Plc1p, Arg82p, and Kcs1p, Enzymes Involved in Inositol Pyrophosphate Synthesis, Are Essential for Phosphate Regulation and Polyphosphate Accumulation in Saccharomyces cerevisiae*

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In Saccharomyces cerevisiae, the phosphate signal transduction PHO pathway is involved in regulating several phosphate-responsive genes such as PHO5, which encodes repressible acid phosphatase. In this pathway, a cyclin-dependent kinase inhibitor (Pho81p) regulates the kinase activity of the cyclin-cyclin-dependent kinase complex Pho80p-Pho85p, which phosphorylates the transcription factor Pho4p in response to intracellular phosphate levels. However, how cells sense phosphate availability and transduce the phosphate signal to Pho81p remains unknown. To identify additional components of the PHO pathway, we have screened a collection of yeast deletion strains. We found that disruptants of PLC1, ARG82, and KCS1, which are involved in the synthesis of inositol phosphates, and ADK1, which encodes adenylate kinase, constitutively express PHO5. Each of these factors functions upstream of Pho81p and negatively regulates the PHO pathway independently of intracellular orthophosphate levels. Overexpression of KCS1, but not of the other genes, suppressed PHO5 expression in the wild-type strain under low phosphate conditions. These results raise the possibility that diphosphoinositol tetrakisphosphate and/or bisdiphosphoinositol triphosphate may be essential for regulation of the PHO pathway. Furthermore, the Δplc1, Δarg82, and Δkcs1 deletion strains, but not the Δphk1 deletion strain, had significantly reduced intracellular polyphosphate levels, suggesting that enzymes involved in inositol pyrophosphate synthesis are also required for polyphosphate accumulation.

Inorganic phosphate is an essential nutrient for all organisms, being required for many metabolic processes such as the biosynthesis of nucleic acids and phospholipids, energy metabolism, and signal transduction. Organisms therefore need efficient regulatory mechanisms for the acquisition, storage, and utilization of phosphate (1). In Saccharomyces cerevisiae, the phosphate signal transduction PHO pathway regulates the expression of a set of phosphate-responsive genes, including PHO5, which encodes repressible acid phosphatase (rAPase),1 in response to changes in intracellular phosphate levels (2–5). The PHO pathway mediates this response by controlling the activity and localization of the transcription factor Pho4p through phosphorylation by the cyclin-cyclin-dependent kinase complex Pho80p-Pho85p. Under conditions of high phosphate, the Pho80p-Pho85p complex phosphorylates and inactivates the transcription factor Pho4p by triggering the association of phosphorylated Pho4p with the nuclear export receptor Mem5p (6, 7). This association leads to the rapid export of Pho4p from the nucleus to the cytoplasm and thus to repression of PHO5 expression. By contrast, when yeast cells are starved of phosphate, the cyclin-dependent kinase inhibitor Pho81p inactivates Pho80p-Pho85p (8, 9), thereby allowing unphosphorylated Pho4p to associate with the nuclear import receptor Pse1p and to re-enter the nucleus to induce expression of PHO5 (10). Pho81p forms a stable complex with Pho80p-Pho85p under both high and low phosphate conditions, but inhibits the kinase activity of Pho85p only under low phosphate conditions, suggesting that the inhibitory activity of Pho81p is regulated post-translationally (8). In addition, Pho81p has been demonstrated to be phosphorylated by Pho80p-Pho85p, and phosphorylation of Pho81p itself seems to be required for its stable interaction with the Pho80p-Pho85p complex (11, 12).

A recent study has shown that PHO5 expression is regulated by intracellular phosphate levels, especially intracellular orthophosphate levels, but not by extracellular phosphate levels (5). Moreover, both inositol polyphosphates and inositol pyrophosphates have been reported to play a role in controlling PHO5 expression, but in different ways. Inositol tetrakisphosphate (IP4) and inositol pentakisphosphate (IP5) are required for modulating the ability of the SWI/SNF and INO80 chromatin-remodeling complexes to induce PHO5 expression under low phosphate conditions (13), whereas inositol pyrophosphates are necessary to maintain the repression of PHO5 expression under high phosphate conditions (14).

