A problem for inositol signaling is to understand the significance of the kinases that convert inositol hexakisphosphate to diphosphoinositol polyphosphates. This kinase activity is catalyzed by Kcs1p in the yeast Saccharomyces cerevisiae. A kcs1Δ yeast strain that was transformed with a specifically “kinase-dead” kcs1Δ mutant did not synthesize diphosphoinositol polyphosphates, and the cells contained a fragmented vacuolar compartment. Biogenesis of the yeast vacuole also required another functional domain in Kcs1p, which contains two leucine heptad repeats. The kinase activity of Kcs1p was also found to sustain cell growth and integrity of the cell wall and to promote adaptive responses to salt stress. Thus, the synthesis of diphosphoinositol polyphosphates has wide ranging physiological significance. Furthermore, we showed that these phenotypic responses to Kcs1p deletion also arise when synthesis of precursor material for the diphosphoinositol polyphosphates is blocked in arg8Δ cells. This metabolic block was partially bypassed, and the phenotype was partially rescued, when Kcs1p was overexpressed in the arg8Δ cells. This was due, in part, to the ability of Kcs1p to phosphorylate a wider range of substrates than previously appreciated. Our results show that diphosphoinositol polyphosphate synthase activity is essential for biogenesis of the yeast vacuole and the cell’s responses to certain environmental stresses.

Environmental and physiological stresses are constantly challenging living organisms. For microorganisms in particular, their immediate environmental conditions, temperature, salinity, and nutrient availability, can fluctuate considerably. The yeast Saccharomyces cerevisiae is a widely used model system for studying the molecular processes that sense and initiate responses to these stressful changes. This work is of general biological interest, because the molecular mechanisms that underlie these processes are highly conserved between yeasts and higher eukaryotes. It has been known for some years that the membrane-bound inositol lipids and their hydrolysis by phospholipase C (Plc1p) both play important roles in adaptations to environmental stress (13). Thus, we have also studied whether Kcs1p has a role in maintaining the integrity of the cell wall. These studies into environmental stress responses represent new areas of research into the function of the KCS1 gene.

One of the functional characteristics of Kcs1p is its diphosphoinositol polyphosphate synthase (DINS) activity (2, 12), which yields a specific class of inositol phosphates distinguished by their diphosphate groups. DINS activity, it also interacts with a guanine nucleotide exchange factor for Rab3A (14). Kcs1p is at least as likely to be multifunctional, since it is much larger in size (120 kDa (15)) than are the mammalian InsP6 kinases (50–60 kDa (2, 16)). Indeed, the KCS1 gene was first identified in a screen for suppressing the temperature sensitivity of the pck1Δ–4 allele (15). Kcs1p also has two groups of four heptad repeats of leucine residues. The latter were suggested to be leucine zippers (15), which in other contexts direct the homo- and heterodimerization of proteins (17). In the current study, we provide the first evidence that there is an important function for

1 The abbreviations used are: DINS, diphosphoinositol polyphosphate synthase; BCIP, 5-bromo-4-chloro-3-indolylphosphate; Ins, inositol; PP-InsP, diphosphoinositol pentakisphosphate; (PP)2-InsP, bis-diphosphoinositol tetrakisphosphate; InsP6, inositol hexakisphosphate; InsP5, inositol pentakisphosphate; InsP4, inositol tetrakisphosphate; InsP3, inositol triphosphate; InsP2, inositol diphosphate; InsP1, inositol monophosphate; OTCase, ornithine carbamoyltransferase; HPLC, high pressure liquid chromatography; DCF, dichlorodihydrofluorescein; DCFDA, dichlorodihydrofluorescein diacetate.

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**In Saccharomyces cerevisiae**, the Inositol Polyphosphate Kinase Activity of Kcs1p Is Required for Resistance to Salt Stress, Cell Wall Integrity, and Vacuolar Morphogenesis*

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Diphosphoinositol Polyphosphate Synthase Activity of Kcs1p

EXPERIMENTAL PROCEDURES

**Strains and Media**—Escherichia coli strain XL1-B was used for plasmid amplification and for in vitro mutagenesis. The BY4709 (MATa, ura3) yeast strain (24) was used to create a series of gene deletions. The long flanking homology strategy (25) was used to perform deletion of ARG82 and KCS1. A long flanking homology replacement cassette was synthesized using a two-step PCR, leading to the konMX4 cassette flanked by about 500 bp corresponding to the promoter and terminator regions of the target genes. The DNA fragments containing the different cassettes were used to transform strain BY4709 on rich medium plates containing 200 μg/ml of Geneticin. The correct targeting of the deletions in G418 transformants was verified by PCR, using whole cells as a source of DNA, and appropriate primers. The following strains were obtained: arg82::konMX4 (03127c) and kcs1::konMX4 (4709 kcs1Δ).

Unless otherwise noted, all yeast strains were grown on minimal medium (pH 6.5), which contained 3% glucose, vitamins, and mineral traces. Unless otherwise stated, the nitrogen source was 0.02 μM (NH₄)₂SO₄ (M.Am medium).

