A Novel Inositol Pyrophosphate Phosphatase in *Saccharomyces cerevisiae*

**Siw14 PROTEIN SELECTIVELY CLEAVES THE β-PHOSPHATE FROM 5-DIPHOSPHOINOSITOL PENTAKISPHOSPHATE (5PP-IP₅)**

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Inositol pyrophosphates are high energy signaling molecules involved in cellular processes, such as energetic metabolism, telomere maintenance, stress responses, and vesicle trafficking, and can mediate protein phosphorylation. Although the inositol kinases underlying inositol pyrophosphate biosynthesis are well characterized, the phosphatases that selectively regulate their cellular pools are not fully described. The diphosphoinositol phosphate phosphohydrolase enzymes of the Nudix protein family have been demonstrated to dephosphorylate inositol pyrophosphates; however, the *Saccharomyces cerevisiae* homolog Ddp1 prefers inorganic polyphosphate over inositol pyrophosphates. We identified a novel phosphatase of the recently recovered more than 20 years ago, inositol pyrophosphates have been implicated in an array of processes, including cellular energetic metabolism, telomere maintenance, oxidative stress response, and vesicle trafficking in animals and fungi (1). Recent work in *Saccharomyces cerevisiae* found that production of inositol pyrophosphates is essential for mounting environmental stress responses (3). Inositol pyrophosphates are thought to be metabolic regulators displaying expanded cellular roles beyond classic second messengers (1). The enzymes that regulate the pools of inositol pyrophosphates are not fully described. The inositol hexakisphosphate kinases synthesize IP₇, the most abundant inositol pyrophosphate in the cell; they add the β-phosphate to IP₆ at the 5th position generating 5PP-IP₅ (2). Yeast possess a single inositol hexakisphosphate kinase gene, *KCS1*, whereas mammals possess three homologous genes encoding inositol hexakisphosphate kinase (Fig. 1, adapted from Ref. 4). IP₈ is synthesized by a PP-IP₅ kinase that adds the β-phosphate to IP₇ at the 1-position to make 1,5PP-IP₄; this enzyme can also use IP₆ as a substrate, generating 1PP-IP₅. Mammalian genomes have two

Inositol pyrophosphates are a novel class of signaling molecules that carry energy-rich diphosphate bonds. In wild-type

yeast, the most abundant isoform, diphosphoinositol pentakisphosphate (PP-IP₅ or IP₆), consists of a fully phosphorylated six carbon *nmyo*-inositol ring further pyrophosphorylated at one of the carbons. Two physiologically relevant isomers of IP₇ include 1PP-IP₅ and 5PP-IP₅, in which the pyrophosphate group is found at the 1-position or the 5-position, respectively. Sequential phosphorylation of IP₇ results in bisdiphosphoinositol tetrakisphosphate (1,5PP-IP₄ or IP₈) that is pyrophosphorylated at both the 1st and 5th positions (1, 2). Since their discovery more than 20 years ago, inositol pyrophosphates have been implicated in an array of processes, including cellular energetic metabolism, telomere maintenance, oxidative stress response, and vesicle trafficking in animals and fungi (1). Recent work in *Saccharomyces cerevisiae* found that production of inositol pyrophosphates is essential for mounting environmental stress responses (3). Inositol pyrophosphates are thought to be metabolic regulators displaying expanded cellular roles beyond classic second messengers (1). The enzymes that regulate the pools of inositol pyrophosphates are not fully described. The inositol hexakisphosphate kinases synthesize IP₇, the most abundant inositol pyrophosphate in the cell; they add the β-phosphate to IP₆ at the 5th position generating 5PP-IP₅ (2). Yeast possess a single inositol hexakisphosphate kinase gene, *KCS1*, whereas mammals possess three homologous genes encoding inositol hexakisphosphate kinase (Fig. 1, adapted from Ref. 4). IP₈ is synthesized by a PP-IP₅ kinase that adds the β-phosphate to IP₇ at the 1-position to make 1,5PP-IP₄; this enzyme can also use IP₆ as a substrate, generating 1PP-IP₅. Mammalian genomes have two

The abbreviations used are: 5PP-IP₅ or IP₇, 5-diphosphoinositol pentakisphosphate; IP₆, inositol hexakisphosphate; 1,5PP-IP₅, bis-diphosphoinositol tetrakisphosphate (also known as IP₈); IP₉, inositol tetrakisphosphate; poly-P, polyphosphate; PI(3,5)P₂, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5,6)P₄, phosphatidylinositol 3,4,5,6-tetraphosphate; Siw14, phosphatase from *S. cerevisiae* (also called Oca3); Kcs1, IP₆ kinase (IP₆K); Vip1, PP-IP₅ kinase (PPIP5K); Ddp1, diadenosine and diphosphoinositol polyphosphate phosphohydrolase; DIPP, diphosphoinositol phosphatase phosphorylase; PTEN, phosphatase and tensin homolog; Nudix, nucleoside diphosphate linked to X phosphorylase; PFA-DSPI, plant-fungal atypical dual-specificity phosphatase; hDIPP1, human DIPP1; pNP, p-nitrophenol phosphatase; 1PP-IP₅, 1-diphosphoinositol pentakisphosphate; SPCP-IP₅, 5-methylene-bisphosphonate inositol pentakisphosphate.