In an effort to understand the mechanism underlying phosphate sensing and signal transduction upstream of the Pho81p-Pho80p-Pho85p complex, we analyzed the Research Genetics collection of yeast deletion mutants. Here, we report that the additional factors Plc1p, Arg82p, Kcs1p, and Adk1p are involved in regulating the PHO pathway upstream of Pho81p. We provide evidence that Plc1p, Arg82p, Kcs1p, and Adk1p negatively regulate the PHO pathway independently of the intracellular polyphosphates, being required for many metabolic processes such as the biosynthesis of nucleic acids and phospholipids, energy metabolism, and signal transduction.
construct pHB96 (ADK1), and grown at 30 °C for an additional 2 days. The APase activity was calculated as described previously (19).

**RESULTS**

**Role of Inositol Pyrophosphates in Phosphate Signaling**

The APase activity was measured by determining the amount of p-nitrophenyl phosphate cleaved during a 10-min incubation at 35 °C. Cleavage was determined by monitoring the absorbance at 420 nm, and the APase activity was calculated as described previously (19).

**RNA Purification and Northern Blot Analysis**—Extraction of total RNA and Northern blot analysis were performed as described previously (17). Cells were precultivated in synthetic high phosphate medium to log phase at 30 °C. Log-phase cultures were inoculated into a specified medium to give an A_{600} of 0.1 and shaken at 30 °C until an A_{600} of 1.0 was reached. Total RNA was extracted, and 15 μg of RNA was loaded per lane. DNA fragments containing the PHO5 open reading frame (+1 to +1404) synthesized by PCR and the 1.0-kb HindIII-XhoI fragment carrying ACT1 prepared from pYA301 (22) were labeled using a random primers DNA labeling kit (Version 2, Takara) with [α-32P]dCTP. Prehybridization, hybridization, and detection were carried out by standard methods.

**31P NMR Spectroscopy**—Yeast strains grown to log phase in synthetic high phosphate medium were inoculated into 100 ml of a specified medium to give an A_{600} of 0.1 and then cultivated at 30 °C until an A_{600} of 1.0 was reached. The cells were harvested by centrifugation, and excess medium was removed to obtain a cell suspension volume of 0.5 ml. The intracellular phosphate concentration in yeast cells was estimated on the basis of the intracellular phosphate concentration was estimated on the basis of the integrated area of the resonance relative to methylene diphosphonate. A haploid cell volume of 70 μm^3 (23) and a cell density of 1.85 × 10^7 cells/ml at an A_{600} of 1 were assumed. Polypolyphosphate concentration is given in terms of phosphate residues.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The 4828 nonessential haploid S. cerevisiae deletion strains generated by the Saccharomyces Genome Deletion Project (15) were obtained from Research Genetics. These strains are on a BY4742 (MATa) background. The isogenic strain BY4741 (MATa) was used for genetic analysis. The double and triple disruption strains were used in this study as well as complete genotype descriptions are listed in Table I. Disruptions of PHO5 in strains BY4741 and BY4742 were generated by a previously described PCR-mediated gene disruption method (16) using Candida glabrata HIS3 or LEU2, respectively, as a reference with no apparent distortion of yeast metabolism. Disruptions were verified by colony PCR. The other double and triple disruptants were generated by standard genetic crossing, sporulation, and tetrad dissection (17). Nutrient (medium containing extract pentone-dextrose-adenine (YPD)), yeast nitrogen base without amino acids and glucose added, with appropriate nutrients (17), and synthetic high and low phosphate (containing 11 mM and 0.22 mM Pi, respectively) media were prepared as described previously (18).