**Genetic Manipulations**—The low copy pFL38 plasmid was used in this work to bear wild type and mutated arg82 (26) and kcs1 genes. Since these proteins are expressed under their native promoter and on a low copy number plasmid (one or two copies in the cell), the amount of each protein is comparable with the amount produced by the genomic copy of the wild type strain. The wild type KCS1 gene was cloned on a pFL38 plasmid by insertion of a 4.95-kb EcoRI-EcoRI fragment of KCS1, synthesized by PCR using appropriate oligonucleotides as primers with EcoRI restriction sites, and genomic wild type DNA as template. Yielding plasmid pPV241. Oligonucleotides were used to create substitution by in vitro mutagenesis on double-stranded DNA from plasmid pPV241 using the QuikChange site-directed mutagenesis kit from Stratagene (Amsterdam, The Netherlands). In this plasmid, the following amino acid changes were created: L794A/L801A/L857A/L864A (pPV198) and S887A/L888A/L899A (pPV217). To check that each mutant protein was stable when expressed, the same Kcs1p proteins were expressed fused at the N terminus to the V5 epitope using the pYES2 plasmid (Invitrogen), with a GAL10 promoter. Plasmids pPV249 (GAL10-KCS1-V5), pPV251 (GAL10-kcs1L794A/L801A/L857A/L864A-V5) and pPV252 (GAL10-kcs1S887A/L888A/L899A-V5) were obtained by insertion in the EcoRI restriction site of pYES2 plasmid of EcoRI KCS1 DNA fragments synthesized by PCR using appropriate oligonucleotides and as templates the plasmids pPV241, pPV217, and pPV198, respectively. We completely sequenced all of the wild type and the different mutated genes to ensure that no additional mutations had been introduced.

To overexpress ARG82 and KCS1 genes from *S. cerevisiae*, we fused their coding sequence to the *tet* promoter present in plasmid pCM262 (gift from E. Herrero, Universitat de Lleida, Spain). For ARG82, a 1070-bp BamHI-BamHI fragment was synthesized by PCR using appropriate oligonucleotides as primers with BamHI restriction sites and DNA from plasmid pPV145 as template, blunt-ended with T4 DNA polymerase and inserted in the *PmEL* restriction site of plasmid pCM262, yielding plasmid pPV186. For KCS1, a 4.15-kb EcoRI-EcoRI fragment was synthesized by PCR using appropriate oligonucleotides as primers with EcoRI restriction sites and DNA from plasmid pPV241 as template, blunt-ended with T4 DNA polymerase and inserted in the *PmEL* restriction site of plasmid pCM262, yielding plasmid pPV187.

Western Analysis—Exponentially growing cells (25 ml) were harvested by centrifugation. The cell pellet was resuspended in 300 μl of buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM ammonium acetate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture tablet (catalog no. 1897498, Roche Diagnostics). Next, 300 μl of 20% trichloracetic acid and 300 mg of glass beads were added, and cells were disrupted in a Bead-Beater. Proteins were extracted by centrifugation and resuspended in 200 μl of gel loading buffer containing 3.5% (w/v) SDS, 80 mM Tris, 8 mM EDTA, 14% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and blue bromophenol. Samples were heat-treated (100°C, 10 min). After electrophoresis on NuPAGE 4–12% Bis-Tris gels, followed by transfer to Highbond membranes, the Kcs1p proteins were visualized using antibodies raised against the V5 epitope. Arg82p proteins were detected using polyclonal antibodies against recombinant GST-Arg82p, which were raised in the mouse, and prepared as previously described using glutathione-Sepharose 4B beads (26). Western blots were performed according to a standard chemiluminescent protocol provided with the WesternBreeze kit from Invitrogen (Merelbeke, Belgium).

**Assays of Inositol Polyphosphates**—To study inositol phosphate lev-

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2 The Arg82p protein has acquired several pseudonyms, including ArgRIII (1), inositol polyphosphate multikinase (Ipmk) (2), and inositol polyphosphate kinase 2 (Ipk2) (3).
els in intact yeast cells, cultures were grown on M. am at 29 °C through at least eight generations in the presence of [3H]inositol (100 μCi/ml; American Radiolabeled Chemicals). The HPLC analysis and identification of [3H]inositol-labeled phosphate in these cells using appropriate standards were performed as described previously, with a Synchropak Q100 column (20); 1-ml fractions were collected. Inositol phosphate isomers were identified by their co-elution with genuine [3H]-labeled standards of Ins(1,4,5)P3, Ins(1,3,4,5,6)P5, InsP5, PP-InsP5, and (PP)2-InsP4 as previously described (20). To study inositol phosphate metabolism by Kcs1p in vitro, the plasmid pFv249 was introduced in the yeast strain 12S16cep4Δ (ura3, leu2, arg5, pep4Δ;kanMX4). An extract was prepared from 1 liter of these cells using a French press, and Kcs1p was purified by Ni2+-nitrilotriacetic acid agarose chromatography and then dialyzed into 50 mM NaCl, 20 mM HEPES, pH 7.0. Kcs1p was incubated with 5000 dpm of either [3H]Ins(1,4,5)P3, [3H]Ins(1,3,4,5,6)P5, or [3H]InsP5 (all purchased from PerkinElmer Life Sciences) at 37 °C in 25–100 μl of medium containing 20 mM HEPES (pH 7.0), 12 mM MgSO4, 10 mM Na2ATP, 20 mM phosphocreatine, 1 mM dithiothreitol, 1 mM EDTA, 360 units of phosphocreatine kinase (Calbiochem), and 0.5 mg/ml bovine serum albumin. Assays were quenched with perchloric acid and neutralized with KCO3 as described previously (20). The first-order rate constant for these assays was determined by HPLC with a 12.5-cm Partipak SAX column as described previously (20).

