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Cell Wall Integrity MAPK Pathway Is Essential for Lipid Homeostasis*[S]

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The highly conserved yeast cell wall integrity mitogen-activated protein kinase pathway regulates cellular responses to cell wall and membrane stress. We report that this pathway is activated and essential for viability under growth conditions that alter both the abundance and pattern of synthesis and turnover of membrane phospholipids, particularly phosphatidylinositol and phosphatidylcholine. Mutants defective in this pathway exhibit a choline-sensitive inositol auxotrophy, yet fully derepress *INO1* and other Opi1p-regulated genes when grown in the absence of inositol. Under these growth conditions, Mpk1p is transiently activated by phosphorylation and stimulates the transcription of known targets of Mpk1p signaling, including genes regulated by the Rlm1p transcription factor. *mpk1*Δ cells also exhibit severe defects in lipid metabolism, including an abnormal accumulation of phosphatidylcholine, diacylglycerol, triacylglycerol, and free sterols, as well as aberrant turnover of phosphatidylcholine. Overexpression of the *NTE1* phospholipase B gene suppresses the choline-sensitive inositol auxotrophy of *mpk1*Δ cells, whereas overexpression of other phospholipase genes has no effect on this phenotype. These results indicate that an intact cell wall integrity pathway is required for maintaining proper lipid homeostasis in yeast, especially when cells are grown in the absence of inositol.

The protein kinase C (PKC)[3] signaling pathway, known in the budding yeast, *Saccharomyces cerevisiae*, as the cell wall integrity mitogen-activated protein kinase pathway (CWI-MAPK), is one of five MAPK cascades identified in this organism (1–4). The signaling proteins in the highly conserved CWI-MAPK pathway include the PKC homolog Pkc1p, the MAP kinase kinase (MEKK) Bck1p, the redundant Thr/Tyr kinases Mkk1p and Mkk2p, and the MAP kinase Mpk1p, also called Slt2p (3, 5–8).

In yeast, PKC signaling plays an essential role in regulating cell wall synthesis. Mutants defective in the CWI-MAPK signaling pathway have phenotypes associated with cell wall defects and show a tendency to lyse at 37 °C, a phenotype that is rescued by osmotic support (9, 10). The *pkc1*Δ mutant exhibits more severe growth phenotypes than the *mpk1*Δ and *bck1*Δ mutants, presumably because Pkc1p controls pathways that are not under Bck1p or Mpk1p control (8). Mutations in the CWI-MAPK pathway severely affect the transcription levels of genes encoding key proteins involved in the biosynthesis of the components of the cell wall (11). Rlm1p, a downstream substrate for Mpk1p phosphorylation (12, 13), regulates the transcription of genes encoding enzymes involved in cell wall organization and biogenesis and members of the Pir family of cell wall proteins (11, 14).

A wide range of external stimuli activate yeast PKC signaling, such as heat stress (15), hypo-osmotic shock (16), oxidative stress (17–19), mating pheromone (1, 20, 21), alkaline stress (22), and endoplasmic reticulum (ER) stress (23, 24). For example, treatment of cells with the ER stress-inducing agents β-mercaptoethanol, dithiothreitol, or tunicamycin, which trigger the unfolded protein response (UPR) pathway, activate the CWI-MAPK pathway (24). Similar to mutants defective in the UPR pathway, CWI-MAPK-defective mutants are hypersensitive to these ER stress-inducing agents, yet the CWI-MAPK pathway functions independently of the UPR in response to ER stress (24).

The UPR is also induced and required for growth in cells grown in the absence of exogenously supplied inositol (25–31), and it is rapidly inactivated upon re-introduction of inositol (32), suggesting that ER stress responses sense changes in phospholipid levels. Given the relationship of PKC signaling with ER stress, the question arises as to whether the CWI-MAPK pathway is sensitive to membrane lipid composition. In mammalian cells, PKC itself is directly activated by binding of 1,2-diacylglycerol (DAG) to a highly homologous cysteine-rich C domain (C1 domain) found in many PKC isoforms (10). DAG produced by phospholipase C-dependent hydrolysis of phosphatidylino-

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[3] The abbreviations used are: PKC, protein kinase C; MAP, mitogen-activated protein; MEKK, MAP kinase kinase; CWI-MAPK, cell wall integrity-mitogen-activated protein kinase; ER, endoplasmic reticulum; UPR, unfolded protein response; PA, phosphatidic acid; DAG, 1,2-diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PBS, phosphate-buffered saline; HA, hemagglutinin; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; GroPCho, glycerophosphocholine; PLB, phospholipase B; PS, phosphatidylserine.