**Plasmids**—Plasmids pH92 (expressing PCL1), pH93 (ARG82), pH94 (KCS1), and pH95 (IPK1) were constructed in the same way using a 5-HindIII PCR primer and a 3-HindIII PCR primer with gene-specific sequences that included the entire open reading frame. PCR products were digested with HindIII and inserted into the HindIII site of pGAD424 (Clontech), which contains the ADH1 promoter. To construct pH96 (ADK1), the ADK1 open reading frame was amplified by PCR using appropriate oligonucleotides as primers with a HindIII restriction site. PCR products and pGAD424 were digested with HindIII, blunt-ended with the Klenow fragment, and ligated together.

**Screening for Phosphate Signaling-defective Deletion Mutants**—Cells were transferred from thawed 96-well microtiter plate stocks to YPD medium plates supplemented with 200 mg/liter Geneticin in 96 place grids using a TK-CP96 96-pin replicator (Tokken Inc.). After incubation at 30 °C for 2 days, strains were stamped onto high and low phosphate plates and grown at 30 °C for an additional 2 days. The APase activity of the yeast strains was determined by staining colonies using o-naphthyl phosphate as a phosphate substrate as described previously (19).

**Acid Phosphatase Assay in Cell Suspension**—Cells were precultured to log phase in synthetic high phosphate medium at 30 °C. Log-phase cultures were inoculated into a specified medium to give an A_{600} of 0.1 and cultivated with shaking at 30 °C until an A_{600} of 1.0 was reached.

**TABLE I**

| Strain     | Relevant genotype | Source   |
|------------|-------------------|---------|
| BY4741     | MATa het31 leu2Δ met15Δ ura3Δ | Ref. 20 |
| BY4742     | MATa het31 leu2Δ lys2Δ ura3Δ | Ref. 20 |
| haploid    | As BY4742, orf::kanMX4 | Ref. 15 |
| PHY77      | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 | This study |
| PHY253     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgHIS3 | This study |
| PHY68      | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 pho81::kanMX4 | This study |
| PHY97      | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 pho81::kanMX4 | This study |
| PHY104     | MATa het31 leu2Δ ura3Δ pho3::CgLEU2 pho81::kanMX4 | This study |
| PHY116     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY121     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 erg5::kanMX4 | This study |
| PHY129     | MATa het31 leu2Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY174     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY179     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 adk1::kanMX4 | This study |
| PHY204     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY240     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 pho81::kanMX4 | This study |
| PHY290     | MATa het31 leu2Δ ura3Δ pho3::CgLEU2 pho81::kanMX4 kcs1::kanMX4 | This study |
| PHY211     | MATa het31 leu2Δ lys2Δ ura3Δ pho3::CgLEU2 pho81::kanMX4 adk1::kanMX4 | This study |
| PHY246     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 adk1::kanMX4 | This study |
| PHY246     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 adk1::kanMX4 | This study |
| PHY290     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY290     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY290     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY290     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY290     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |
| PHY290     | MATa het31 leu2Δ met15Δ ura3Δ pho3::CgLEU2 plc1::kanMX4 | This study |

a orf, open reading frame; CgLEU2, C. glabrata LEU2; CgHIS3, C. glabrata HIS3.

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centration, resulting in a negative APase phenotype. By contrast, the expression of PHO5 in mutants of negative regulators would be induced constitutively; thus, these mutants should show a constitutive APase phenotype.

From the three screens, we identified 12 mutants exhibiting the constitutive APase phenotype and 10 mutants exhibiting the negative APase phenotype (Fig. 1B). Interestingly, among the genes known to be involved in the synthesis of soluble inositol polyphosphate, deletion of the Kcs1p, Arg82p, and Adk1p mutants were higher under the low phosphate conditions than under the high phosphate conditions, whereas the APase activity of the Δkcs1 mutant was almost the same as that in the Δpho80 strain under both high and low phosphate conditions (Fig. 1B). Interestingly, among the genes known to be involved in the synthesis of soluble inositol polyphosphate, deletion of only the IPK1 gene, which encodes IP5 kinase, did not result in the constitutive APase phenotype (Fig. 1, A and B). We therefore focused the following studies on the role of Plc1p, Arg82p, Kcs1p, and Adk1p in regulating the PHO pathway.

