Pil1p and Lsp1p Negatively Regulate the 3-Phosphoinositide-dependent Protein Kinase-like Kinase Phk1p and Downstream Signaling Pathways Pkc1p and Ypk1p*

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The Saccharomyces cerevisiae homologs, Phk1/2p, of the mammalian 3-phosphoinositide-dependent protein kinase 1 (PDK1) regulate the Pck1-MAP kinase cascade and the partially parallel Ypk1/2p pathway(s) that control growth and cell integrity. Mammalian PDK1 is regulated by 3-phosphoinositides, whereas Phk1/2p are regulated by sphingolipid long-chain bases (LCBs). Recently Phk1/2p were found to complex with two related proteins, Pil1p (Ygr086) and Lsp1p (Ypl004). Because these two proteins are not related to any known protein we sought to characterize their functions. We show that Phk1p phosphorylates both proteins in vitro in a reaction that is only weakly regulated by LCBs. In contrast, LCs inhibit phosphorylation of Pil1p by Phk2p, whereas LCs stimulate phosphorylation of Lsp1p by Phk2p. We find that Pil1p and Lsp1p down-regulate resistance to heat stress and, specifically, that they down-regulate the activity of the Pck1-MAP and Ypk1p pathways during heat stress. Pil1p and Lsp1p are thus the first proteins identified as regulators of Phk1/2p. An unexpected finding was that the level of Ypk1p is greatly reduced in pck1Δ cells, indicating that Pck1p controls the level of Ypk1p. Homologs of Pil1p and Lsp1p are widespread in nature, and our results suggest that they may be negative regulators of PDK-like protein kinases and their downstream cellular pathways that control cell growth and survival.

Factors that regulate cell growth and survival often activate the 3-phosphoinositide-dependent protein kinase, termed PDK11 (1). PDK1 then activates specific members of the AGC protein kinase family that regulate diverse cellular functions necessary for growth and survival (reviewed in Refs. 2–4). PDK1 homologs are found in most organisms, including two, Phk1p and Phk2p, in Saccharomyces cerevisiae where they have partially overlapping functions and at least one of the proteins is necessary for growth (5). One target of Phk1p and Phk2p is the Pck1-MAP kinase pathway that regulates cell wall maintenance and integrity (6). In a large-scale analysis of protein complexes, Ho et al. (7) found the predicted proteins Ygr086c and Ypl004 in a complex with either Phk1p or Phk2p. Here we present the results of experiments that examine the biochemical and functional roles of these two uncharacterized proteins in pathways regulated by Phk1p and Phk2p. Based upon our results, we assigned the name Pil1p (phosphorylation is inhibited by long chain bases) to Ygr086p and the name Lsp1p (long chain bases stimulate phosphorylation) to Ypl004p.

Phk1p and Phk2p show 52% amino acid identity and 69% similarity and nearly the same level of identity and similarity to human PDK1, with the similarities confined mostly to the amino-terminal protein kinase domain of the proteins. The yeast and mammalian proteins also are functionally similar, because expression of human PDK1 restores growth to inviable phk1Δphk2Δ mutant cells (5). One known target of Phk1p and Phk2p is Pkc1p, which controls an MAP kinase pathway necessary for regulating the integrity of the cell wall (8, 9) and for repolarization of the actin cytoskeleton during heat stress (10). Phk2p was shown to phosphorylate Pkc1p in vitro and Pkc1p isolated from a phk1Δphk2Δ mutant strain had reduced kinase activity, indicating that the Phk proteins function upstream of Pkc1p (6). Additional studies showed that the Phk proteins were necessary for preventing cell lysis due to defects in the cell wall and for repolarization of the actin cytoskeleton following a heat shock (6). Thus, the Phk proteins are one of two pathways for regulating the Pck1-MAP kinase cascade during heat stress. The other pathway includes membrane proteins of the WSC family that sense stress in the plasma membrane and activate the guanine nucleotide exchange factor Rom2p, which regulates the GTPase Rhop1p. Active Rhop1p then turns on Phk1p-Phk2p-Phk3p-Phk4p MAP kinase cascade (reviewed in Ref. 11).

Another downstream target of Phk1p and Phk2p is the protein kinase Ypk1p, a member of the AGC kinase family, which has been shown both in vitro and in vivo to be phosphorylated by Phk1p (5). Ypk1p and its closely related paralog, Ypk2p (Ykr2p), perform redundant functions necessary for growth, because a ypk1 ypk2 double mutant is not viable. The sphingolipid long chain bases (LCBs), dihydro sphingosine (DHS), and phytosphingosine (PHS) are the only regulators of Phk1p identified to date (12). LCBs are particularly important regulators during heat stress, because their concentration transiently increases (13, 14). Ypk1p and probably Ypk2p are downstream targets of this PHS-regulated signal transduction pathway that is necessary for growth of vegetative cells. Other studies have shown that the Phk proteins and LCBs are required for endocytosis. A temperature-sensitive strain (lcb1–100) blocked in endocytosis 37 °C turned out to be defective in lcb1 (15), which encodes a subunit of serine palmito...
find that both Pkh1p and Pkh2p phosphorylate Pil1p and Lsp1p show no sequence similarity to known pro-

to the actin cytoskeleton, nor were they able to phosphorylate

to otherwise (19).