TABLE 1
Strains used in this study

Strain	Genotype	Source or Ref.
DL100	*MATa leu2-3,12 trp1-1 ura3-52 his4 can1^r*	9
DL253	*MATα leu2-3,12 trp1-1 ura3-52 his4 can1^r bck1Δ::URA3*	3, 5–8
DL376	*MATa leu2-3,12 trp1-1 ura3-52 his4 can1^r pkc1Δ::LEU2 ts*	9
DL455	*MATα leu2-3,12 trp1-1 ura3-52 his4 can1^r mpk1Δ::TRP1*	3, 5–8
LN106	*MATα leu2-3,12 trp1-1 ura3-52 his4 can1^r mpk1Δ::TRP1 opi1Δ::KanMX4*	This study
LN284	*MATa leu2-3,112 trp1-1 ura3-52 his4 can1^r ino1Δ::KanMX*	This study
LN285	*MATα leu2-3,112 trp1-1 ura3-52 his4 can1^r mpk1Δ::TRP1 ino1Δ::KanMX6*	This study
MRY2061	*MATα leu2-3,12 trp1-1 ura3-52 his4 can1^r bck1Δ::URA3 opi1Δ::KanMX4*	50
MRY2062	*MATa leu2-3,12 trp1-1 ura3-52 his4 can1^r opi1Δ::KanMX4*	50
MRY2065	*MATα leu2-3,12 trp1-1 ura3-52 his4 can1^r pkc1Δ::LEU2 opi1Δ::KanMX4 ts*	50
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	31
SJY425	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ino1Δ::HIS3MX6*	This study
SHY932	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mpk1Δ::KanMX6*	Invitrogen (record no. 10993)
SHY933	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 rlm1Δ::KanMX6*	Invitrogen (record no. 12739)
SHY934	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 nte1Δ::KanMX6*	Invitrogen (record no. 10511)
SHY227	*MATa ade1 ino1–13/MATα ade1 ino1–13*	2–54

sitol (4,5)-bisphosphate (33–38) or during sphingolipid metabolism (39, 40) leads to PKC activation in mammalian cells and the fungus *Cryptococcus neoformans*, respectively.

Although *S. cerevisiae* Pkc1p contains the conserved C1 domain (41), attempts to explore DAG activation of PKC signaling in *S. cerevisiae* have produced only negative or inconclusive results. Several *in vitro* studies found that purified yeast Pkc1p is not activated by DAG, phorbol esters, or phospholipids (42, 43). Nevertheless, other lipids may play indirect roles in PKC activation in yeast via the Rho1 GTPase, the master regulator of this pathway. Production of phosphatidylinositol (4,5)-bisphosphate at the plasma membrane recruits Rom2p, the GTPase exchange factor for Rho1p, which leads to PKC activation (44). In addition, the mitochondrial anionic phospholipids, phosphatidylglycerol and cardiolipin, appear to regulate PKC signaling, possibly by affecting Rho1p membrane association (45). At present, however, a direct connection between PKC activation and lipid homeostasis has not been firmly established in yeast.

To explore the linkage between lipid metabolism and PKC signaling, we investigated the sensitivity of the CWI-MAPK pathway to the phospholipid precursors inositol and choline. The presence of these precursors in the medium of actively growing yeast profoundly alters both the abundance as well as the pattern of synthesis and turnover of membrane phospholipids, neutral lipids, and sphingolipids (46–48). These changes in lipid metabolism are accompanied by alterations in the expression levels of hundreds of genes (31, 32), suggesting regulation by multiple signaling pathways. In this study, we report that mutants in the CWI-MAPK pathway, including *pkc1*Δ, *bck1*Δ, and *mpk1*Δ, exhibit an inositol auxotrophy phenotype associated with alterations in phospholipid metabolism, particularly when choline is present in the growth medium. Growth in the absence of inositol results in Mpk1p phosphorylation and induction of CWI-MAPK target gene expression, which is absent in *mpk1*Δ and *rlm1*Δ cells. Surprisingly, *mpk1*Δ cells exhibit normal derepression of *INO1* and other inositol-regulated genes that are repressed by Opi1p. However, the *mpk1*Δ mutant exhibits aberrant phospholipid metabolism, including defective phosphatidylcholine (PC) turnover and accumulation. Our results indicate that PKC signaling is activated by growth in the absence of inositol and is essential for lipid homeostasis and cell viability.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—Yeast strains were maintained on liquid YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) or chemically defined synthetic complete media described elsewhere (31). Where indicated, synthetic complete media were supplemented with 75 μM inositol (I^+) and/or 1 mM choline (C^+). Solid media maintained the same composition plus 2% agar. Where indicated, 1 M sorbitol or 0.5 M KCl was added as osmotic support. Yeast strains were grown at 30 °C unless otherwise indicated.

