetic medium in the presence of [³H]inositol (40 μCi/ml) until late logarithmic phase. Soluble cell lysates were prepared and separated by HPLC to detect specific IP species. Extracts from wild-type scIpk1 cells yielded a strong IP₆ peak (Fig. 5A), but showed no accumulation of the precursor IP₅ or the isomer PP-IP₄. In all the mutant sc-iplk1 strains (Fig. 5), IP₆ production was greatly reduced, and the levels of PP-IP₄ and IP₅ were coincidentally increased. However, there were distinct differences between the mutant strains. There was no detectable IP₆ production in the sc-iplk1-2 and sc-iplk1-4 mutants (Fig. 5, C and E), respectively, and the profiles were similar to the profile of the previously reported sc-iplk1 null strain (Fig. 3A). In contrast, low levels of IP₆ were detected in the sc-iplk1-1 and sc-iplk1-3 mutant strains (Fig. 5, B and D, respectively). The sc-iplk1-5 strain yielded the highest relative IP₆ production level among the mutants (Fig. 5F).

To more accurately assess their activity, large-scale purifications were conducted for the GST-scIpk1-1/3, GST-scIpk1-2, and GST-scIpk1-5 proteins. Roughly equivalent amounts of mutant protein (~20 ng/μl) and various concentrations of wild-type protein were incubated under the above conditions for 1- and 10-hour time periods. The wild-type protein was catalytically active for at least 160 min under these assay conditions. In the 10-hour incubation, IP₆ production was detected for the GST-scIpk1-1/3 and GST-scIpk1-2 proteins; however, the rates of production were less than ~4 and ~1% of the wild-type protein, respectively (Fig. 6B). Surprisingly, despite the apparent function of the mutant protein in vivo (Fig. 5), the GST-scIpk1-5 protein was catalytically inactive in vitro under all assay conditions tried to date. The lack of in vitro activity may reflect an instability or folding defect when GST-scIpk1-5 is expressed and purified from bacteria compared with the endogenous scIpk1-5 protein.

DISCUSSION

Previous molecular and biochemical analysis of inositol kinases and phosphatases has revealed high degrees of functional and structural conservation among respective enzyme family members across species. For example, similar inositol 5-phosphatases have been identified in mammals, S. cerevisiae, plants, Caenorhabditis elegans, and Drosophila (35). We recently reported the first molecular analysis of a gene encoding an IP₅ 2-kinase, that from S. cerevisiae, termed scIpk1 (6). Here we establish that genes encoding putative IP₅ 2-kinases with homology to scIpk1 exist in at least two other organisms, S. pombe and C. albicans. S. pombe protein is a bona fide IP₅ 2-kinase and is designated spIpk1. This conclusion is based on at least three pieces of evidence independent of the sequence homology. Biochemical characterization of purified recombinant GST-spIpk1-C showed that the protein was an in vitro catalyst for converting IP₅ and ATP to IP₆. Expression of spIPK1-C in S. cerevisiae cells lacking scIpk1 restored the production of IP₆ in vivo. Finally, expression of spIPK1-C com-
implemented the synthetic lethality of a glel-2 sc-ipk1-4 double mutant. Thus, spIp1k functioned in vivo and in vitro in a manner analogous to scIp1k. Given the phylogenetic span between S. pombe and S. cerevisiae, this conservation suggests that specific IP₅₂-kinase enzymes for generating the second messenger IP₆ may exist in all eukaryotes.

Despite sharing only 24% sequence identity, the recombinant scIp1k and spIp1k proteins have similar substrate selectivities and catalytic efficiencies (Table II). The apparent kinetic parameters for scIp1k and spIp1k-C are also within the range of the reported parameters for the reported IP₅₂-kinase activity fractionated from immature soy beans: apparent $K_m$ values for IP₅ and ATP of 2.3 $\mu$M and 8.4 $\mu$M, respectively, with an apparent $V_{max}$ of 0.243 $\mu$mol/min/mg (36). Although others had documented that activities for producing IP₆ were present in cell extracts and fractions from S. pombe and C. albicans cells (14, 37), the genes encoding the respective enzymes had not been identified, and the kinetic parameters for purified recombinant proteins had not been established.

The homology between the S. pombe, S. cerevisiae, and C. albicans proteins reveals at least two blocks of highly conserved amino acid sequence. We have designated these Box A (E(i/l/m)KPKWL(t/y)) and Box B (LXMTLRDV(t/g)(l/c)(f/y)I). These two motifs are separated by similar lengths of amino acid spans (93 residues for S. cerevisiae, 78 for S. pombe, and 104 for C. albicans). The Box A and B motifs in Ip1k enzymes may form the catalytic and/or substrate-binding sites.

The location of the sc-ipk1 mutant alleles also highlights amino acid residues that are potentially important for catalysis or structure. In particular, the two point mutants that result in
a complete loss of in vitro catalysis, C139Y and N10D, are conserved in sclp1 and putative calp1k. Three of the mutant sc-ipk1 alleles are C-terminal truncations of various lengths. The longest truncation (sc-ipk1-4) removed Box B and eliminated all detectable IP₅ 2-kinase activity in vivo and in vitro. In contrast, the truncations that retained both Boxes A and B possessed some in vivo activity. This supports the role of the conserved motifs in catalysis. Interestingly, the removal of only the C-terminal 14 residues of sclp1 (allele sc-ipk1-1/3) resulted in a significant decrease in catalytic activity. However, the sc-ipk1-5 mutant, with a further truncation and missing the C-terminal 45 residues, is actually more active in vivo in terms of IP₅ production. This suggests that the region between the sc-ipk1-1/3 and sc-ipk1-5 truncations (residues 237–268) is an inhibitory domain whose removal allows for a higher rate of activity.