Roles for Ypk1p and Ypk2p in the cell wall integrity pathway

TABLE I

| Strain                          | Genotype                        | Reference |
|---------------------------------|---------------------------------|-----------|
| 15Dau                           | MATα ura3Δ leu2Δ3 trp1Δ his3Δ ade1Δ | (6)       |
| IN106-3B                        | MATα ura3Δ leu2Δ3 trp1Δ his3Δ ade1Δ pkh1Δ1000x pkh2Δ1000x | (6)       |
| RCD520                          | MATα ura3Δ leu2Δ3 trp1Δ his3Δ ade1Δ pkh1Δ1000x pkh2Δ1000x | This study |
| IN106-3B and RCD368             |                                 | This study |
| RCD521                          | MATα ura3Δ leu2Δ3 trp1Δ his3Δ ade1Δ pkh1Δ1000x pkh2Δ1000x pil1Δ:::KAN, made from cross of IN106-3B and RCD364 | This study |
| RCD522                          | MATα ura3Δ leu2Δ3 trp1Δ his3Δ ade1Δ pkh1Δ1000x pkh2Δ1000x pil1Δ:::KAN, made from cross of IN106-3B and RCD364 | This study |
| BY4741                          | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | (37)      |
| BY4742                          | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | (37)      |
| RCD364                          | MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 pil1Δ::KAN | This study |
| RCD368                          | MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ2 pil1Δ::KAN | This study |
| RCD369A                         | MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ pil1Δ::KAN | This study |
| RCD449                          | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SLT2::HA::HIS3, made from BY4742 | This study |
| RCD450                          | MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ pil1Δ::KAN SLT2::HA::HIS3, made from RCD364 | This study |
| RCD451                          | MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ pil1Δ::KAN SLT2::HA::HIS3, made from RCD364 | This study |
| DL500                           | MATα leu2Δ0 trp1Δ ura3Δ0 HIS3Δ1::HA  | D. E. Levin |
| DL523                           | MATα ura3Δ0 trp1Δ his4Δ1 can1Δ::pck1::LEU2  | D. E. Levin |
| RCD504                          | MATα ura3Δ0 trp1Δ his4Δ1 can1Δ::pck1::LEU2 pil1Δ::KAN, made from cross of DL523 and RCD364 | This study |
| RCD505                          | MATα ura3Δ0 trp1Δ his4Δ1 can1Δ::pck1::LEU2 pil1Δ::KAN, made from cross of DL523 and RCD368 | This study |
| RCD506                          | MATα ura3Δ0 trp1Δ his4Δ1 can1Δ::pck1::LEU2 pil1Δ::KAN, made from cross of DL523 and RCD368 | This study |

Experimental Procedures

Yeast cells, transformed with a multicopy vector expressing the desired gene under control of the GAL1 promoter (Table II), were grown overnight at 30°C to an A600 nm of 0.4 in S-Ura medium containing 2% sucrose and 0.1% glucose. Protein overproduction was induced by adding galactosel to 2.5% and incubating the culture for 6 h. A cell-free protein extract was prepared as described previously (6), and 10 mg was mixed with an equal volume of wash buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM imidazole). The mixture was incubated for 1 h with shaking at 4°C with 500 μl of nickel-nitrotriacetic acid-agarose (Qiagen) and then loaded into a column. The flowthrough was passed through the column again, and then 5 ml of wash buffer was passed through the column prior to elution with 2.5 ml of elution buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 50 mM imidazole). His-tagged proteins were also purified by immunoprecipitation from cell-free yeast extracts as described previously (19).

Immunoblotting—Protein samples were subjected to 10% SDS-PAGE followed by electroblotting onto an Immobilon-P membrane (Millipore). Membranes were blocked by incubation for 1 h at room temperature in 1% blocking solution (1% casein in Tris-buffered saline (TBS), Roche Applied Science). Membranes were then shaken with anti-His

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Table II
Plasmids used in this study

| Plasmids | Description |
|----------|-------------|
| pGAL-LSP1 | A 1.0-kb KpnI-Hhol fragment of DNA containing the coding region of LSP1 cloned into pYES2/NTA (2μm, URA3), LSP1 controlled by the GAL1 promoter |
| pGAL-PIL1 | A 1.0-kb KpnI-Xhol fragment of DNA containing the coding region of PIL1 cloned into pYES2/NTA (2μm, URA3), PIL1 controlled by the GAL1 promoter |
| pGAL-PKH1 | A 2.5-kb EcoRI-Xhol fragment containing the coding region of PKH1 cloned into pYES2/NTA (2μm, URA3), PKH1 controlled by the GAL1 promoter |
| pGAL-PKH1 (KD) | A 2.5-kb EcoRI-Xhol fragment containing the coding region of PKH1 (KD) cloned into pYES2/NTA (2μm, URA3), PKH1 (KD) controlled by the GAL1 promoter |
| pGAL-PKH2 | A 3.2-kb BamHI-Xhol fragment containing the coding region of PKH2 cloned into pYES2/NTA (2μm, URA3), PKH2 controlled by the GAL1 promoter |
| pGAL-PKH2 (KD) | A 3.2-kb BamHI-Xhol fragment containing the coding region of PKH2 (KD) cloned into pYES2/NTA (2μm, URA3), PKH1 (KD) controlled by the GAL1 promoter |
| pPIL1 | 2.0-kb BamHI-Sall DNA fragment extending from 500 bp upstream to 440 bp downstream of PIL1 and inserted in YEps32 (2μm, URA3) |
| pLSP1 | 2.1-kb BamHI-Sall DNA fragment extending from 540 bp upstream to 480 bp downstream of LSP1 and inserted in YEps32 (2μm, URA3) |