Plc1p, Arg82p, Kcs1p, and Adk1p Are Required for the Regulation of PHO5 Expression—In S. cerevisiae, there are two types of APase: one is a constitutive APase encoded by PHO3, and the other is a rAPase encoded by PHO5 and its homologs PHO11 and PHO12 (19, 30). Expression of rAPase is controlled by the PHO pathway in response to intracellular phosphate concentrations (2–5). To investigate whether Plc1p, Arg82p, Kcs1p, and Adk1p function as negative regulators in the PHO pathway, we knocked out the PHO3 gene in the Δplc1, Δarg82, Δkcs1, and Δadk1 strains and examined the resulting double disruptants for rAPase activity. We found that deletion of PHO3 in the Δplc1, Δarg82, Δkcs1, and Δadk1 strains did not affect the increased APase activity in these mutants (Fig. 1B).

To determine whether the increased rAPase activity in these disruptants is a consequence of an increase in transcription of the PHO5 gene, we examined PHO5 transcript levels in the Δplc1Δpho3, Δarg82Δpho3, Δkcs1Δpho3, and Δadk1Δpho3 strains by Northern blot analysis. We found that the PHO5 transcript levels in these double disruptants were increased even under the high phosphate conditions compared with the
wild-type strain (Δpho3) and correlated with the levels of rAPase activity (Fig. 1C), indicating that Plc1p, Arg82p, Kcs1p, and Adk1p are all involved in negative regulation at the level of PHO5 transcription. It should be noted that the PHO5 transcript levels in the Δadk1Δpho3 strain, but not in the other double disruptants, was higher under the low phosphate conditions than under the high phosphate conditions, indicating considerable residual regulation by the phosphate concentration in this strain. These observations suggest that Adk1p is only indirectly involved in regulating the PHO pathway. We also examined PHO5 transcription in the Δpho3Δpho81 strain and found that PHO5 transcription in this strain, which differed from that in the Δpho3Δpho82, Δarg82Δpho3, and Δkcs1Δpho3 strains, was induced only under the low phosphate conditions, as in the wild-type strain (Δpho3) (Fig. 1B and C).

Plc1p, Arg82p, Kcs1p, and Adk1p function upstream of the Pho81p-Pho80p-Pho85p Complex—Because the signal transduction mechanisms that function upstream of the Pho81p-Pho80p-Pho85p complex are still unknown, we tested whether Plc1p, Arg82p, Kcs1p, and Adk1p function upstream or downstream of Pho81p by analyzing the epistasis relationship between pho81 and the deletion mutants of these candidate regulators of the PHO pathway. If the deletion mutants are constitutive in a pho81 mutation, then deletion of PHO81 in the disruptants should result in an uninducible PHO5 expression phenotype. However, if the deletion mutants are hypostatic to a pho81 mutation, then deletion of PHO81 in the disruptants still displaying a constitutive PHO5 expression phenotype. We therefore generated triple disruptants of Δpho81 and Δplc1Δpho3, Δarg82Δpho3, Δkcs1Δpho3, and Δadk1Δpho3 and examined them for expression of PHO5 by the rAPase assay. The rAPase activity in each of the triple disruptants was diminished, similar to what was observed in the Δpho81Δpho3 strain (Fig. 2), indicating that pho81 is epistatic to each of the mutants. These results suggest that Plc1p, Arg82p, Kcs1p, and Adk1p function as negative regulators of the PHO pathway upstream of Pho81p.

Plc1p, Arg82p, Kcs1p, and Adk1p regulate the PHO pathway independently of the intracellular orthophosphate concentration—Previous studies demonstrated that PHO5 expression is closely correlated with intracellular orthophosphate concentrations (5, 31). To examine whether the induction of PHO5 expression in the Δplc1Δpho3, Δarg82Δpho3, Δkcs1Δpho3, and Δadk1Δpho3 strains under the high phosphate conditions was due to a reduction in intracellular orthophosphate, we measured the intracellular orthophosphate concentration in these double disruptants by in vivo 31P NMR spectroscopy. Surprisingly, we found that, under the high phosphate conditions, the Δplc1Δpho3 and Δarg82Δpho3 strains had significantly reduced intracellular polyphosphate levels, viz. 14 and 24%, respectively, of the level in the wild-type strain (Δpho3). Moreover, intracellular polyphosphate was reduced to an undetectable level in the Δkcs1Δpho3 strain.