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5 The abbreviations used are: 5PP-IP₅ or IP₇, 5-diphosphoinositol pentakisphosphate; IP₆, inositol hexakisphosphate; 1,5PP-IP₅, bis-diphosphoinositol tetrakisphosphate (also known as IP₈); PI(3)P₅, inositol tetrakisphosphate; poly-P, polyphosphate; PI(3,5)P₂, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5,6)P₄, phosphatidylinositol 3,4,5,6-tetraphosphate; Siw14, phosphatase from *S. cerevisiae* (also called Oca3); Kcs1, IP₆ kinase (IP₆K); Vip1, PP-IP₅ kinase (PPIP5K); Ddp1, diadenosine and diphosphoinositol polyphosphate phosphohydrolase; DIPP, diphosphoinositol phosphatase phosphorylase; PTEN, phosphatase and tensin homolog; Nudix, nucleoside diphosphate linked to X phosphorylase; PFA-DSPI, plant-fungal atypical dual-specificity phosphatase; hDIPP1, human DIPP1; pNP, p-nitrophenol phosphatase; 1PP-IP₅, 1-diphosphoinositol pentakisphosphate; SPCP-IP₅, 5-methylene-bisphosphonate inositol pentakisphosphate.
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Adapted from Hatch and York 2010

FIGURE 1. Inositol pyrophosphate synthesis and degradation in yeast. Soluble inositol polyphosphates and inositol pyrophosphates are generated by phosphorylation of myo-inositol-1,4,5-trisphosphate by the inositol phosphate multikinase Arg-82 that produces inositol-1,3,4,5,6-pentakisphosphate after two phosphorylation steps, and inositol phosphate kinase Ipk1 phosphorylates the 2-position to produce IP₅. The inositol pyrophosphates are synthesized by adding a β-phosphate to the 5-position preferentially by the IP₅ kinase Kcs1 (indicated by the heavier arrows; see Ref. 5), and then at the 1-position by the PP-IP₅ kinase Vip1. Our data show that Siw14 opposes the action of Ksc1 by preferentially hydrolyzing the β-phosphate at the 5-position; Ddp1 preferentially hydrolyzes the β-phosphate at the 1-position (6). This schematic was modified from Hatch and York (4).

Experimental Procedures

Yeast Strains and Plasmids

Yeast strains were purchased from Open Biosystems and Invitrogen, including parental strain BY4741 (MATa his3Δ leu2Δ met15Δ ura3Δ) and isogenic mutant strains siw14Δ::KanMX, vip1Δ::KanMX, and ddp1Δ::KanMX. The double mutants vip1Δ::KanMX siw14Δ::URA3 (siw14Δ vip1Δ) and ddp1Δ::KanMX siw14Δ::URA3 (siw14Δ ddp1Δ) were constructed by replacing SIW14 with siw14Δ::URA3 using homologous recombination after amplification of the allele using the primers 5’-AGTTCCGGCTTCTTTATCTACTCTCTTTCTGAACTCAATTTCATCATCATTTT-3’ and 5’-TATCAATAAACATCATTTTCTTCGAAGAGACTAGTTACGTAAGGTAATCAGTCTACATATTACCATTAGTTTGCAGCC-3’. Transformation of Saccharomyces used the protocol of Ito et al. (9).

Plasmid pR492 carries the SIW14 gene with its native promoter in the centromeric pRS316 vector (10) for expression in Saccharomyces. It was cloned after amplification using primers SIW14 forward (5’-ACTCTTCAAGCTTTTATCTACTCTTCAAGCTTGCTATAGATGGA-GCT3’) and SIW14 reverse (5’-AGCTCTCAGGCCTATCGCATTTAGACCTG-3’), BY4741 genomic DNA as template, and PrimeStar HS polymerase (Clontech). PCR fragments were purified (Qiagen clean-up kit), digested with HindIII and XhoI, and ligated into pRS316. The catalytically dead allele, SIW14-C214S (11), was made by fusion PCR (12). Initial synthesis of fragments was completed using PrimeSTAR® HS DNA polymerase (Clontech), following the manufacturer’s instructions, pR492 as template, and primer pairs SIW14 forward with C214S reverse (5’-CAACCGGACTGATACTAGTAAATAGGAGGAAACATAGAACGCGCGGAG-3’) and C214S forward (5’-CTTTCATGTTTGCCTCTATTACTATGATCATCAGTGTTG-3’) with SIW14 reverse. Fragments were purified and combined as templates for the fusion PCR using the SIW14 forward and reverse primers. The fusion product was purified, digested, and ligated as above, and its DNA sequence was determined, generating plasmid pR496.

For expression of proteins in Escherichia coli, plasmid vectors pGEX-4T-1 and pGEX-4T-SIW14 were obtained (7). The mutant allele of SIW14 (SIW14-C214S) was constructed using fusion PCR (12), as described above, using primers pGEX forward (5’-GCAAGGCTGGCAAGGCAC-3’) and reverse (5’-GAAGAGGAGCGAGGAGGCAGATC-3’) in place of the SIW14 primers. Fusion products were purified, digested with BamHI and XhoI (New England Biolabs), ligated into pGEX-4T-1, and confirmed by DNA sequencing.