Antibody (1:3000 dilution, Amersham Biosciences, catalogue no. 27-4710-0) overnight at 4 °C. After two washes with TBS-T (0.1% Tween) and two washes with 0.5% blocking solution, membranes were incubated for 2 h with alkaline phosphatase-linked anti-mouse antibody (1:5000, Sigma). Membranes were washed three times with TBS-T, exposed to ECF substrate (Amersham Biosciences), and fluorescent signals were analyzed by using a PhosphorImager (Storm 860, Amersham Biosciences).

Ypk1 was immunoblotted (12) using anti-Ypk1 protein antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Total protein extracts were prepared by growing cells to an A600nm of 0.3 at 30 °C, adding myriocin as indicated in the text, switching cells to 34 °C and harvesting after 6 h. Cells were centrifuged and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin and pepstatin, 50 mM NaF, 1 mM Na2VO4, 1.5 mM MgCl2, 150 mM NaCl). One-half volume of acid-washed glass beads were added followed by vortexing for 3 min at 4 °C. Unbroken cells and debris were removed by centrifugation at 4000 × g for 10 min. A 150-μg sample of the supernatant fluid was separated on a SDS-PAGE.

SLt2p Activation Assay—Quantitation of phosphorylated SLt2p was done by immunoblotting of an SDS-PAGE loaded with 150 μg of protein. Membranes were probed with rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (New England Biolabs, 1:2000 dilution in 0.5% blocking solution). Total HA-tagged SLt2p was detected by SDS-PAGE (60 μg of protein) and immunoblotting with rat anti-HA antibody (Sigma, 1:3000 dilution). Blots were incubated for 2 h with alkaline phosphatase-linked anti-rat (Sigma) or anti-rabbit (Chemicon) antibody (diluted 1:3000 in 0.5% blocking solution).

Stress Resistance—Heat stress resistance of log phase cells (A600nm of 0.3) was performed as described previously (28) as was the resistance of stationary phase cells to heat stress (27). Other stress-resistance experiments were performed as described by Huang et al. (28).

Alkaline Phosphatase Treatment of Ypk1p—Immunoprecipitates were prepared by incubating 5 μl of anti-Ypk1 protein antibodies with 20 μl of a 50:50 slurry of protein G-Sepharose and 300 μg of cell-free extract for 1–2 h at 4 °C. The beads were processed as described previously (12) and subjected to SDS-PAGE.

Ypk1 Kinase Assay—Cells grown as described for Ypk1p immunoblotting in medium containing 1 μM myriocin, were centrifuged and rinsed once with ice-cold IP buffer (20 mM Tris-HCl, pH 7.5, 125 mM potassium acetate, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM diethiothreitol, 1 μg/ml leupeptin, and pepstatin, 0.1% Triton X-100, and 12.5% glycerol. Cells were broken with glass beads as described for Ypk1 immunoblotting. The lysate was clarified by centrifugation at 14,000 × g at 4 °C for 15 min and Ypk1p was immunoprecipitated from the supernatant fluid and used in a kinase assay with Cross-tide (GRPRTSSFAEG, BIOSOURCE Int.) as substrate as described previously (23). Kinase reactions were terminated by spotting a 45-μl portion of the reaction mixture onto a small square of phosphocellulose paper, which was washed and counted in a liquid scintillation spectrometer (29).

Miscellaneous Assays—Protein concentrations were measured by using the Bio-Rad DC kit with bovine serum albumin as a standard. Pkh1p kinase activity was assayed in vitro as described previously (19). Phosphorylated Pkl1p and Lsp1p was identified and quantified by using a PhosphorImager.

RESULTS
Pkl1p Is Phosphorylated by Pkh1p and Pkh2p—To begin to understand why Pkl1p complexes with Pkh1p and Pkh2p we determined if it was a kinase substrate. The three proteins were tagged at their amino terminus with six histidines and individually overproduced in yeast by using the galactose-inducible GAL1 promoter. Proteins were partially purified by nickel affinity chromatography and used in equal amounts for in vitro phosphorylation reactions. Phosphorylated proteins were examined by SDS-PAGE and quantified by phosphorimaging analysis. In preliminary experiments we determined that phosphorylation of Pkl1p by Pkh1p was linear up to 60 min (data not shown). Therefore, we used 30-min incubations for all experiments shown here.

In the absence of any LCB, Pkh1p and Pkh2p phosphorylated Pkl1p (Fig. 1A, lanes 3 and 7, respectively). Contrary to expectation, phosphorylation of Pkl1p by either protein kinase was not stimulated by LCBs but was actually reduced by inclusion of 50 μM LCBs in the reaction (Fig. 1A, compare lane 3 with lane 4 and lane 7 with lane 8, and see below). Specifically, Pkl1p phosphorylation by Pkh1p was noticeably inhibited by the biological isomer erythro-DHS and to a lesser extent by sphingosine, the major LCB found in mammals (Fig. 1A, lane 4). Pkl1p phosphorylation by Pkh2p was strongly inhibited by PHS and sphingosine and inhibited to a lesser extent by erythro-DHS (Fig. 1A, lane 8). The related but chemically different lipids C18-DHS-phosphate and C16-ceramide were used as controls for lipid specificity and neither inhibited phosphorylation. Stearylamine, a long-chain amine also did not inhibit phosphorylation.