In contrast to the polyphosphate levels, intracellular orthophosphate levels in these double disruptants were ~1.6-fold higher than that in the wild-type strain (Fig. 3A). These observations indicate that, despite the increase in intracellular orthophosphate levels, expression of PHO5 in the Δplc1Δpho3, Δarg82Δpho3, and Δkcs1Δpho3 strains is constitutive; this differs from its expression in the wild-type strain, where it is repressed (Fig. 1B). Therefore, these findings further suggest that Plc1p, Arg82p, and Kcs1p regulate the PHO pathway.
independently of intracellular orthophosphate levels.

On the other hand, the levels of both intracellular orthophosphate and polyphosphate in the \( \Delta adk1 \Delta pho3 \) strain were similar to those in the wild-type strain (Fig. 3B). Previous studies showed that the constitutive expression of \( \text{PHO5} \) in the \( \Delta pho3 \) strain, a disruptant for a high affinity phosphate transporter, could be suppressed by increasing intracellular orthophosphate levels through the deletion of \( \text{PHM3}, \text{PHM4} \), or both \( \text{PHM1} \) and \( \text{PHM2} \) (5, 31). We therefore knocked out \( \text{PHM3} \) in the \( \Delta adk1 \Delta pho3 \) strain and found that the resulting triple mutant showed defects in accumulating intracellular polyphosphate and thus contained higher levels of intracellular orthophosphate compared with the wild-type strain (Fig. 3B). Under the high phosphate conditions, expression of \( \text{PHO5} \) was increased in the \( \Delta adk1 \Delta pho3 \Delta phm3 \) strain, as it was in the \( \Delta adk1 \Delta pho3 \) strain (Fig. 3C), suggesting that, in addition to enzymes involved in inositol pyrophosphate synthesis, \( \text{Adk1p} \) is involved in regulating \( \text{PHO5} \) expression in an orthophosphate-independent manner.

Finally, we confirmed that, as expected, deletion of the \( \text{PHM3} \) gene in the \( \Delta plc1 \Delta pho3 \), \( \Delta arg82 \Delta pho3 \), and \( \Delta kcs1 \Delta pho3 \) double disruptants diminished intracellular polyphosphate levels, but had little effect on intracellular orthophosphate levels; in addition, the constitutive \( \text{PHO5} \) expression phenotype of the double disruptants was not affected by the deletion of \( \text{PHM3} \) (data not shown).

Overexpression of \( \text{KCS1} \) Suppresses \( \text{PHO5} \) Expression under Low Phosphate Conditions—Because enzymes involved in inositol pyrophosphate synthesis appear to be essential for the regulation of \( \text{PHO5} \) expression independently of intracellular orthophosphate levels, we considered that overexpressing each of these enzymes might be sufficient to suppress \( \text{PHO5} \) expression. We therefore overexpressed \( \text{PLC1}, \text{ARG82}, \text{KCS1}, \) and also \( \text{IPK1} \) in the wild-type strain (\( \Delta pho3 \)) under the control of the \( \text{ADH1} \) promoter, a highly expressed constitutive promoter. We found that the rAPase activity in the wild-type strain overexpressing \( \text{KCS1} \) grown in low phosphate medium was decreased to approximately one-third of that in the wild-type strain transformed with empty vector (Fig. 4). In contrast to \( \text{KCS1} \), overexpression of \( \text{PLC1}, \text{ARG82}, \) and \( \text{IPK1} \) in the wild-type strain did not affect rAPase activity (Fig. 4), suggesting that overexpression of these genes is not sufficient to repress \( \text{PHO5} \) expression. Based on these observations, it is possible that the products of these enzymes, but not the enzymes themselves, may play a role in regulating the \( \text{PHO} \) pathway.