For expression in mammalian cells, the SIW14 gene was cloned into the donor vector plasmid pDONR221 using BP Clonase II and sequentially transferred to a Gateway compatible N-terminally tagged pCMV-Myc destination vector using LR Clonase II, following the manufacturer’s directions in the Gateway Cloning Technology Instruction Manual (Life Technolo-
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Plasmids pGEX-4T-1, pGEX-4T-SIW14, and pGEX-4T-SIW14ΔC214S were transformed into the E. coli strain Rosetta™(DE3) pLysS (EMD Millipore). Overnight bacterial cultures were inoculated 1:20 into fresh LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with 100 µg/µl ampicillin and 34 µg/µl chloramphenicol. Cells were grown at 37 °C with shaking for 2 h (−0.3 OD₆₀₀), and protein expression was induced at room temperature for 3 h with 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Transformed cells were lysed as described (7), substituting Tris-buffered saline (19.98 mM Tris-HCl, pH 8.0, 135 mM NaCl) containing 0.1% Triton X-100, 5 mM dithiothreitol (DTT), and protease inhibitors (Halt®, ThermoScientific) as the lysis buffer. Proteins were purified using glutathione-agarose (Pierce) resin following the manufacturer’s instructions with the following change: resin with bound protein was incubated in wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 28 mM reduced glutathione (Sigma) for 5 min at room temperature to elute. Three elutions were combined, and proteins were concentrated using Amicon ultracentrifugal 30-kDa filters for 15 min at 4000 g. Proteins were examined using SDS-PAGE (Any kD™ Mini-PROTEAN® TGX™ Gel, Bio-Rad) followed by Coomassie Blue staining or immunoblot analysis with rabbit α-GST-HRP antibody (Abcam) at a 1:5000 dilution in phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 137 mM NaCl, 2.7 mM KCl), 2% milk, and 0.01% Tween 20 and were compared with Kaleidoscope Prestained Standards (Bio-Rad).

**Phosphatase Activity Assays**

**Substrates**—p-Nitrophenol phosphate (pNPP) was purchased from Sigma. The phosphatidylinositol phosphatases phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 5-phosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃) each with caprylic acid (8:0) as the two acyl chains, were purchased from Echelon Biosciences. IP₆ was purchased from Sigma. Four isoforms of IP₅ (5-diphosphoinositol pentakisphosphate (5PPIP₃), non-hydrolyzable 5-methylene-bisphosphonate inositol pentakisphosphate (5PCP-IP₃), 1PP-IP₃, and non-hydrolyzable 1-methylene-bisphosphonate inositol pentakisphosphate (1PCP-IP₃)) and IP₆ (3,5-bisphosphoinositol tetrakisphosphate (3,5PPIP₃)) were synthesized following the methods described previously (13–15).

[³²P]Polyphosphate (poly-³²P) was prepared as described previously (16) using purified recombinant *E. coli* polyphosphate kinase (gift of E. Crooke) and 5 µCi of [δ-³²P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences). Substrate preparations were analyzed by scintillation counting to calculate the concentration of the poly-P synthesized.

**Detection of Orthophosphate**—Orthophosphates cleaved from the phosphatidylinositol phosphatases were detected with the EnzChek phosphate assay kit (Life Technologies, Inc.) following the manufacturer’s instructions, except reaction volumes were scaled down to 100 µl and the absorbance at 360 nm was measured using a NanoDrop ND-1000. The orthophosphates cleaved from the inositol pyrophosphates were measured using the Malachite Green Phosphate Assay kit (Cayman Chemical Co.) following the manufacturer’s guidelines. Assay Conditions with pNPP and Phosphatidylinositol Phosphates (PIPₙ)—Purified recombinant Siw14 (10 µg) was incubated with 10 mM of pNPP in 25 mM HEPES, pH 6.8, 50 mM NaCl, 10 mM MgSO₄, and 1 mM DTT for 90 min at 37 °C. pNPP assays were analyzed by the colorimetric change at 420 nm using the GloMax Multi Detection System (Promega). Purified recombinant Siw14 (50 µg) was incubated with 100 µM of the mono-, di-, and triphosphorylated phosphatidylinositol phosphates PIPₙ in reaction buffer (0.1 mM Tris-HCl, pH 6.8, 10 mM DTT, 40 mM NaCl) for 90 min at 37 °C (7). PTEN (0.08 µg; Echelon Biosciences) was used with PI(3,4,5)P₃. The specific activity of both Siw14 and PTEN with PIPₙ was normalized to the activities with pNPP.

**Assay Conditions with Soluble Inositol Pyrophosphates (IPₙ)—**Purified recombinant Siw14 (10 µg) was incubated with 600 µM ³²P in the form of poly-³²P for 90 min at 37 °C in 50 mM HEPES-KOH, pH 7.0, 5 mM MgCl₂, 0.4% β-mercaptoethanol, and 2% glycerol. As a positive control, 2.5 µg of *E. coli* polyphosphate phosphatase (a gift from E. Crooke) was incubated for 15 min under the same conditions. Reaction products were separated by electrophoresis in 35% acrylamide gels in TBE buffer (89 mM Tris, pH 8.0, 89 mM boric acid, 2 mM EDTA) and stained with toluidine blue (20% methanol, 0.1% toluidine blue, and 3% glycerol) as described (6, 17).