Other Enzyme Assays—Ornithine carbamoyltransferase (OTCase) was assayed as described previously (27). For this enzyme assay, the repression factor for OTCase corresponds to the ratio of OTCase-specific activities of the arg82Δ strain grown on M. am versus OTCase-specific activities of the different strains grown on M. am plus 1 mg/ml arginine. Alkaline phosphatase release from cells was recorded on plates that contained M. am plus 40 μg/ml BCIP (Sigma). The hydrolysis of the dye yields a blue stain.

Vacuole Analyses—Cells were grown at 29 °C in YPD medium (2% peptone, 1% yeast extract, 2% glucose). Cells from 5 ml of early logarithmic phase cultures (A600 = 0.4–0.6) were collected and resuspended in 100 μl of medium containing 50 mM sodium citrate buffer, pH 5.0, 2% glucose, 10 μg/ml carboxy-DCFDA. Cells were incubated at room temperature for 20 min, whereupon the membrane-permeable carboxy-DCFDA enters the vacuoles, the acetate groups are hydrolyzed, and the non-membrane-permeable and fluorescent carboxy-DCF highlights the vacuolar compartment. Intracellular localization of carboxy-DCF was examined with a Zeiss LSM510 microscope with a 100× oil immersion objective (NA = 1.4) under transmitted light or with epifluorescence (488-nm excitation with an argon laser, HFT 488-nm dichroic, and 505-nm long pass filter). The images that are shown in this study are representative of at least 100 cells from every strain.

RESULTS

The Effect of Deletion of the KC81 Gene upon DNP Activity and Vacuolar Morphology—We initially studied the consequences of deleting the entire KC81 gene. Since Kcs1p has diphosphoinositol phosphate kinase activity, we monitored the effect of the gene deletion upon the cellular levels of PP-InsP5 and (PP)2-InsP4. Individual inositol phosphates were resolved by HPLC of yeast cell extracts that had been radiolabeled to equilibrium, their saturation phosphate pools were radiolabeled to equilibrium, their saturation levels of PP-InsP5 and (PP)2-InsP4 as previously described (20). To study inositol phosphate metabolism by Kcs1p in vitro, the plasmid pFV249 was introduced in the yeast strain 12S16cep4Δ (ura3, leu2, arg5, pep4Δ;kanMX4). An extract was prepared from 1 liter of these cells using a French press, and Kcs1p was purified by Ni2+-nitrilotriacetic acid agarose chromatography and then dialyzed into 50 mM NaCl, 20 mM HEPES, pH 7.0. Kcs1p was incubated with 5000 dpm of either [3H]Ins(1,4,5)P3, [3H]Ins(1,3,4,5,6)P5, or [3H]InsP5 (all purchased from PerkinElmer Life Sciences) at 37 °C in 25–100 μl of medium containing 20 mM HEPES (pH 7.0), 12 mM MgSO4, 10 mM Na2ATP, 20 mM phosphocreatine, 1 mM dithiothreitol, 1 mM EDTA, 360 units of phosphocreatine kinase (Calbiochem), and 0.5 mg/ml bovine serum albumin. Assays were quenched with perchloric acid and neutralized with KCO3 as described previously (20). The first-order rate constant for these assays was determined by HPLC with a 12.5-cm Partipak SAX column as described previously (20).

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The vacuolar compartment of S. cerevisiae was identified by fluorescence microscopy. Wild-type cells were found to have the normal complement of one large vacuole (Fig. 3). In contrast, the vacuolar compartment in the kcs1Δ strain was fragmented...
following strains were analyzed: wild type strain; kcs1Δ; kcs1Δ + pKCS1; kcs1Δ + pKCS1L1L2−AAA; kcs1Δ + pKCS1SLL−AAA; wild-type and mutant forms of kcs1p upon inositol phosphate pools in S. cerevisiae

Inositol phosphate levels were determined by HPLC as described in the legend to Fig. 2. Data are means ± S.E. from 5–7 experiments.

| Wild type     | InsP3 58 ± 20 | InsP6 1230 ± 125 | PP-InsP5 28.3 ± 6 | (PP)2-InsP4 31.5 ± 6 |
|---------------|---------------|------------------|-------------------|----------------------|
| kcs1Δ         | 55 ± 14       | 1767 ± 392       | 2 ± 0.3           | 2.2 ± 0.3            |
| kcs1Δ + pKCS1 | 41 ± 8        | 1042 ± 86        | 10.5 ± 1.8        | 20.7 ± 3.7           |
| kcs1Δ + pKCS1L1L2−AAA | 50 ± 12 | 1119 ± 54 | 0.99 ± 0.1 | 0.61 ± 0.44 |
| kcs1Δ + pKCS1SLL−AAA | 65 ± 9 | 1968 ± 99 | 28.5 ± 5 | 41.4 ± 5.5 |

leakage of intracellular alkaline phosphatase into the extracellular milieu (7, 30). We also observed phosphatase leakage when the yeast cells were grown on media with a less stressful pH of 6.5 (Fig. 5B). The addition of 1 M sorbitol elicited an osmoremedial effect that reduced the leakage of phosphatase from inside the cell (Fig. 5, A and B), indicating that in the kcs1Δ strain, there was a defect in the maintenance of cell wall structure rather than a defect in the plasma membrane (see Refs. 30 and 31).