To demonstrate that Pkh1p and Pkh2p were responsible for phosphorylation rather than some contaminating protein kinase, we used variant Pkh1p (K154R) and Pkh2p (K208R), which lack kinase activity (KD mutants). Phosphorylation of Pkl1p by either KD variant was either eliminated or greatly reduced in the presence or absence of LCB (Fig. 1A, lanes 5, 6, 9, and 10). We conclude from these data that Pkl1p can be phosphorylated by Pkh1p and Pkh2p.

Phosphorylation of Pkl1p by Pkh1p and Pkh2p Is Reduced by LCBs—We examined LCB inhibition of Pkl1p phosphorylation in more detail by varying the concentration and type of LCB. Phosphorylation of Pkl1p by Pkh1p began to be inhibited by 50 μM PHS and was about 70% inhibited at the highest concentration of PHS (Fig. 1B). This concentration of PHS is about what we estimate the concentration of LCBs to be in cells grown at 25 °C (13). Erythro-DHS started to inhibit at 20 μM, and sphingosine started to inhibit at 50 μM, whereas the non-
biological isomer threo-DHS only inhibited at the highest concentration, which is most likely due to a 5% contamination with erythro-DHS as determined by HPLC (data not shown). The long-chain amine stearylamine only showed inhibition at the highest concentration used, whereas C18-DHS-phosphate and C6-ceramide showed no inhibition. Similar trends were seen for inhibition of Pil1p phosphorylation by Pkh1p (Fig. 1C) except that (i) the concentration of LCB needed to inhibit were slightly different, (ii) a greater level of inhibition, more that 90%, was obtained with several LCBs, and (iii) stearylamine inhibited better than in the reactions containing Pkh1p (Fig. 1B). We conclude from these data that LCBs inhibit phosphorylation of Pil1p by both Pkh1p and Pkh2p, with Pkh2p being more strongly inhibited.

**Fig. 1.** Phosphorylation of Pil1p by Pkh1p and Pkh2p. Partially purified wild type and kinase dead (KD) His6-Pkh1p and His6-Pkh2p were used in protein kinase assays containing partially purified His6-Pil1p. A, reactions were done in the presence or absence of the indicated sphingolipids (50 μM). The 32P-labeled Pil1p was analyzed by SDS-PAGE and quantified by using a PhosphorImager. Results are from one of three separate experiments. Pluses (+) indicate the present and minuses (−) indicate the absence of the agent in the kinase reaction. The ability of different lipids to stimulate phosphorylation of Pil1p by Pkh1p (B) or Pkh2p (C) was determined. Numbers below each lane indicate the level of phosphorylated Pil1p relative to the lane with no lipid. Results are representative of one experiment performed three times.

Lsp1p Is Phosphorylated by Pkh1p and Pkh2p—Lsp1p, like Pil1p, was phosphorylated by Pkh1p in the absence of LCBs, and LCBs slightly stimulated phosphorylation (Fig. 2A, lanes 3 and 4). In contrast to Pkh1p, Pkh2p only weakly phosphorylated Lsp1p in the absence of LCBs but phosphorylation was strongly stimulated by 50 μM LCBs (Fig. 2A, compare lanes 7 and 8, and see below). For example, Lsp1p phosphorylation by Pkh2 was stimulated by PHS, the biological isomer erythro-DHS and by sphingosine (Fig. 2A, compare lanes 7 and 8). The non-biological isomer threo-DHS only stimulated phosphorylation slightly relative to the other LCBs. Stearylamine stimulated phosphorylation, but the other control lipids C18-DHS-phosphate and C6-ceramide did not, indicating that LCBs and
long-chain amines stimulate while related but chemically different sphingolipids do not. Kinase dead variants of Pkh1p (K154R) and Pkh2p (K208R) did not phosphorylate Lsp1p (Fig. 2A, lanes 5, 6, 9, and 10). We conclude from these experiments that LCBS are not required for Pkh1p to phosphorylate Lsp1p, although they do stimulate phosphorylation. On the other hand, LCBS are required for Pkh2p to phosphorylate Lsp1p.

Phosphorylation of Lsp1p by Pkh1p and Pkh2p was examined in more detail by varying the concentration LCBS. Phosphorylation of Lsp1p by Pkh1p began to be stimulated by 20 μM PHS and peak stimulation of 2.6-fold occurred at 50 μM (Fig. 2B). A similar trend was seen with erythro-DHS while sphingosine only stimulated at 50 μM or higher. The non-biological isomer threo-DHS showed weak stimulation at the highest concentrations used, and this stimulation is most likely due to a 5% contamination with erythro-DHS. Stimulation by stearylamine was similar to that seen erythro-DHS. Neither of the other control lipids C18-DHS-phosphate and C6-ceramide showed stimulation.

Maximal stimulation of phosphorylation by Pkh2 occurred at 20–50 μM PHS, erythro-DHS, and sphingosine, and the fold stimulation ranged between 5 and 10 (Fig. 2B). Stearylamine also stimulated phosphorylation, but only at higher concentrations, and neither C18-DHS-phosphate nor C6-ceramide stimulated phosphorylation.