**Assay Conditions with Polyphosphate**—Recombinant Siw14 (20 µg) was incubated with 600 µM ³²P in the form of poly-³²P for 90 min at 37 °C in 50 mM HEPES-KOH, pH 7.0, 5 mM MgCl₂, 0.4% β-mercaptoethanol, and 2% glycerol. As a positive control, 2.5 µg of *E. coli* polyphosphate phosphatase (a gift from E. Crooke) was incubated for 15 min under the same conditions. Reaction products were spotted onto thin layer chromatography plates and assayed as described (16). Plates were exposed to phosphorimaging screens, and the density of each spot on the TLC plate was measured using ImageQuant software on a STORM 840 instrument (Molecular Dynamics).

**Extraction of ³²H]Inositol Phosphates (³²H-IPₙ) and HPLC Analysis**—myo-Inositol pools in yeast cells were uniformly radiolabeled by culturing strains overnight in YPD (1% yeast extract, 2% peptone, and 2% dextrose), and 0.005 OD₆₀₀ cells were inoculated into 10 ml of synthetic complete (SC) medium lacking inositol (0.17% yeast nitrogen base-inositol (MP Biologicals), 0.5% ammonium sulfate, 0.069% complete supplement mixture (CSM) – Leu amino acid mix (MP Biomedical), 0.76 mM leucine, and 2% dextrose) and containing 75 µCi of myo-[³²H]inositol (>60 Ci/mmol, PerkinElmer Life Sciences). Cultures were grown for 20–24 h until cells were in mid-log phase (~0.6 OD₆₀₀). Tritiated soluble inositol phosphates ([³²H]-IPₙ) were extracted as described (18), except that cells
were broken via a vortex in the presence of glass beads for four cycles of 2 min with cooling in-between for 2 min on ice. The inositol phosphates in extracts were separated using HPLC using a Hitachi 110 × 4.6-mm Partisisphere strong anion exchange column and collecting 1-ml fractions per min for 90 min. The counts/min of each fraction was measured by scintillation counting (Beckman Coulter) in Ultima-Flo AP (PerkinElmer Life Sciences) scintillation fluid. All inositol phosphate profiles were corrected for baseline counts/min before analysis.

**Extraction of [3H]inositol Phosphates ([3H-IP]n) from Mammalian Cells and HPLC Analysis**

One ml of HEK-293T cells was seeded in 6-cm well plates at 0.2 × 10⁶ cells/ml confluency in medium (1× DMEM (Corning CellGro), 10% FBS (HyClone), 1% l-alanyl-l-glutamine (Life Technologies, Inc.)) containing 1% penicillin/streptomycin (Lonza) and 0.1 mg/ml Normocin (InvivoGen). The following day, the medium was exchanged with 1 ml of inositol-free medium supplemented with myo-[3H]inositol (1× DMEM without l-glutamine and without inositol (MP Biomedicals), 10% FBS (HyClone), 1% l-alanyl-l-glutamine (Life Technologies, Inc.), 1% penicillin/streptomycin (Lonza), 0.1 mg/ml Normocin (InvivoGen), and 20 μCi/ml myo-[3H]inositol (PerkinElmer Life Sciences)) and labeled for 72 h. Cells were transfected in duplicate with mammalian expression plasmids that express Myc alone (pCMV-Myc), Myc-tagged Siw14 (Myc-Siw14), or Myc-tagged Siw14-C214S; transfection occurred for 48 h in 2 ml of the myo-[3H]inositol labeling medium with Lipofectamine 2000 (Invitrogen) per the manufacturer’s instructions. Soluble [3H]inositol phosphates were extracted as described (18) with the modifications that 250 μl of extraction buffer was added to each well, and duplicate transfected wells were combined and neutralization occurred for 2 h on ice.

**Extraction of Poly-P and Quantification**

Poly-P was extracted as described (6) and ~150,000 cpm of poly-[32P] was added to the lysis buffer per sample to measure extraction efficiency; recovery was measured by scintillation counting. Cells were lysed for 8 min in 2-min cycles. To measure the total poly-P pools, poly-P was converted to ATP using 3.4 μg of polyphosphate kinase, 10 mM ADP in 50 mM Tris-HCl, pH 7.0, 4 mM MgCl₂, 40 mM (NH₄)₂SO₄ for 2 h. Reactions were stopped with 4 mM EDTA and 80 mM Tris-HCl, pH 8.0. ATP was measured using an ATP luciferase assay kit (Invitrogen) on a Victor³ 1420 MultiLabel counter (PerkinElmer Life Sciences) plate reader. Twelve biological replicates were analyzed over four experiments; averages were calculated after samples had been normalized to extraction efficiency.