We sought an independent means of examining cell wall integrity. It has been reported that perturbations to the integrity of the cell wall can decrease the degree of resistance of the cells to the toxic effects of caffeine (see Ref. 31 and references therein). These experiments were performed on YPD media, since the toxicity of caffeine is enhanced, even in wild-type cells, when they are grown on minimal medium (data not shown). In the absence of caffeine, the greater nutritional support of the YPD media provided the kcs1Δ cells with some protection against their growth defect, compared with cells grown on minimal media (compare Figs. 4A and 5C). Nevertheless, the growth phenotype of kcs1Δ cells was still evident at 37 °C in YPD media, and they were more sensitive to 10 mM caffeine than were wild-type cells (Fig. 5C). Osmotic stabilization of the plasma membrane by 1 M sorbitol largely protected against this sensitivity to caffeine (Fig. 5C); this osmotic remedial response is typical of a defect in cell wall integrity (31). Clearly, Kcs1p has far more wide ranging consequences for cell function than has been appreciated from earlier studies (12, 15).

**DIPS Activity of Kcs1p Is Required for Vacular Biogenesis, Protection against Salt Stress, and Cell Wall Synthesis**—Since Kcs1p is probably pleiotropic (see the Introduction), an important objective in the current study was to investigate the specific role of diphosphoinositol polyphosphate synthesis. Therefore, we transformed kcs1Δ cells with a plasmid encoding wild-type Kcs1p, which restored PP-InsP5 and (PP)2-InsP4 levels to 40–70% of those in wild-type cells (Table I). This was sufficient to rescue vacuolar morphology (Fig. 3), cell wall integrity (Fig. 6B), protective adaptations to salt stress (Fig. 6B), and normal growth rates (Fig. 6A).

We next transformed the kcs1Δ cells with a plasmid encoding a catalytically inactive form of Kcs1p. To achieve this goal, we took note that all three mammalian diphosphoinositol polyphosphate synthases possess a SLL consensus, which is essential for catalytic activity (16). Multiple sequence alignments indicate that the corresponding sequence in Kcs1p is Ser-887, Thr-888, and Leu-889. We transformed kcs1Δ cells with a plasmid encoding a mutant form of kcs1p, in which these three residues were changed to Ala. In this yeast strain, designated kcs1Δ + pKCS1SLL−AAA, PP-InsP5 and (PP)2-InsP4 were virtually eliminated (Table I). We also confirmed that the kcs1Δ + pKCS1L1L2−AAA mutant protein was stable when expressed in these cells (Fig. 6C). In this kcs1Δ + pKCS1L1L2−AAA strain, the vacuolar space remained fragmented (Fig. 3), the growth rate was impaired (Fig. 6), there was a defect in cell wall integrity, and the cells were sensitive to salt stress (Fig. 6). This is the
YPD, and, where indicated, 10 mM caffeine or 1 M sorbitol was added to
the strains indicated were plated and incubated for 3 days at 37
ther. We introduced into
zippers (15), but this idea was not subsequently pursued fur-
Leu-871, and Leu-878 (15). These were proposed to be leucine
808, and Leu-815, and the second comprises Leu-857, Leu-864,
of leucine residues; the first comprises Leu-794, Leu-801, Leu-
The Kcs1p protein contains two groups of four heptad repeats
both cases, the cells showed a wild-type phenotype (data not
assay solution containing 0.05 M glycine-NaOH (pH 9.8), 1% agar, and
no addition, 40
30m M BCIP. Sorbitol (1 M) was present where indicated.
FIG. 6. The effect of the diphosphoinositol polyphosphate syn-
thesis activity of Kcs1p upon cell growth, cell wall integrity, and
resistance to salt stress. A, 10-fold serial dilutions of cells were plated
and incubated at 30 °C for 3 days on M.am. Strain kcs1Δ (4709 kcs1Δ) was
transformed with the following plasmids: pURA3 (pFL38), pKCS1 (pFV241), pKcs1L1L,-AA (pFV198), and pKcs1L1L,-AAA (pFV217). Similar
results were obtained when pFV241, pFV198, and pFV217 were
substituted by pFV249, pFV251, and pFV252, respectively. B, the 4709
kcs1Δ strains transformed with plasmids expressing wild type and
mutated kcs1p proteins listed. Cells were plated onto (from left to right)
M.am plus uracil; M.am plus uracil plus 0.8 M NaCl; or M.am plus uracil
plus 40 µg/ml BCIP. Plates were incubated at 30 °C for 3 days. C,
mutated cells that were similar in
kcs1Δ (vector without insert); lane 2, kcs1Δ plus pKCS1 (pFV249); lane 3, kcs1Δ plus
kcs1Δ + pKcs1L1L,-AA (pFV252); lane 4, kcs1Δ plus pKcs1L1L,-AA (pFV251). The
apparent molecular mass of these kcs1p fusion proteins was approxi-
ately 165 kDa.