Because overexpression of \( \text{KCS1} \) is supposed to result in enhanced conversion of \( \text{IP}_6 \) to \( \text{PP-IP}_4 \) and \( (\text{PP})_2-\text{IP}_3 \), leading to overproduction of \( \text{IP}_5 \) and \( (\text{PP})_2-\text{IP}_3 \), we propose that \( \text{IP}_5 \) and/or \( (\text{PP})_2-\text{IP}_3 \) may be essential for the regulation of the \( \text{PHO} \) pathway.

Previous studies have shown that overexpression of \( \text{KCS1} \) in the \( \Delta arg82 \) strain rescues the repression of some phosphate-responsive genes (\( \text{PHO11} \) and \( \text{VTC3} \)) (14), probably because it rescues the synthesis of higher inositol polyphosphates such as \( \text{IP}_5 \) and \( (\text{PP})_2-\text{IP}_3 \) (32). To investigate whether overexpression of a gene encoding an enzyme involved in the synthesis of inositol polyphosphates could repress \( \text{PHO5} \) expression in the disruptants for the other enzymes, we overexpressed \( \text{PLC1}, \text{ARG82}, \text{KCS1}, \) and \( \text{IPK1} \) individually in the \( \Delta plc1 \Delta pho3 \), \( \Delta arg82 \Delta pho3 \), and \( \Delta kcs1 \Delta pho3 \) strains and examined the resulting strains for rAPase activity. We found that overexpre-
sion of PLC1, ARG82, or KCS1 could restore the repression of PHO5 in the \( \Delta plc1 \Delta pho3, \Delta arg82 \Delta pho3, \) and \( \Delta kcs1 \Delta pho3 \) strains, respectively, but not in the other double disruptants. However, overexpression of IPK1 did not repress PHO5 expression in any of the double disruptants (data not shown). These results suggest that a defect in the kinase activity of one inositol-polyphosphate kinase cannot be restored by overexpressing one of the other inositol-polyphosphate kinases to repress PHO5 expression.

Because adenylate kinase, like enzymes involved in inositol pyrophosphate synthesis, also seems to regulate PHO5 expression independently of intracellular orthophosphate levels, we overexpressed ADK1 in the wild-type strain (\( \Delta pho3 \)) and examined the rAPase activity. We found that, in contrast to KCS1, overexpression of ADK1 could not suppress PHO5 expression under phosphate starvation conditions (Fig. 4), suggesting that even an excessive amount of Adk1p is not effective at repressing PHO5 expression. To explore the relationship between Adk1p and the production of PP-IP4 and/or (PP)\(_2\)-IP3 in regulating the PHO pathway, we overexpressed PLC1, ARG82, KCS, and IPK1 as well as ADK1 in the \( \Delta adk1 \Delta pho3 \) strain to examine whether the defect in phosphate signaling in this strain could be rescued by overexpressing enzymes involved in the synthesis of inositol polyphosphates such as inositol-polyphosphate kinase. We found that overexpression of ADK1, but not of PLC1, ARG82, KCS1, or IPK1, suppressed expression of PHO5 in the \( \Delta adk1 \Delta pho3 \) strain (data not shown), suggesting that Adk1p regulates the PHO pathway, but not via modulation of the synthesis of PP-IP4 and/or (PP)\(_2\)-IP3.

**Inositol Polyphosphate Is Involved in Regulating Polyphosphate Accumulation**—Because the \( \Delta plc1 \Delta pho3, \Delta arg82 \Delta pho3, \) and \( \Delta kcs1 \Delta pho3 \) strains, which are deficient in the production of all inositol pyrophosphates (PP-IP\(_4\), (PP)\(_2\)-IP\(_3\), PP-IP\(_5\), and (PP)\(_2\)-IP\(_4\)), are defective in polyphosphate accumulation, we considered that inositol polyphosphates might be essential for synthesizing polyphosphates. To determine whether PP-IP\(_4\) and (PP)\(_2\)-IP\(_3\) or PP-IP\(_4\) and (PP)\(_2\)-IP\(_4\) are required for polyphosphate accumulation, we measured intracellular polyphosphate levels in the \( \Delta ipk1 \Delta pho3 \) strain, which is impaired only in the production of PP-IP\(_4\) and (PP)\(_2\)-IP\(_4\). We found that, in contrast to the other strains, the \( \Delta ipk1 \Delta pho3 \) strain accumulated intracellular polyphosphate to a level similar to that accumulated by the wild-type strain (\( \Delta pho3 \)) (Fig. 3A).