The most striking difference between sclp1 and sclp1 is the unique N-terminal region of the S. pombe protein. We reanalyzed any potential open reading frames in the promoter region of scIPK1 and confirmed that there are no potential coding regions that would have homology to the N-terminal region of sclp1 or that would extend the N-terminal portion of sclp1. In addition, a chromosomally expressed, epitope-tagged version of sclp1 was generated by homologous recombination at the C terminus. This results in expression of a fusion protein of the correct predicted molecular mass with the designated initiation methionine for sclp1 (data not shown). Therefore, we believe that this is a true difference between these Ip1 enzymes. However, as the S. pombe genome is not yet completely sequenced, it is possible that there are other IP₅ 2-kinases in S. pombe.

The N-terminal domain of sclp1 contains a region that is predicted to form a coiled-coil structure and that is homologous to the coiled-coil domains of the mammalian amphiphysin and yeast Rvs161/167 proteins. The coiled-coil regions of the Rvs/ amphiphysin proteins have been shown to mediate dimerization (28–32). We predict that the homology to sclp1 reflects a conserved structural fold and that this region of sclp1 is a protein-protein interaction domain for homo- or heterodimerization. It will be interesting to determine if this domain has any effect on the catalytic activity of sclp1 and to identify protein interaction partners.

The amphiphysin and Rvs proteins have been implicated in a number of cellular processes, including endocytosis, actin function, and signaling (reviewed in Refs. 30, 38, and 39). There are also several intriguing possible connections between these proteins and inositol metabolism and IP signaling. Cells lacking scRvs167 or scRvs161 have altered phospholipid compositions (40). The role of amphiphysin-1 in clathrin-mediated endocytosis during synaptic vesicle reformation involves interactions with synaptotagmin-1 (an inositol-polyphosphate 5-phosphatase), clathrin, and AP-2 (28, 41–46). Interestingly, AP-2 has been characterized in vitro as an IP₅-binding protein (47–49), and IP₅ alters the in vitro binding of AP-2 to synaptotagmin (50). Based on these reports, it was possible that scRvs161 and scRvs161 might have some function in the IP pathway. We therefore analyzed IP levels in S. cerevisiae cells lacking Rvs167 or Rvs161; however, no changes were observed (data not shown).

Our previous studies in S. cerevisiae have defined an in vivo role for IP₅ production in mediating mRNA export through the essential nuclear pore complex-associated factor Gle1 (6). In this report, we have conducted a complete in vivo and in vitro analysis of the IP₅ 2-kinase activity for all the sc-ipk1 mutant alleles that were isolated in the original gle1 synthetic lethal screen. All showed marked in vivo reductions in IP₅ levels. However, the low residual production of IP₅ by the sc-ipk1-5 mutant was surprising. This may indicate that the spatial and temporal regulation of IP₅ production is important to the mechanism of regulating mRNA export. A role for IP₅ in mRNA export in other eukaryotes has not yet been established. The existence of a human GLE1 (19) and a probable S. pombe Gle1 homologue (GenBank™/EBI accession number CAB39139.1) supports the hypothesis that the interrelationship between IP₅ production and Gle1 will be conserved.

The mechanism by which scIp1 kinase activity and presumably IP₅ production regulate Gle1-mediated mRNA export is unknown. IP₅ levels have been shown to be relatively stable in cells, although recent work in yeast suggests that levels change in response to stress and other cellular perturbations (14). The turnover of IP₅ has not been carefully analyzed in S. cerevisiae, and it is not known whether or not Ip1 functions as an ATP synthetase in vivo (the reverse reaction). It has also been demonstrated that IP₅ is the precursor for diphosphorylated inositol in both yeast and vertebrate cells (3, 10). Others have suggested that production of PP-IP₅ or bis(diphospho)IP₅(IP₂- IP₅) may be required for mRNA export. However, the gene encoding an IP₅ kinase (KCS1) was not identified in the gle1 synthetic lethal screen (6), and mutations in KCS1 do not have defects in mRNA export (10). Thus, it remains possible that IP₅ functions as a classic signaling molecule that directly binds an effector protein in the mRNA export machinery.

In summary, our characterization of sclp1 and the new spIp1 has defined their in vitro kinetic parameters, pinpointed residues important for catalysis, and documented cross-species functionality. The conservation of Ip1 across species strongly indicates that the production of IP₅ is an important cellular function. Based on these results, we propose that a family of conserved IP₅ 2-kinase enzymes exists in all eukaryotes. Given the low degree of sequence identity between the S. cerevisiae, S. pombe, and C. albicans enzymes, it may be difficult to identify putative mammalian IP₅-2 kinases based on BLAST data base searches. Our results showing heterologous complementation of an sc-ipk1 mutant background by expression of sclp1 argues that genetic strategies to clone by heterologous complementation will be potentially powerful approaches for isolating other cross-species family members.

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