FIG. 5. The effect of KCS1 and ARG82 gene deletions upon cell
wall integrity. A, the strains indicated were plated for 2–4 days at
30 °C, and then the plates were overlaid for 50 min with a phosphatase
first demonstration, in yeast, that the synthesis of an inositol
phosphate is necessary for the homeostatic responses to salt
stress and the maintenance of cell wall integrity.
The Importance of the Leucine Heptad Repeats in Kcs1p—
The Kcs1p protein contains two groups of four heptad repeats
of leucine residues; the first comprises Leu-794, Leu-801, Leu-808, and Leu-815, and the second comprises Leu-857, Leu-864, Leu-871, and Leu-878 (15). These were proposed to be leucine
zippers (15), but this idea was not subsequently pursued fur-
ther. We introduced into kcs1Δ cells a plasmid encoding a
mutant kcs1p protein in which two Ala residues were substi-
tuted for either Leu-794 and Leu-801 in the first putative
zipper or Leu-857 and Leu-864 in the second putative zipper. In
both cases, the cells showed a wild-type phenotype (data not
shown). Next, we expressed in the kcs1Δ strain a plasmid
encoding a form of kcs1p in which all four Leu residues were
changed to Ala. The corresponding protein was stable when
expressed in cells (Fig. 6C). This mutation did not affect the
synthesis of diphosphoinositol polyphosphates (Table I). These
cells (kcs1Δ + pKcs1L1L,-AA) were also resistant to salt stress
(Fig. 6B), but cell wall integrity was compromised (Fig. 6B).
The vacuolar space was divided into several smaller compart-
ments, typically 3–5 in number (Fig. 3). This was a less severe
defect in vacuolar morphology than was observed in the
kcs1Δ cells (Fig. 3). Nevertheless, these data emphasize the multi-
functional nature of Kcs1p and validate our goal of identifying
the individual molecular contributions made by different
domains in the protein.

Vacuolar Morphology, Cell Wall Integrity, and Protection
against Salt Stress All Require Adequate Precursor Pools
for Diphosphoinositol Polyphosphate Synthesis—Arg82p has
inositol phosphate kinase that converts Ins(1,4,5)P3 to
Ins(1,3,4,5,6)P5 (Refs. 3 and 20; see Fig. 1). Thus, when the
ARG82 gene is deleted, levels of Ins(1,4,5)P3 increase (Fig. 7),
as previously shown (3, 20). InsP2 levels were also elevated,
prolonging some increased dephosphorylation of
InsP3 (Fig. 7). The arg82Δ cells cannot synthesize appropriate
levels of Ins(1,3,4,5,6)P5, without which InsP6 synthesis is also
compromised (Table II; see also Fig. 1 and Refs. 3, 20, and 28).
Adequate turnover of the diphosphoinositol polyphosphates
requires an abundant supply of their InsP5 and InsP6 precursors
(Fig. 1), so in arg82Δ cells, the synthesis of diphosphoinositol
polyphosphates was greatly reduced (Table II). We discovered
phenotypic consequences for arg82Δ cells that were similar in
to those of kcs1Δ cells. Thus, arg82Δ cells display de-
fective vacuolar morphology (Fig. 8), increased leakiness of
cellular phosphatase, and poor resistance to salt stress (Figs. 4 and 5). Sorbitol had a substantial osmoremedial effect upon phosphatase leakage (Fig. 5), indicating that the main cause was a defect in cell integrity (30, 31). The phenotype was rescued by transforming cells with a plasmid containing the \textit{ARG82} gene (Figs. 8 and 9). It is important to note that \textit{ARG82} has not previously been reported to be involved in any of these phenotypes.

Separating the Roles of Arg82p in Synthesizing Precursors for Diphosphoinositol Polyphosphates from Regulation of Expression of Genes in the Arginine Metabolic Pathway—Arg82p has been studied for many years for its role in the transcrip-

![FIG. 7](image_url)

**FIG. 7.** Inositol phosphate levels in cells expressing wild-type and mutated arg82p. The \textit{arg82Δ} strains were transformed with the plasmids as indicated in each panel, and each strain was labeled with \[^{3}H\]inositol and analyzed by HPLC as described in the legend to Fig. 2. The inset to each panel shows the levels of arg82p present in 20-μg extracts of total cell protein, as determined by Western analysis. The apparent molecular mass of these arg82p proteins was approximately 52 kDa, except the \textit{arg82Δ}–282–303 deletion mutant, which was 47 kDa.

| Strain | Vacuole space | Sensitivity to salt stress | Cell wall | OTCase repression factor | \textit{InsP}_6 | PP-\textit{InsP}_5 | (PP)\textit{InsP}_4 |
|--------|---------------|---------------------------|-----------|-------------------------|--------------|----------------|----------------|
| Wild type | Normal | No | Intact | 6 | 1406 ± 308 | 18.1 ± 0.8 | 22.3 ± 7 |
| \textit{arg82Δ} + p\textit{ARG82} | Fragmented | Yes | Leaky | 1 | 0 | 1.9 ± 0.6 | 0.4 ± 0.2 |
| \textit{arg82Δ} + \textit{parg82Δ282-303} | Normal | No | Intact | 9 | 1271 ± 155 | 17.4 ± 2.7 | 20 ± 1.7 |
| \textit{arg82Δ} + \textit{parg82W65R} | Partly fragmented | No | Intact | 1 | 1048 ± 82 | 13.9 ± 1.3 | 21.2 ± 0.8 |
| \textit{arg82Δ} + \textit{parg82G135A} | Partly fragmented | No | Intact | 7 | 568 ± 71 | 20 ± 2.6 | 21 ± 3.6 |
| \textit{arg82Δ} + \textit{parg82D131A} | Fragmented | Yes | Leaky | 12 | 1019 ± 67 | 12.8 ± 2.4 | 16.2 ± 2.4 |
| \textit{arg82Δ} + \textit{parg82K133A} | Fragmented | Yes | Leaky | 9 | 23.4 ± 1.6 | 17.7 ± 1.3 | 3.3 ± 0.5 |

The vacuolar space was analyzed as described in the legend to Fig. 8. Cell wall integrity and the response to salt stress was assessed as described in the legend to Fig. 9. Some of the data for arginine-dependent repression of OTCase were taken from Ref. 26, and the remainder were determined as described under “Experimental Procedures.” Inositol phosphate levels (means ± S.E. from three experiments) were determined by HPLC as described in the legend to Fig. 7.
We created a third category of yeast strains (arg82Δ + parg82ΔG135A and arg82Δ + parg82ΔK133A) with gross perturbations to their inositol phosphate profiles (Fig. 7), including stress were all normal (Figs. 8 and 9; Table II). These results show that the role of Arg82p in regulating expression of enzymes in the arginine metabolic pathway is separate from the processes that regulate vacuole morphogenesis.