Deletion of PIL1 or LSP1 Enhances Heat Stress Resistance—Because the Pkh1/2 kinases have been shown to regulate the Pkc1p-MAP kinase pathway, and because this pathway controls cellular processes necessary for surviving heat stress, we determined if deletion of PIL1 or LSP1 or both genes influenced heat stress resistance in log and stationary phase cells. Log phase pil1Δ and lsp1Δ cells were 2-fold more resistant to heat stress than wild type cells (Fig. 3). Stationary phase pil1Δ cells were 3-fold more resistant than wild type cells to heat stress, and lsp1Δ cells were nearly 4-fold more resistant (Fig. 3). The double mutant cells were slightly less resistant than the single mutants, suggesting that the two proteins regulate the same or similar cellular processes that mediate heat stress. We conclude that Pil1p and Lsp1p act to down-regulate heat stress resistance.

If Pil1p and Lsp1p act to down-regulate heat stress resistance, then overproduction of either protein should produce the opposite effect and reduce heat stress resistance. Stationary phase cells with PIL1 on a multicopy vector were 7-fold less resistant to heat stress compared with cells transformed with the empty vector (Fig. 3), which is consistent with our hypothesis. On the other hand, multicopies of LSP1 slightly increased heat stress resistance, indicating that Lsp1p has a more complex mechanistic role in heat stress resistance than does Pil1p.

Pil1p and Lsp1p Down-regulate the Pck1p-MAP Kinase Pathway—To examine in more detail the hypothesis that Pil1p and Lsp1p down-regulate the Pck1p-MAP kinase pathway we measured phosphorylation of Slt2p, the terminal or MAP kinase in this pathway. Activation of Slt2p can be monitored with a polyclonal antibody that only recognizes Slt2p activated by dual phosphorylation of a threonine and a tyrosine residue (30, 31). We found a 3-fold increase in the basal level of Slt2p phosphorylation in log phase pil1Δ cells grown at 25 °C compared with wild type cells (Fig. 4A). This difference persisted following heat shock by transfer of cells from 25 °C to 39 °C. We conclude that Pil1p acts to down-regulate the basal and heat-induced activity of the Pck1-MAP kinase pathway.

The basal level of phospho-Slt2 in lsp1Δ cells grown at 25 °C was the same as in the wild type cells. But when cells were heat-shocked by transfer from 25 °C to 39 °C, the level of phospho-Slt2 was 2- to 3-fold higher in the mutant than it was in wild type cells (Fig. 4B). These data support the hypothesis that Lsp1p is a negative regulator of the Pck1-MAP kinase pathway during heat stress but that it has little if any control over basal level activity of the pathway.

Pil1p and Lsp1p Work Together to Negatively Regulate Pkh1p Activity in a Pathway Controlled by Long-chain Bases—We hypothesize that Pil1p and Lsp1p regulate Pkh1p kinase activity, because both proteins form a complex with Pkh1p (7), they both are phosphorylated by Pkh1p in vitro in an LCB-sensitive reaction (Figs. 1 and 2). To examine this hypothesis we first deleted either pil1 or lsp1 or both genes in an otherwise wild type strain background and examined phenotypes that are known to be associated with loss of or reduced Pkh1p activity. Only very small (2-fold or less) changes were observed (data not shown) and we reasoned that this was due to redundancy in some functions of Pkh1p and Pkh2p, with one of the two kinases being less sensitive to loss of Pil1p or Lsp1p.

To circumvent the redundancy issue, we used a strain having phkh2 deleted and the wild type PKh1 allele replaced with a temperature-sensitive allele (strain INA106-3B, phkh1D398Gphkh2Δ) so that the activity of Pkh1p could be varied by changing the temperature of the incubation medium. Variants of strain INA106-3B were made with pil1, lsp1, or both genes deleted. At the permissive temperature of 30 °C the lsp1Δ cells (phkh1D398Gphkh2Δlsp1Δ) grew the slowest of all strains, whereas the double mutant pil1Δlsp1Δ cells (phkh1D398Gphkh2Δpil1Δlsp1Δ) grew as well as the wild type (Fig. 5, left panel, compare the top and bottom rows), showing that Pil1p and Lsp1p are negatively regulating Pkh1p activity.

We next examined growth at the partially restrictive tem-
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At a partially restrictive temperature (34°C) strain INA106-3B (pkh1ts pkh2ts) will contain a level of Pkh1p that is limiting for growth, and the strain should be very sensitive to myriocin because a drop in LCBS will further reduce the activity of Pkh1p and further impede growth. In contrast, in the wild type strain 15Dau the basal (not dependent upon LCBS for activity) level of Pkh1p and Pkh2p activity should remain high enough in the presence of a low dose of myriocin to promote normal growth. These predictions were verified as shown in Fig. 5. At 0.2 μM myriocin wild type 15Dau cells grew as well as they did in the absence of drug, whereas growth of INA106-3B cells was nearly completely inhibited by myriocin (Fig. 5, compare the center and right panels).

Having established conditions where growth of INA106-3B cell was dependent upon LCBS, we determined if deletion of PIL1 or LSP1 or both genes promoted growth, as predicted if they negatively regulate Pkh1p and act downstream of a pathway controlled by LCBS. The pil1Δ and lsp1Δ cells failed to grow just like the parental INA106-3B cells, but the double pil1Δlsp1Δ mutant cells grew, although not as well as the wild type 15Dau cells (Fig. 5, right panel, compare the top and bottom rows). We conclude from the results with the double deletion mutant that Pil1p and Lsp1p work together to negatively regulated Pkh1p activity in a pathway that requires LCBS for activity.