Previous work has shown that polyphosphate accumulation is influenced by vacuolar H\(^+\)-ATPase (V-ATPase) activity (33, 34), but that this activity is not strictly essential (35). Among the known mutants that are defective in polyphosphate accumulation, the \( \Delta plc1 \) mutant strain, which has a mutation in a subunit of V-ATPase, is completely deficient in V-ATPase activity (34), whereas the \( \Delta phm3 \) and \( \Delta phm4 \) strains retain V-ATPase activity (35). Mutants deficient in V-ATPase activity are characteristically sensitive to media containing 60 mM CaCl\(_2\) at pH 7.5 (34). To determine whether the defect in polyphosphate accumulation in the \( \Delta plc1, \Delta arg82, \) and \( \Delta kcs1 \) strains is a consequence of a defect in V-ATPase activity, we tested these disruptants for growth in the presence of CaCl\(_2\) at pH 7.5. The \( \Delta arg82 \) and \( \Delta kcs1 \) strains grew normally on YPD medium (pH 7.5) supplemented with 60 mM CaCl\(_2\), whereas the \( \Delta plc1 \) strain did not grow under these conditions (Fig. 5). These results suggest that the \( \Delta arg82 \) and \( \Delta kcs1 \) strains retain V-ATPase activity, whereas the \( \Delta plc1 \) strain does not; thus, PP-IP\(_4\) and/or (PP)\(_2\)-IP\(_3\) may not influence polyphosphate accumulation through the regulation of V-ATPase activity, whereas IP\(_3\) may be necessary for V-ATPase activity.

Therefore, we examined whether the inositol pyrophosphates might be involved in regulating the PHO pathway by screening a collection of mutant strains, including \( \Delta plc1, \Delta arg82, \) and \( \Delta kcs1 \) strains, for repression of PHO5 expression in the wild-type strain (data not shown). These results suggested that overexpression of \( \Delta plc1, \Delta arg82, \) and \( \Delta kcs1 \) strains could restore the repression of PHO5 expression, whereas the \( \Delta plc1 \) strain did not; thus, PP-IP\(_4\) and/or (PP)\(_2\)-IP\(_3\) may be necessary for regulating the PHO pathway. The ADK1 strain could be rescued by overexpressing enzymes involved in inositol polyphosphate synthesis. Furthermore, deletion of IPK1, which encodes an IP\(_5\) kinase that converts IP2 to IP0, did not affect the repression of PHO5. Taking these observations together, we do not favor the idea that each of these enzymes has an individual role in regulating the PHO pathway. Rather, it is likely that the products of these enzymes, PP-IP\(_4\) and/or (PP)\(_2\)-IP\(_3\), play a role in regulating the PHO pathway.

Inositol polyphosphates are required for numerous cellular processes. IP\(_3\) is a critical second messenger needed for regulating the release of Ca\(^{2+}\) from intracellular stores in animal cells (36, 37). In plants, IP\(_6\) may play a role both as an antioxidant (38) and as a phosphate store (39). In animal cells, IP6 interacts with several proteins that regulate endocytosis (40–43), synaptic vesicle trafficking (44–46), and receptor desensitization (47). In yeast, IP\(_3\) is required for the efficient export of mRNA from the nucleus (48, 49). Inositol pyrophosphates regulate endocytic trafficking (50) and are required for vacuole biogenesis (28) and cell wall integrity and resistance to salt stress (32). Recently, inositol pyrophosphates have been reported to be essential for the expression of both genes regulated by the quality of the nitrogen source and phosphate-responsive genes (14). Our present results provide evidence that PP-IP\(_4\) and/or (PP)\(_2\)-IP\(_3\), but not PP-IP\(_5\) or (PP)\(_2\)-IP\(_4\), may be involved in PHO5 expression independently of intracellular orthophosphate levels, raising the intriguing possibility that these molecules may function as phosphate signals. It has been shown recently that IP\(_4\) and IP\(_5\), but not IP\(_3\) or pyrophosphates, are required for regulating the SWI/SNF and INO80 chromatin-remodeling complexes to induce the transcription of PHO5 (13). Thus, the inositol polyphosphates IP\(_3\) and IP\(_4\), and the pyrophosphates PP-IP\(_4\) and/or (PP)\(_2\)-IP\(_3\), may regulate the expression of PHO5 at different stages in the pathway and in opposite ways.