We also created two yeast strains (arg82Δ + parg82ΔG135A, arg82Δ + parg82ΔW69R), which displayed slight metabolic defects, namely elevated levels of InsP₂, InsP₃, and InsP₄ (compare Figs. 7 and 2), and slightly impaired synthesis of InsP₆ (Table II). Both strains contained nearly normal levels of diphosphoinositol polyphosphates (Table II) but exhibited a vacuolar defect (Fig. 8), albeit more minor than that seen in arg82Δ or kcs1Δ cells. The size of the vacuolar space in both arg82Δ + parg82ΔG135A cells and arg82Δ + parg82ΔW69R cells was approximately equal to that of wild-type cells but typically comprised between two and four separate compartments (Fig. 8). Further evidence that this phenotype is relatively mild comes from data showing the cells had a normal response to salt stress (Fig. 9), and there was no enzyme leakage through the cell wall (Fig. 9). This phenotype was observed irrespective of whether OTCase was properly repressed (as in arg82Δ + parg82ΔG135A cells) or not (as in arg82Δ + parg82ΔW69R cells). Again, these data show that the role of Arg82p in regulating OTCase expression is separate from the inositol kinase activity that is necessary for synthesizing precursors for diphosphoinositol polyphosphates.

We created a third category of yeast strains (arg82Δ + parg82ΔD131A and arg82Δ + parg82ΔK133A) with gross perturbations to their inositol phosphate profiles (Fig. 7), including
abnormally low levels of PP-InsP5 and (PP)2-InsP4 (Table II). These two strains showed normal arginine-dependent repression of OTCase (Fig. 2). However, overexpression of KCS1 in the arg82Δ background rescued vacuolar morphology (Fig. 7). Nevertheless, overexpression of KCS1 in the arg82Δ background rescued vacuolar morphology (Fig. 8) and resistance to salt stress (Fig. 9). An important conclusion to derive from these data is that disrupting the inositol phosphate kinase activity of Arg82p yields more extensive phenotypic consequences than have previously been recognized.

Overexpression of Kcs1p Partially Suppresses Some Cellular Defects of the arg82Δ Strain—The arg82Δ cells do not contain the InsP5 and Ins(1,3,4,5,6)P6 substrates for Kcs1p (Fig. 7). However, overexpression of KCS1 in the arg82Δ background rescued vacuolar morphology (Fig. 8) and resistance to salt stress (Fig. 9). Compared with arg82Δ cells, phosphatase leakage was less pronounced in arg82Δ + pTet-KCS1 cells (Fig. 9), indicating that cell wall integrity was improved but not fully restored. The growth rate of the arg82Δ + pTet-KCS1 cells was improved compared with the arg82Δ strain (Fig. 9), but was still less than that of the wild-type cells. The arginine-dependent expression of OTCase and arginase, which is defective in arg82Δ cells (21), was not rescued by Kcs1p expression (data not shown).

We next investigated whether the mechanism by which Kcs1p rescued the arg82Δ phenotype involved phosphorylation of the Ins(1,4,5)P3 and Ins(1,3,4,5)P4, which are present in arg82Δ cells (see Ref. 20 and Fig. 7). We found that recombinant Kcs1p phosphorylated Ins(1,4,5)P3 and Ins(1,3,4,5)P4 in vitro; the first-order rate constants for these reactions were 0.0058 μmol min⁻¹ and 0.0056 μmol min⁻¹, respectively, compared with 0.064 μmol min⁻¹ for [3H]InsP6. In addition, we found that the arg82Δ + pTet-KCS1 cells contained an array of inositol polyphosphates, including substantial levels of (PP)2-InsP4 and PP-InsP5 (Fig. 10). Clearly, higher inositol polyphosphates are synthesized in the arg82Δ cells when Kcs1p is overexpressed; presumably Kcs1p is aided by the actions of the endogenous InsP5 2-kinase and the PP-InsP5 kinase (see Fig. 1).

DISCUSSION

The four most important conclusions to arise from this study are as follows. First, we have shown that PP-InsP5 and (PP)2-InsP4 synthesis by Kcs1p is a specific functional aspect of this protein that is critical for biogenesis of the yeast vacuole. Second, we have shown that diphosphoinositol polyphosphates are also necessary for stability of the cell wall and for adaptive responses to salt stress. Thus, the significance of PP-InsP5 and (PP)2-InsP4 is far more wide ranging than previously appreciated. Indeed, no inositol phosphates have previously been implicated in regulating these physiological processes. Third, we have demonstrated that the leucine zipper motifs within Kcs1p also contribute to vacuolar morphogenesis and cell wall stability. Finally, we showed that cell wall stability, cell growth, adaptation to salt stress, and vacuolar morphogenesis were all impaired in cells expressing kinase-defective arg82p mutants, due to the absence of precursor material for the synthesis of diphosphoinositol polyphosphates.