**Extraction of Phosphatidyl[3H]inositol Phosphates (P[3H]IP₅) and HPLC Analysis**

Cells were grown overnight in YPD, and 0.005 OD₆₀₀ cells were inoculated into SC-inositol medium containing 75 μCi of [3H]inositol. Cultures were grown for 20–24 h until cells were in log phase (~0.6 OD₆₀₀). Tritiated phosphatidylinositides (P[3H]IP₅) were extracted and deacylated using (19, 20) as guidelines. Briefly, cells were killed by mixing them with 2 volumes of methanol/HCl (100:1) for 5 min on ice and harvested by centrifugation at 6000 × g for 10 min at 4 °C. Lysis was accomplished by 16 rounds of vortexing in 300 μl of H₂O/methanol/HCl (66:100:1) with glass beads for 30 s and resting on ice for 1 min between rounds. Phosphatidylinositol phosphates were isolated by extracting the samples with 1.33 ml of ice-cold chloroform and 470 μl of methanol/HCl (100:1) on ice for 10 min. Samples were mixed with 20 μg of nonradioactive bovine brain phosphatidylinositol phosphate as a carrier (Sigma) and extracted with 0.8 ml HCl, 5 ml tetrabutylammonium hydrogen sulfate, and 1 ml EDTA. The lower layer was dried in a vacuum dryer and resuspended in 750 μl of methanol. To deacylate, the PIP₅ samples were incubated with methylamine reagent (Sigma) at 53 °C for 50 min, cooled to room temperature, and dried under vacuum. Samples were washed with water, and then the lower aqueous phase was removed using n-butanol/petroleum ether/ethyl formate (20:4:1) two times and dried again. Once deacylated, the IP₅ were separated using a Waters 1525 Binary HPLC pump with a Hitachi 125 × 4.6-mm Partisisphere strong anion exchange column as described above except that 120 fractions of 1 ml each were collected. The [3H]inositol was quantified for each elution as described above.

**Statistical Analysis of Data**

Enzyme assays were performed in triplicate except for the PIP₅, which were performed in duplicate; this is noted in the figure legends. Experiments with radiolabeled cells and extraction of soluble or lipid inositol phosphates were performed between four and nine times, as indicated in the figure legends, using an overnight culture that had been inoculated with a freshly grown colony of *S. cerevisiae* with the indicated genotype. For the mammalian transfection experiments, three to five samples containing HEK-293T cells were transfected with the indicated constructs and were analyzed for inositol polyphosphates. The mean and standard error of the mean are reported graphically. The paired Student’s t test was used to determine significance, and p values are reported using the following scheme: *, p value ≤0.05; **, p value ≤0.01; ***, p value ≤0.001; and ****, p value ≤0.0001.

**Results**

**Biochemical Characterization of Siw14** —The *SIW14* gene of *S. cerevisiae* encodes a phosphatase with a basic and shallow active site (7, 21). To characterize substrates of the yeast *SIW14*-encoded phosphatase, GST-tagged Siw14 was expressed in *E. coli* and purified by affinity chromatography. The predicted mass of the recombinant fusion protein was 52 kDa, and it migrated at ~50 kDa by SDS-PAGE (Fig. 2A). The purified recombinant protein displayed activity using the generic phosphatase substrate *p*-nitrophenyl phosphate (*pNPP*, Fig. 2B). We performed site-directed mutagenesis to generate a phosphatase-inactive mutant of Siw14 by mutating the conserved cysteine within the putative active site (position 214) to serine (11). The activity of the purified GST-Siw14-C214S mutant protein was undetectable (Fig. 2B), confirming that the phosphatase activity detected in the purification of GST-Siw14 was due to Siw14 itself. We determined that the optimal conditions for the Siw14 phosphatase reaction are 37 °C in a buffer of pH 6.0 (Fig. 2C, and data not shown). Treatment with thrombin
to remove the GST moiety had no effect on activity toward pNPP (data not shown). The activity of Siw14 for pNPP was low, with a $K_m$ of $3 \times 10^{-7}$ M, a $k_{cat}$ of $4.4 \times 10^{10}$ M$^{-1}$ s$^{-1}$, and a specificity constant of $1.5 \times 10^{10}$ M$^{-1}$ s$^{-1}$ (Fig. 3, A and B; Table 1).

Siw14 Has a Low Activity toward Poly-P and Phosphatidylinositol Phosphates (PIP$_n$)—To identify the substrate(s) of Siw14, we tested polyphosphate and lipid inositol phosphates (7, 21). Siw14 is homologous with the Arabidopsis thaliana protein AtPFA-DSP1 that is able to cleave short chain poly-P molecules (21). Siw14 cleaved orthophosphate from radiolabeled poly-P but only with the use of more protein and longer reaction times as compared with pNPP and with a control phosphatase, E. coli polyphosphate phosphatase (data not shown). Thus, we concluded that poly-P is a poor substrate for Siw14 and is not its specific substrate.

We tested the ability of Siw14 to dephosphorylate phosphatidylinositol phosphates (PIP$_n$) because the purified enzyme was previously described to act upon PI(3,5)P$_2$ (7). We used each of the mono-, di-, and triphosphorylated forms of phosphatidylinositol phosphates with two caprylic acid (8:0) acyl chains as potential substrates and measured the release of orthophosphate. As shown in Fig. 4A, we found that the recombinant Siw14 protein had only minimal activity with each of the PIP$_n$, showing no clear preference for any of these substrates; the greatest activity occurred with PI(3,5)P$_2$ as substrate. We compared Siw14 with PTEN, a well characterized PI(3,4,5)P$_3$ phosphatase, after normalizing their activities to pNPP as a common substrate. The specific activity of Siw14 for PI(3,5)P$_2$ was nearly 20-fold lower than that of PTEN for PI(3,4,5)P$_3$ (Fig. 4A). Although we could replicate the results of Romá-Mateo et al. (7), these data suggested to us that none of the PIP$_n$ are the authentic physiological substrates for Siw14.

To investigate this further, we radiolabeled cells with myo-[3H]inositol, extracted phosphatidylinositol phosphates from wild-type and siw14Δ mutant cells, and examined the deacylated products by HPLC; using this method, we can detect the
inositol headgroups from PI(3,5)P$_2$ and PI(4,5)P$_2$. We found no difference in the levels of tritiated PI(3,5)P$_2$ or PI(4,5)P$_2$ in the siw14/H9004 mutant as compared with the isogenic wild-type strain, consistent with the in vitro experiments (Fig. 4, B and C).