Pil1p and Lsp1p Negatively Regulate the Ypk1p Cell Integrity Pathway—Because Pil1p and Lsp1p negatively regulate the Pkh1-MAP kinase cascade, we wanted to determine if they also regulate the parallel Ypk1p2p pathway, given that Pkh1p2p are also upstream regulators of this pathway (5, 23). For these experiments the Pck1-MAP kinase pathway was inactivated by deleting pck1 so that growth would be dependent upon the Ypk1p2p pathway. Growth of pck1Δ cells with pil1, lsp1, or both genes deleted was examined. All of the mutant strains grew as well as wild type cells at 30°C, but at 34°C their growth was slightly reduced relative to wild type (Fig. 6). We reasoned that deletion of pil1 or lsp1 produced no change in growth of the pck1Δ cells, because the level of LCBS was sufficient to activate the Pkh1p2p-Ypk1p2p pathway and compensate for loss of Pck1p. To test this idea a low concentration of myriocin was used to try and reduce the intracellular concentration LCBS so that growth of pck1Δ cells would be reduced without affecting wild type cells. These desired effects were seen with 0.5 μM myriocin in the agar medium (Fig. 6). Deleting pil1, lsp1, or both genes in the pck1Δ cells produced a striking enhancement of growth. In the presence of 1 μM myriocin only the pck1Δpil1Δ or the pck1Δpil1Δlsp1Δ mutant cells grew, and they grew even better than wild type cells. We conclude that Pil1p has a more pronounced regulatory role than Lsp1p on the activity of a pathway that is necessary for growth. Evidence that the pathway involves Ypk1p is presented below.

To establish that the results shown in Fig. 6 were not an artifact of growing cells on agar medium, we performed the growth tests in liquid medium. Cells were grown to early log phase at 30°C and then switched at time zero to 34°C. Following the switch, pck1Δ cells divide about two times before growth stops (Fig. 7A). The same growth pattern was seen for pck1Δpil1Δ, pck1Δlsp1Δ, and pck1Δpil1Δlsp1Δ mutant cells. Thus, these results differ from those conducted with agar medium where all strains grew at 30°C (Fig. 5).

Addition of 0.5 μM myriocin to liquid medium produced two changes. First, all the mutant cells went through three or four cell divisions. Second, pck1Δlsp1Δ cells grew to a slightly higher density than parental pck1Δ cells, whereas pck1Δpil1Δ and pck1Δpil1Δlsp1Δ mutant cells grew to even higher densities, with the pck1Δpil1Δ cells nearly reaching the density of wild type cells (Fig. 7B). These results are similar but not identical to those observed with agar medium.

**Fig. 4. Pil1p and Lsp1p down-regulate the Pkc1p-MAP kinase pathway.** The phosphorylation level of Slt2p in wild type (BY4742) and pil1Δ cells (RCD364) (A) or wild type and lsp1Δ cells (RCD368) (B) before and at 30, 60, 90, and 120 min after heat shock at 39°C was measured by immunoblotting with an antibody specific for phospho-Slt2p. Another antibody that recognizes any form of Slt2p was used to measure total Slt2p. Phosphorylation was quantitated by Phosphor-Imager analysis, and the values were plotted as a percentage of the wild type value at the 0-min time point. Values represent the average ± S.D. of two separate experiments. The two strains in A behaved differently over the course of the experiment (p = 0.027) as determined by using a generalized F-test (MIXED, SAS software package) with the degrees of freedom (1 or 2) calculated by using Satterthwaite’s approximation. The two strains in B behaved differently at the 30-, 60-, and 90-min time points (p < 0.05) as determined by using the Student’s t test.
Pil1p and Lsp1p negatively regulate the LCB-dependent Ypk1p pathway. Wild type strain 15Dau, mutant strain INA106-3B (pkh1Δpkh2Δ), and derivatives of it were grown to saturation in YPD medium at 30 °C. Then 5-fold serial dilutions were prepared and spotted onto YPD plates, which were incubated for 3 days at the indicated temperature. Representative results from one of three independent trials are shown.

To directly determine if deletion of pil1, lsp1, or both genes up-regulated Ypk1p activity in pck1Δ cells, we performed immunoblotting studies using anti-Ypk1p antibody. It has been shown previously that phosphorylated Ypk1p (the most active species) migrates slower than the non-phosphorylated, less active form and that LCB stimulation of phosphorylation is mediated by Pkh1/2. We anticipated that deletion of pil1 would generate more phospho-Ypk1p. The observed results were quite different. First, we found that only the non-phosphorylated, faster migrating species was present in cells grown at 30 °C (Fig. 8, left panel). Second, following a shift to 34 °C and growth for 6 h, wild type cells contained both phosphorylated and non-phosphorylated species (Fig. 8, compare the left and center panels), but unexpectedly, all of the pck1Δ mutant strains had a greatly reduced level of Ypk1p. Finally, addition of 1 μM myriocin caused the faster migrating, non-phosphorylated species of Ypk1 to increase to a level that was slightly higher in the pck1Δpil1Δ cells compared with wild type cells while in pck1Δpil1Δlsp1Δ cells the level increased to slightly less than in wild type cells (Fig. 8, right panel). As a control to demonstrate that the upper, slower migrating band of Ypk1p is phosphorylated we treated immunoprecipitated protein with calf-intestinal phosphatase (Fig. 8, CIP). These data show that deletion of pck1 causes the level of Ypk1p to decrease when cells are given a mild heat shock, but the level can be restored if the first step in sphingolipid synthesis is partially blocked by treating cells with myriocin and if pil1 is deleted. This restoration of Ypk1 protein explains why pck1Δpil1Δ and pck1Δpil1Δlsp1Δ cells are able to grow, whereas pck1Δ and pck1Δlsp1Δ cells do not grow at 34 °C in the presence of 1 μM myriocin.