To ascertain the specific importance of the DINS activity of Kcs1p, we first created a strain of yeast from which the entire KCS1 gene had been deleted. We then rescued this strain by transformation with wild-type kcs1p, or we transformed the cells with a mutant form of kcs1p in which the DINS activity was specifically eliminated. The latter cells (kcs1Δ + pKCS1SSL→AAA) showed defective vacuolar biogenesis, resulting in the formation of small, fragmented vacuoles. The importance of creating a yeast strain with such a specific metabolic defect was borne out by additional experiments in which we discovered the significance of the two leucine heptad repeats in Kcs1p. When both repeats were mutated, the vacuolar space was fragmented, and cell wall integrity was compromised. This result provides the first evidence that these leucine residues are functionally significant, perhaps as leucine zippers, which promote functionally significant homo- and heterodimerization of proteins (17). Indeed, yeast two-hybrid analysis has indicated that Kcs1p may associate with Bmh2 (33), a homologue of the mammalian 14-3-3 family (34). The latter modulate interactions between proteins that regulate signal transduction processes and cell cycle control (34). In any case, our work demonstrates that the contributions of Kcs1p to vacuole morphogenesis involve coordinating the activities of more than one functional domain within this protein.

We also created specific mutant strains of yeast in which the inositol phosphate kinase activity of Arg82p was rendered inactive. These cells displayed impaired vacuolar biogenesis, cell wall integrity was compromised, and there was a defective response to salt stress. Our data indicate that these phenotypes, which have not previously been recognized to arise from arg82p mutations, are largely due to inadequate synthesis of diphosphoinositol polyphosphates, as a result of the elimination of the InsP5 and InsP6 precursor pools. Furthermore, the specific absence of DINS activity in the kcs1Δ + pKCS1SSL→AAA strain elicited a general growth defect (Fig. 6). The same growth phenotype was seen in the arg82Δ strain (Figs. 4 and 5 and Ref. 26), since these cells also cannot synthesize diphosphoinositol polyphosphates (Table II). Our validation of the general growth defect of arg82Δ cells contrasts with an alternate proposal that growth impairment in this strain is nutritionally dependent (3). This is an important point. Recognition that arg82Δ cells have a general growth defect undermines the hypothesis that the inositol phosphate kinase activity of Arg82p directly empowers a transcriptional complex to regulate expression of enzymes in the arginine metabolic pathway (see Ref. 32 for a detailed explanation). When this transcriptional role for the kinase activity of Arg82p was first proposed (3), PP-InsP5 and (PP)2-InsP4 were not considered to be relevant, and InsP6 was stated as being the end point of the inositol phosphate signaling pathway. In contrast, our current work shows that diphosphoinositol polyphosphates are not only required for cell growth and integrity of the cell wall, but these polyphosphates also direct the very survival of the yeast cell in the face of environmental stress.

In yeast, cell wall biosynthesis is controlled by integrating inputs from several regulatory pathways (13), any of which might not function appropriately in kcs1Δ cells. Nevertheless, the osmotic remedial sensitivity of the cell wall to caffeine, as is the case in our kcs1Δ cells (Fig. 4), raises the more specific
possibility that a protein kinase C/mitogen-activated protein kinase signaling pathway is perturbed (31). In fact, the KCS1 gene was first identified in a screen for suppressing the temperature sensitivity of the pck1–4 allele (15). As for the impaired response to salt stress in kcs1Δ cells, one clue to the underlying mechanism comes from an earlier observation that genes encoding the subunits of the vacuolar H⁺-ATPase are dramatically up-regulated following salt stress (35); the electrochemical gradient generated by this ATPase is crucial to sequestration of sodium into the vacuole. It is therefore possible that, in the arg82Δ and kcs1Δ strains, the defective response to salt stress may be molecularly linked to the deformity of the vacuolar compartment.

What might be the link between diphosphoinositol polyphosphates and biogenesis of the yeast vacuole? Vacuoles derive from the fusion of cytoplasm-derived vesicles and from clathrin-coated and non-clathrin-coated vesicles derived from the endocytic apparatus and the trans-Golgi network (36, 37). It has been known for several years that diphosphoinositol polyphosphates bind with high affinity to those clathrin-dependent and non-clathrin-dependent adaptor proteins, such as AP-180 and coatomer, that regulate vesicle traffic in both yeasts and mammalian cells (19, 38, 39). Furthermore, recent studies indicate that a mammalian diphosphoinositol polyphosphate synthase associates with proteins that regulate vesicle exocytosis (14). To rationalize these effects, it has been suggested that PP-InsP₃ and (PP)₂-InsP₄ might be phosphate donors for protein phosphorylation (see Ref. 16). Alternately, when the diphosphoinositol polyphosphates bind to target proteins, the phosphotransferase reaction (when the diphosphate ligand is either phosphorylated or dephosphorylated) may act as a molecular switch (2, 40, 41). This idea may be viewed as being analogous to G-proteins that function as a binary switch (275, 286–325).