**Soluble Inositol Pyrophosphates Are the Physiological Substrates for Siw14**—We considered whether soluble inositol polyphosphates might be the natural substrates for Siw14 because of their structural similarity to phosphatidylinositol phosphates for which Siw14 had low preference and the shallow and basic active site of Siw14 that might accommodate them (7). Purified recombinant GST-Siw14 was tested with commercially prepared IP$_6$, two different isoforms of chemically synthesized IP$_7$ (5-diphosphoinositol pentakisphosphate (5PP-IP$_5$) and 1-diphosphoinositol pentakisphosphate (1PP-IP$_5$)). The human DIPP1 (hDIPP1) enzyme was used as a positive control during quantitation because it is a known inositol pyrophosphate phosphatase (5, 6).

As shown in Fig. 5, A and B, Siw14 exhibited no detectable activity with IP$_6$ as a substrate and very low activity with 1PP-IP$_5$ as a substrate. However, Siw14 dephosphorylates 5PP-IP$_5$ to IP$_6$ as shown by the change in mobility of bands on a 35% acrylamide gel (Fig. 5, A and B). We quantified these reactions using a malachite green assay (Fig. 5C), and found that Siw14 exhibited greater activity with 5PP-IP$_5$ than with 1PP-IP$_5$ or with IP$_6$. We also assessed activity from purified recombinant GST-Siw14 and GST-Siw14-C214S proteins with 5PP-IP$_5$ as substrate; we detected no orthophosphate product formed from the C214S mutant protein (Fig. 5E). These data showed that 5PP-IP$_5$ is a preferred substrate for Siw14.

To confirm that we were detecting cleavage of the pyrophosphate of 5PP-IP$_5$, we tested the ability of Siw14 to utilize analogs of IP$_7$ that contain non-hydrolyzable methylene-bisphosphonate moieties (13, 14). In these analogs, carbon replaces the oxygen in the anhydride linkage to the β-phosphate. In semi-
quantitative gel assays, GST-Siw14 hydrolyzed 5PP-IP₅ to IP₆, but was unable to use 5PCP-IP₅ as a substrate (Fig. 5B). This finding is consistent with Siw14 hydrolyzing the /H₉₂₅²-phosphate at the 5-position. To quantify this, malachite green assays were performed with GST-Siw14 using 5PP- and 1PP-isomers of IP₇ as well as the bisphosphonate analog; hDIPP₁ was used as a positive control. As can be seen in Fig. 5C, we detected no product formed when 5PCP-IP₅ and 1PCP-IP₅ were used as substrates for GST-Siw14, indicating that Siw14 is unable to cleave any of the phosphates from these molecules. Together with the above data presented in Fig. 4, our results provide support that Siw14 is capable of dephosphorylating the /H₉₂₅²-phosphate of inositol pyrophosphates as the physiological substrate, preferring 5PP-IP₅ over 1PP-IP₅, to produce IP₆.

We characterized the biochemical properties of GST-Siw14 using 5PP-IP₅. We examined the activity of Siw14 under different pH conditions and found that it has highest activity at a pH of 6.0 (Fig. 5D), similar to pNPP. We determined the affinity Siw14 for 5PP-IP₅ and assayed its kinetic parameters under these conditions (Fig. 5, E and F). We found a $K_m$ of 34 $\mu$M, a $k_{cat}$ of $2.5 \times 10^{-1}$ s⁻¹, and a specificity constant of 74 M⁻¹ s⁻¹ using Hanes-Woolf plot analysis. We note that the $K_m$ for 5PP-IP₅ is ~100 times lower than for pNPP, and the $k_{cat}$ for 5PP-IP₅ is ~6000 times faster, supporting an enhanced kinetic efficiency of 5 orders of magnitude with 5PP-IP₅ (compare the specificity constants of 74 M⁻¹ s⁻¹ for 5PP-IP₅ versus $1.5 \times 10^{-4}$ M⁻¹ s⁻¹ for pNPP) (Table 1).

**Siw14 Modulates IP₇ Levels in Vivo—**Our in vitro studies demonstrated Siw14 to be a 5PP-IP₅ phosphatase. To evaluate the in vivo relevance of these results, we examined the intracellular inositol polyphosphate levels in *siw14Δ* mutant strains. The relative inositol phosphate levels were quantified by radio-