A prediction based upon the results shown in Fig. 8 is that Ypk1p enzyme activity should be restored in the pck1Δpil1Δ and pck1Δpil1Δlsp1Δ cells but not in the pck1Δ and pck1Δlsp1Δ cells even though the Ypk1p protein migrates like the faster, non-phosphorylated form. These predictions were verified using Ypk1p immunoprecipitated from cells grown the same way as was done for the experiments described in Fig. 8 and assayed for kinase activity using a specific peptide substrate (Cross-tide). Enzyme activity was as high in pck1Δpil1Δ cells as in wild type and was about half this level in pck1Δpil1Δlsp1Δ cells, which could explain why the two mutant strains grew (Figs. 6 and 7). Enzyme activity was very low in both the strains, pck1Δ and pck1Δlsp1Δ, that failed to grow and that contained a low level of Ypk1p protein.

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**FIG. 5.** Pil1p and Lsp1p down-regulate Pkh1p activity. Wild type strain 15Dau, mutant strain INA106-3B (pkh1Δpkh2Δ), and derivatives of it were grown to saturation in YPD medium at 30 °C. Then 5-fold serial dilutions were prepared and spotted onto YPD plates, which were incubated for 3 days at the indicated temperature. Representative results from one of three independent trials are shown.

**FIG. 6.** Pil1p and Lsp1p negatively regulate the LCB-dependent Ypk1p pathway. 5-fold serial dilutions of wild type (WT, strain DL100) or the indicated variants of a pck1Δ strain (DL523) were spotted on YPD plates, with or without myriocin, and incubated at the indicated temperature for 2 days. Representative results from one of three independent trails are shown.

**FIG. 7.** Pil1p and Lsp1p negatively regulate the LCB-dependent Ypk1p pathway. The strains described in the legend to Fig. 6 were grown to an A_600nm of about 0.3 in YPD medium, and then the cultures were switched to 34 °C at time zero. Myriocin was added at time zero to the indicated final concentration (B and C). A, no drug control.

Addition of 1 μM myriocin to liquid medium enabled pck1Δpil1Δ and pck1Δpil1Δlsp1Δ cells to grow to nearly the same density as the wild type (Fig. 7B). pck1Δlsp1Δ cells did not grow much better than the parental pck1Δ cells. These results are very similar to those observed using agar medium, and together the two sets of data establish that Pil1p is a negative regulator of a pathway (Ypk12p, see below) that is necessary for cell growth. We conclude that in pck1Δ cells Pil1p works with Lsp1p to create a even stronger negative regulator of this pathway than does Pil1p by itself. Most significantly, Pil1p and the Pil1p-Lsp1p combination work downstream of a pathway regulated by LCBs.
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FIG. 8. A diminished level of Ypk1p in pkc1Δ cells can be restored by deletion of PIL1 and myriocin treatment. Cells grown as described in the legend to Fig. 7 were harvested at 6 h, cell-free extracts were prepared, and 150 μg was used for immunoblotting with anti-Ypk1p antibody. Anti-phosphoglycerokinase (PKG) served as an internal standard for protein concentration. Samples shown in the lower right corner were treated (right lane) or not treated (left lane) with calf-intestinal alkaline phosphatase before immunoblotting. Only lane 1 in the center panel of the blots shown at the top has the slower migrating, phospho-Ypk1p species. All of the other lanes contain the faster migrating, non-phosphorylated Ypk1p species.

FIG. 9. Ypk1p protein kinase activity is restored in pkc1Δ cells by deletion of PIL1 and myriocin treatment. Cells were grown as described in the legend to Fig. 7 for 6 h at 34 °C in the presence of 1 μM myriocin and cell-free extract were prepared. Ypk1p was precipitated from 1 mg of each extract with anti-Ypk1p antibody. Immunoprecipitated protein kinase activity was measured using Cross-tide as substrate as described under “Experimental Procedures.” The average amount of radioactive phosphate incorporated into Cross-tide ± S.D. for three trials is shown.

FIG. 10. Model of Pil1p and Lsp1p action during heat stress. This model depicts Pil1p and Lsp1p as negative regulators (inhibitors) of the Pkh1/2p protein kinases when cells are not heat-stressed. We speculate that phosphorylated Pil1p is inhibitory. Upon heat stress, the transient increase in LCBs acts to inhibit phosphorylation of Pil1p especially by Pkh2p, because it is more responsive to LCBs than is Pkh1p. Non-phosphorylated Pil1p no longer inhibits Pkh1p and Pkh2p, allowing these LCB-activated enzymes to phosphorylate and activate Pil1p and Ypk1p. Further details about the model are presented under “Discussion.”