REFERENCES

1. Qiu, H. F., Dubois, E., Broen, P., and Messenguy, F. (1990) Mol. Genet. Genomics 222, 192–200
2. Saiardi, A., Erdjument-Bromage, H., Snowman, A., Tempest, P., and Snyder, S. H. (1999) Curr. Biol. 9, 1323–1326
3. Odom, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000) Science 287, 2026–2029
4. Foti, A., Audhya, A., and Emr, S. D. (2001) Mol. Biol. Cell 12, 2396–2411
5. Stole, L. E., Huynh, C. V., Thornor, J., and York, J. D. (1998) Genetics 148, 1715–1729
6. Gary, J. D., Wurmsner, A. E., Bonangeli, C. J., Weissman, L. S., and Emr, S. D. (1998) J. Cell Biol. 143, 65–79
7. Audhya, A., Foti, A., and Emr, S. D. (2000) Mol. Biol. Cell 11, 2873–2889
8. Murguia, J. R., Belles, J. M., and Serrano, R. (1996) J. Biol. Chem. 271, 29029–29033
9. Mons, M. C., Real, E., Wojda, I., Behabebel, J.-P., Magier, W. H., and Sidierius, M. (2001) Mol. Microbiol. 41, 717–730
10. Nass, R., and Rao, R. (2001) Microbiology 145, 3221–3228
11. Darley, C. P., van Wysewinkel, O. C. M., van der Woude, K., Magier, W. H., and de Boer, A. H. (2000) Biochem. J. 351, 241–249
12. Saiardi, A., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (2000) J. Biol. Chem. 275, 24686–24692
13. Smits, G. J., van den Ende, H., and Klio, F. M. (2001) Microbiology 147, 781–794
14. Luo, H. R., Saiardi, A., Nagata, E., Ye, K., Yu, H., Jung, T. S., Luo, X., Jain, S., Sawa, A., and Snyder, S. H. (2001) Neuron 31, 439–451
15. Huang, K. N., and Symington, L. S. (1995) Genetics 141, 1275–1285
16. Saiardi, A., Nagata, E., Luo, H. R., Snowman, A., and Snyder, S. H. (2001) J. Biol. Chem. 276, 39179–39185
17. Landschulz, W. H., Johnson, P., and McKnight, S. L. (1988) Science 240, 1759–1764
18. Metnitz, F. S., Miller, R. N., Putney, J. W., Jr., and Shears, S. B. (1993) J. Biol. Chem. 268, 3850–3856
19. Ali, N., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (1995) Biochemistry 310, 279–284
20. Saiardi, A., Caffrey, J. J., Snyder, S. H., and Shears, S. B. (2000) FEBS Lett. 488, 28–32
21. Dubois, E., and Messenguy, F. (1994) Mol. Gen. Genet. 243, 315–324
22. Aram, N., Messenguy, F., El Bakkoury, M., and Dubois, E. (2000) Mol. Cell. Biol. 20, 2087–2097
23. El Bakkoury, M., Dubois, E., and Messenguy, F. (2000) Mol. Microbiol. 35, 15–31
24. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Yeast 14, 115–132
25. Wach, A. (1996) Yeast 12, 255–265
26. Dubois, E., Dewste, V., Vences, C., and Messenguy, F. (2000) FEBS Lett. 486, 300–304
27. Messenguy, F., Pinneckx, M., and Wisme, J. M. (1971) Eur. J. Biochem. 22, 277–286
28. York, J. D., Odom, A. R., Murphy, R., Ives, E. B., and Wente, S. R. (1999) Science 285, 96–100
29. Caffrey, J. J., Scanes, Y. T., Yang, X., and Shears, S. B. (2000) J. Biol. Chem. 275, 12730–12736
30. Paravicini, G., Cooper, M., Friedl, L., Smith, D. J., Carpentier, J.-L., Kligr, L. L., and Payton, M. A. (1992) Mol. Cell. Biol. 12, 4986–4995
31. Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C., and Molina, M. (2000) J. Biol. Chem. 275, 1511–1519
32. Shears, S. B. (2000) Bioessays 22, 786–789
33. Uetz, P., Glott, L., Cagney, G., Mansfield, T. A., Jueden, R. S., Knight, J. R., Lockshon, D., Narayan, V., Strivivasan, M., Pochart, P., Qureshi-Emill, A., Li, Y., Godwin, B., Conover, D., Kahlifisch, T., Vajidadamadar, G., Yang, M., Johnston, M., Fields, S., and Rothberg, J. M. (2000) Nature 403, 623–627
34. Baldin, V. (2000) Prog. Cell Cycle Res. 4, 49–60
35. Posas, F., Chambers, J. R., Heyman, J. A., Hoffert, J. P., de Nadal, E., and Artino, J. (2000) J. Biol. Chem. 275, 17249–17255
36. Bryant, N. J., and Stevens, T. H. (1998) Microbiol. Mol. Biol. Rev. 62, 230–247
37. Shaw, J. D., Cummings, K. H., Huyer, G., Michaelis, S., and Wendland, B. (2001) Exp. Cell Res. 271, 1–9
38. Ye, W., Ali, N., Bembeneck, M. E., Shears, S. B., and Lafer, E. M. (1995) J. Biol. Chem. 270, 1564–1568
39. Fleischer, B., Xie, J., Mayrleitner, M., Shears, S. B., Palmer, D. J., and Fleischer, S. (1994) J. Biol. Chem. 269, 17826–17832
40. Voglmaier, S. M., Bembenek, M. E., Kaplin, A. I., Dormán, G., Olszewski, J. D., Prestwich, G. D., and Snyder, S. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4305–4310
41. Shears, S. B. (2001) Cell. Signal. 13, 151–158
42. Flick, J. S., and Thorner, J. (1993) Mol. Cell. Biol. 13, 5861–5876
43. Safrany, S. T., Ingram, S. W., Cartwright, J. L., Falck, J. R., McLennan, A. G., Barnes, L., and Shears, S. B. (1999) J. Biol. Chem. 274, 21735–21740