If PP-IP\(_4\) and/or (PP)\(_2\)-IP\(_3\) is an authentic phosphate signaling molecule, then what is the phosphate sensor(s) that could...
directly sense the level of this molecule? Deletion of the genes encoding phosphate sensors is thought to lead to a complete defect in the phosphate signal transduction that regulates the expression of phosphate-responsive genes such as PHO5; thus, disruptants of phosphate sensors should exhibit a phenotype of either completely constitutive or completely inducible PHO5 expression.

Our systematic screening for additional components of the PHO pathway identified ADK1, which encodes an adenylyl kinase, in addition to the genes involved in inositol pyrophosphates synthesis (Fig. 1, B and C). Because PHO5 expression in the Δadk1 strain was slightly increased under the high phosphate conditions and significantly induced during phosphate starvation (Fig. 1C), we do not favor the idea that Adk1p is a phosphate sensor. Because we did not identify any genes encoding phosphate sensors in our screening of single deletion mutants in nonessential genes, it seems possible that phosphate sensors may be encoded by essential genes or, alternatively, that there might be multiple phosphate sensors encoded by nonessential genes that have a redundant function in phosphate sensing.

Because Adk1p functions in the interconversion of AMP and ATP to two ADP molecules, it is possible that Adk1p may influence PHO5 expression through its function in maintaining energy metabolism. However, disruptants of other genes involved in the homoeostasis of energy, such as those encoding ATP synthase and the ADP/ATP transporter, were found to have normal PHO5 expression in our systematic screening; thus, Adk1p may not control the PHO pathway through the regulation of energy metabolism. It has recently been shown that ADO1, which encodes an adenosine kinase (51), negatively regulate PHO5 expression (52). Adenosine kinase catalyzes the phosphorylation of adenosine to AMP. Because inorganic phosphate is also essential for adenosine nucleotide metabolism, it is possible that the PHO pathway may be partially controlled by adenosine nucleotide metabolism. However, we did not notice that deletion of other genes involved in nucleotide metabolism such as YNK1, which encodes nucleoside-diphosphate kinase, did not affect PHO5 expression (data not shown). Because Adk1p has been found to be a member of a Pho85p-associated complex by systematic mass spectrometry (21), Adk1p may regulate the PHO pathway by modulating the kinase activity of the Pho80p-Pho85p complex.

Our study has also suggested that PP-IP$_4$ and/or (PP)$_2$-IP$_3$ is required for polyphosphate accumulation in a manner that differs from its regulation through V-ATPase activity (Figs. 3A and 5). It is not clear, however, how PP-IP$_4$ and/or (PP)$_2$-IP$_3$ regulates polyphosphate accumulation; it is possible that these inositol pyrophosphates may affect polyphosphate accumulation by regulating the activity of polyphosphate synthetase or the vacuolar phosphate transporter. By contrast, IP$_3$ seems to be necessary for V-ATPase activity (Fig. 5), possibly by regulating the assembly or stability of the V-ATPase complex.

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Plc1p, Arg82p, and Kcs1p, Enzymes Involved in Inositol Pyrophosphate Synthesis, Are Essential for Phosphate Regulation and Polyphosphate Accumulation in Saccharomyces cerevisiae

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