DISCUSSION

Our results are the first to show that Pil1p and Lsp1p are negative regulators of heat stress resistance (Fig. 3), and the PKD1-like kinase Pkh1p, along with its downstream targets, the Pkh1p-MAP kinase cascade and the Ypk1p pathway. These two pathways work in parallel to control cell wall integrity during unstressful and stressful times (23). Our conclusions are based on the observation that the basal and heat-induced level of phospho-Slt2p, the species indicative of an activated Bcr1p kinase, is lower in pil1Δ cells (Fig. 4A) and that the heat-induced level of phospho-Slt2p is elevated in lsp1Δ cells (Fig. 4B). In addition, we showed that deletion of pil1 or both pil1 and lsp1 enhanced growth of INA106-3B (pkh1Δ/pkh2Δ) cells under moderate heat stress conditions where Pkh1p activity limits growth (Fig. 5, 34 °C). Because LCBs activate Pkh1p and Pkh2p, we determined if Pil1p and Lsp1p function downstream of an LCB-regulated pathway by treating cells with low concentrations of myriocin to partially inhibit synthesis of LCBs. If Pil1p and Lsp1p are negative regulators, then abolishing them should increase Pkh1p activity and promote growth in the presence of myriocin. Deleting pil1 or deleting both pil1 and lsp1 did improve growth of INA106-3B cells (Fig. 5, 34 °C panel) as predicted for negative regulators. However, myriocin did not stimulate growth (Fig. 5, compare 34 °C panels with or without myriocin). Myriocin probably failed to stimulate growth in this experiment, because Pkh1p phosphorylates Pil1p and Lsp1p quite well in vitro in the absence of LCBs (Fig. 1), and, thus, the phosphorylated forms were probably present and are likely to be the ones that negatively regulate Pkh1p. Because Pkh2p is more responsive to LCBs (Figs. 1 and 2) we predict that during heat stress the transient increase in LCBs in pil1Δ cells (Fig. 4A) and, thus, the phosphorylated Pil1p activity, presumably via stimulation of Pkh1/2p activity. Deleting pil1, or both pil1 and lsp1, enabled pck1Δ cells to grow in the presence of 0.5 μM myriocin (Fig. 6), a strong indication that Pil1p and Lsp1p negatively regulate Pkh1/2p activity. Doubling the concentration of myriocin to 1 μM moderately inhibited growth of wild type cells but had almost no effect on pck1Δpil1Δ or pck1Δpil1Δlsp1Δ cells. These results strongly support the conclusion that Pil1p is a negative regulator of the LCB → Pkh1/2p → Ypk1/2p pathway (Fig. 10). Although Lsp1p has a similar function, it is less important in this pathway than Pil1p. These conclusions were supported also by the results of cells grown in liquid cultures (Fig. 7). One interesting and unexpected effect of myriocin in the liquid cultures was that pck1Δ and pck1Δlsp1Δ cells went through one or two more cell doublings than they did when myriocin was omitted. These results suggest that LCBs or another sphingolipid intermediate play a role in the decision to enter a new cell cycle.

To directly demonstrate the proposed role of Pil1p as a negative upstream regulator of Ypk1p activity, immunoblots were probed with anti-Ypk1p antibody. Wild type cells contain two forms of the protein with the slower migrating form being more phosphorylated than the faster migrating form (Fig. 8) (12, 23). Unexpectedly, pck1Δ cells had a very reduced level of Ypk1p when grown at 34 °C, which was not restored to the wild type
level by deleting pil1 or lsp1 (Fig. 8, 34 °C panel). However, treatment with myriocin restored the Ypk1 protein level (Fig. 8, right panel) and enzyme activity (Fig. 9) in pck1pil1Δ and partially restored them in pck1pil1lsp1Δ cells. This unexpected ability of myriocin to restore Ypk1 to the wild type level explains why pck1pil1Δ or pck1pil1lsp1Δ cells grew well in the presence of myriocin (Figs. 6 and 7). It also supports the hypothesis that Pil1p negatively regulates an LCB-controlled pathway (Pkh1/2p) that governs Ypk1p and perhaps Ypk2p.

How myriocin restores Ypk1p in pck1Δ cells and how the level of Ypk1p depends upon the Pck1-MAp kinase cascade remain to be determined. But our results could be related to an observation made by Schmelzle et al. (22). These authors found that the basal and heat-induced levels of phospho-Slt2p were reduced in ypk1Δ cells. Together the two sets of data argue that there is a reciprocal interaction between the Pck1-MAp cascade and the Ypk1 pathway such that each is dependent upon the other: it is not known if Ypk2p is also dependent upon the Pck1-MAp cascade.

Our data add a new level of control and complexity to LCB-regulated signal transduction pathways by showing that Pil1p and Lsp1p negatively regulate Pkh1p and its downstream effector pathways during heat stress (Fig. 10). Although we have not directly demonstrated in vitro that Pil1p and Lsp1p also regulate Pkh2p, they most likely do, because they both form a complex with many proteins besides Pkh1/2p (7) and so they are not similar to any known amino acid sequences, although highly related (72% amino acid similarity), are not similar to any known protein or domain and, thus, provide no clues about molecular identity and 84% similarity), are not similar to any known protein or domain and, thus, provide no clues about molecular identity.

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