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**FIGURE 5. Siw14 dephosphorylates IP₇.** A and B, reactions containing 10 $\mu$g of GST-Siw14 (+) or water (−) were incubated with 400 $\mu$M IP₆, 1PP-IP₅, 5PP-IP₅, or the non-hydrolyzable analog 5PCP-IP₅ (as indicated) and then separated on a 35% acrylamide gel. The mobility of IP₆, IP₇, and IP₈ is indicated. Representative gels are shown; experiments with 1PP-IP₅ and 5PP-IP₅ were repeated three times; experiments with 5PCP-IP₅ were repeated twice. C, reactions containing 10 $\mu$g of GST-Siw14 or 1 $\mu$g of hDIPP₁ were incubated with 50 $\mu$M IP₆, 5PP-IP₅, 1PP-IP₅, or the non-hydrolyzable analogs 5PCP-IP₅ or 1PCP-IP₅. The cleaved orthophosphate was quantified using a malachite green assay. Reactions were performed in triplicate and were performed with three separate enzyme preparations (data shown are from one of the preparations). D, GST-Siw14 activity with 5PP-IP₅ in buffers with the pH varying from pH 4 to pH 10. The liberated orthophosphate was quantified using malachite green. E, activity of purified recombinant GST-Siw14 or GST-Siw14-C214S with 5PP-IP₅; F, Michaelis-Menten; G, Hanes-Woolf plots assessing the affinity and kinetics of Siw14 with 5PP-IP₅. Bars or points represent the average of triplicate reactions normalized to the no enzyme control for each substrate. Error bars for graphs in C–E represent the standard error of the mean.
Siw14 Cleaves the Pyrophosphate Moiety of SPP-IP$_5$

![FIGURE 6. In vivo IP$_7$ and IP$_8$ levels increase in the Siw14 yeast deletion mutant. A, representative inositol polyphosphate profiles of WT and siw14Δ. B, ratios of IP$_7$ to IP$_8$ in yeast siw14Δ, normalized to WT. Average values are from nine replicates; error bars represent the standard error of the mean. Significance was determined by Student’s t test; **, p ≤ 0.01; ****, p ≤ 0.0001.](image)

levels of inositol pyrophosphates do not result in higher levels of poly-P.

**Siw14 and Other Enzymes That Metabolize IP$_7$ Act Independently**—A number of mutant yeast strains have been shown to accumulate IP$_7$ (Fig. 1) (6, 23, 24). Vip1 is the kinase that adds the β-phosphate at the 1-position to convert IP$_7$ (SPP-IP$_5$) to IP$_8$ (1,5PP-IP$_4$) (23). DDP1 encodes the Saccharomyces homolog of hDIPP1. In vitro, the Ddp1 enzyme is able to dephosphorylate the β-phosphate from IP$_7$ only at prolonged time points or at non-physiological concentrations; however, in vivo IP$_7$ levels increase in the ddp1 mutant (6). We measured the levels of IP$_7$ in the ddp1Δ and the vip1Δ mutants and compared them with the siw14Δ mutant. We found that the increase in IP$_7$ levels for the siw14Δ mutant (6.5 ± 1.0-fold) was of the same magnitude as the accumulation in the ddp1Δ (7.0 ± 1.1-fold) and vip1Δ (4.0 ± 1.7-fold) mutants (Fig. 8, A–C), as had been seen previously (6).

We wondered whether these enzymes acted independently. We assessed accumulation of IP$_7$ in the double siw14Δ ddp1Δ and siw14Δ vip1Δ mutants. Both double mutants accumulate higher levels of IP$_7$ than either single mutant (Fig. 8, A–C); we found a 21.3 ± 3.2-fold increase for the siw14Δ ddp1Δ mutant that was slightly more than additive, and an 11.8 ± 2.6-fold increase for the siw14Δ vip1Δ mutant that was additive.

**Discussion**

In this study we have discovered Siw14 to be a physiological regulator of the soluble inositol pyrophosphate 5PP-IP$_5$ (see Fig. 1). Siw14 displays pyrophosphate-specific phosphatase activity both in vitro and in vivo. The Siw14 enzyme has a strong preference for inositol pyrophosphates over other previously described putative substrates (such as poly-P and PI(3,5)P$_2$) (7, 21). Siw14 is a member of the plant and fungi atypical-dual specificity phosphatase (PFA-DSP) subfamily (7). This subfamily has members in plants, fungi, kinetoplastids, and slime molds (7), and there is only limited homology with the broader dual specificity phosphatase family at the signature motif C(X)$_n$R in the catalytic center (21). The proteins within the PFA-DSP subfamily have not been well studied, and the evolutionary conservation of their inositol pyrophosphate phosphatase activity remains unknown. However, DSP
enzymes are distinct from other characterized inositol pyrophosphate phosphatases that are of the Nudix hydrolase family. The *A. thaliana* genome encodes five isoforms of this protein, and *S. cerevisiae* has three isoforms (7, 8). The structure of the *A. thaliana* AtPFA-DSP1 was determined by x-ray crystallography (21), and the yeast Siw14 ortholog was modeled based on this structure (7). The active site is predicted to be basic and shallow, consistent with our finding that the substrate is large and negatively charged (7, 21). Siw14 and AtPFA-DSP1 are highly conserved throughout the catalytic core and share 61% identity and 76% similarity (7). Although the AtPFA-DSP1 protein was shown to dephosphorylate phosphotyrosine, phosphatidylinositol mono-, di-, and triphosphates, and short chain inorganic polyphosphate (poly-P) (7, 21), it was not tested with soluble inositol pyrophosphates, and thus it is unclear what substrates it would prefer. A potentially important role in plants for these PFA-DSP enzymes is implied given the genomic expansion of this phosphatase subfamily.

Despite apparently similar roles *in vivo*, we found that recombinant Siw14 had a lower specific activity toward 5PP-IP$_5$ when compared with hDIPP1, the previously characterized inositol pyrophosphate phosphatase from humans. The specificity constant for 5PP-IP$_5$ from our Siw14 data (74 M$^{-1}$ s$^{-1}$) is significantly lower than the previously reported data for hDIPP1, which ranges from 2.5 $\times$ 10$^8$ to 4.76 $\times$ 10$^7$ M$^{-1}$ s$^{-1}$ (5, 25).

There are several possible reasons to explain this difference. The first is that the enzymes belong to different classes as follows: Siw14 is a member of the atypical dual specificity phosphatase family (EC 3.1.3, phosphoric monoester hydrolase), and hDIPP1 is a member of the Nudix family (EC 3.6.1, acting on phosphorus-containing anhydrides). Second, this difference may be specific to the recombinant form of Siw14 enzyme. It is possible that the activity of Siw14 is regulated by a post-translational modification; it has two putative phosphorylation sites (26) and *E. coli* is unable to make these post-translational modifications. Alternatively, the purification process may have yielded substantial inactive protein or that Siw14 may require additional partner proteins for full activity. Using two-hybrid and affinity purification approaches, several high throughput studies found that Siw14 is in a complex with Oca1 and Oca2 (27–30). It is possible that full activity from Siw14 may require these (or other) partner proteins. Ongoing experiments are being performed to address the role(s) for these proteins in their interactions with Siw14.

Consistent with the *in vitro* biochemical assays, IP$_7$ levels rise in the *siw14* mutant. The increase in IP$_7$ in the *siw14* mutant (Fig. 6) was similar to that observed in the *vip1* and *ddp1* deletion mutants (6, 23, 24). The IP$_7$ that we detected is likely the 5PP-IP$_5$ isoform given the presence of Kcs1 (the kinase specific for the 5-position). We see a small but significant increase in the accumulation of IP$_8$ in the *siw14* mutant, likely due to the increased amount of substrate for the Vip1 kinase.
we can also detect a 2.8-fold increase in IP₈ in the siw14/H9004/ddp1/H9004 double mutant (data not shown). Our data are consistent with a model that indicates that the primary pathway from IP₆ to IP₈ is through the addition of a β-phosphate to the 5-position of IP₆ by Kcs1 prior to the addition at the 1-position by Vip1 (Fig. 1). Because the siw14/H9004/vip1/H9004 deletion mutant has twice the IP₇ levels as either of the single mutants, this finding suggests an additive effect on the IP₇ pools due to independent activity by these enzymes. We cannot rule out additional pathway regulation in the siw14/H9004/ddp1/H9004 mutant because the IP₇ increase was slightly more than additive in that strain.

Inositol pyrophosphates have been linked to cellular stress response, vesicle trafficking, ribosome biogenesis, telomere length, energy charge, and starvation (3, 31–34). The Sudbery laboratory showed that the siw14Δ mutant does not respond appropriately to nutrient deprivation in stationary phase and is defective in fluid phase endocytosis (11). Our laboratory noted an up-regulation of the environmental stress response in unstressed siw14Δ mutants compared with the wild-type, consistent with the results of Worley et al. (3). The siw14Δ mutant of Saccharomyces also has actin clumps and cytoskeletal defects during stationary phase (11); Schizosaccharomyces pombe strains with a mutation in the asp1/H11001 gene, a member of the VIP1 family, also exhibit cytoskeleton structure defects (35). We note that yeast strains with mutations in SIW14, VIP1, and KCS1 genes share similar phenotypes such as resistance to H₂O₂ and other stresses (3, 24). The intrinsic levels of or the cycling through of inositol pyrophosphates might be important for the regulation of the stress response in yeast, although it has yet to be determined whether the accumulation of the

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**FIGURE 8. Levels of IP₇ increase in single and double kinase and phosphatase mutants.**

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|---|---|---|
| A | representative inositol polyphosphate profiles of WT, and single and double mutants of siw14Δ and ddp1Δ. | B | representative inositol polyphosphate profiles of WT, and single and double mutants of siw14Δ and vip1Δ. | C | levels of IP₇ in each mutant, normalized to WT and IP₆, over four separate experiments. Error bars represent standard error of the mean, and significance was determined by Student’s t test. Each mutant was significantly different than the WT, p ≤ 0.0001; *, p = 0.05; **, p = 0.01. |
different isoforms of IP$_7$ have the same or overlapping phenotypes. IP$_7$ levels are highly dynamic in mammalian cells (36); whether and how Siw14-like functionality is conserved in higher organisms remain to be defined. Our identification of a novel inositol pyrophosphate phosphatase provides additional support for what is likely to emerge as a highly regulated signaling network characterized by this novel high energy molecule class whose cellular pools are combinatorially altered by phosphotransfer to proteins, kinase-mediated synthesis, and dephosphorylation.

Author Contributions—E. A. S. purified proteins, performed site-directed mutagenesis, designed, performed, and analyzed enzyme assays, performed poly-P experiments, including synthesizing poly-$^{32}$P, extracted soluble inositol polyphosphates and poly-P, performed and analyzed the mammalian expression studies, analyzed inositol polyphosphate, PIP, HPLC data, and poly-P data, and wrote the paper. L. S. C. designed, performed, and analyzed the mammalian studies, performed the yeast lipid extractions, performed HPLC analyses and scintillation counting, and analyzed the data. M. W. synthesized all isoforms of IP$_7$. E. C. designed and assessed the poly-P analyses and scintillation counting, and analyzed the data. M. W. designed, performed, and analyzed the mammalian expression studies, analyzed inositol polyphosphates, performed and analyzed enzyme synthesis, and dephosphorylation.

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