Yeast Strains—*S. cerevisiae* strains used in this study are described in Table 1. The *mpk1*Δ *opi1*Δ double mutant strain LN106 was constructed using standard tetrad analysis by crossing MRY2062 with DL455. The other *opi1*Δ double mutant strains MRY2061, MRY2062, and MRY2065 were constructed by PCR-mediated gene replacement of the entire *OPI1* open reading frame in DL253, DL100, and DL376 using the template plasmid pFA6-kanMX4 (49) and primers OPI1-S1 and OPI1-S2 as described previously (50). Construction of *ino1*Δ single and double mutants LN284, SJY425, and LN285 was performed by PCR-mediated gene replacement of the entire *INO1* open reading frame in DL100, BY4742 (31), and DL455 using template plasmids pFA6a-KanMX6 or pFA6a-HIS3MX6 (51) and primers INO1F1 (AGC TTT CGT CAC CTT TTT TTG GCT TGT TCT GTT GTC GGG TTC CTA CGG ATC CCC GGG TTA ATT AA) and INO1R1 (TTT ATA GGT AGG CGG AAA AAG AAA AAG AGA GTC GTT GAA ATG AGA GAA TTC GAG CTC GTT TAA AC). Other strains used in this study were either purchased from Invitrogen or as described previously (52–54).

Analysis of Ino$^-$ and Opi$^-$ Phenotypes—For the analysis of inositol auxotrophy (Ino^-) phenotype (54), yeast strains were grown to mid-log phase in synthetic complete media containing 75 μM inositol, harvested, and washed with water. Following growth cells were resuspended at a concentration of 1.0 OD_{660}/ml, serially diluted in 10-fold increments, spotted onto plates containing 75 μM inositol (I^+) or no inositol (I^-) and/or 1 mM choline (C^+) or no choline (C^-), and incubated at the indicated temperature for 2–3 days. For the analysis of overproduction of inositol (Opi^-) phenotype (63), yeast cells were spotted on chemically defined synthetic complete I^- media and incubated for 2 days at 25 °C. A suspension of the AID indicator strain

TABLE 2
Plasmids used in this study

Name	Description or relevant genotype	Source or Ref.
pFA6-kanMX4	Template for PCR-mediated gene replacement	49
pFA6a-kanMX6	Template for PCR-mediated gene replacement	51
pFA6a-HIS3MX6	Template for PCR-mediated gene replacement	51
UAS_{INO}-lacZ	*UAS_{INO}-CYC1-lacZ*, 2μ, *LEU2*	50
pJH310-INO1	*INO1* in pGEM1 for riboprobe	59
pGEM-KAR2	*KAR2* in pGEM1 for riboprobe	31
pGEM-MLP1	*MLP1* in pGEM1 for riboprobe	This study
pGEM-YRR078C	*YRR078C* in pGEM1 for riboprobe	This study
pFL44-SLT2HA	*SLT2*(*MPK1*)-HA, 2μ, *URA3*	21
pFL44-slt2^{K54R}HA	*slt2^{K54R}*-HA, 2μ, *URA3*	21
pRS426-NTE1	*NTE1*, 2μ, *URA3*	83
YEp352-PLB1	*PLB1*, 2μ, *URA3*	O. Merkel
YEp351-PLB2	*PLB2*, 2μ, *LEU2*	69
URA3-2μ-SPO14	*SPO14* (*PLD1*), 2μ, *URA3*	J. Engebrecht
pFV139	*PLC1*, 2μ, *URA3*	84
pRS426	Empty, 2μ, *URA3*	85

(SHY227), which is homozygous for *ino1* and *ade1* mutations, was sprayed on the plates and incubated for 2 days at 30 °C.

Growth Curves and Viability Assays—Yeast strains were grown to mid-log phase in I^+C^- medium, harvested, and washed with water. Cells were inoculated at an initial concentration of 0.1 OD_{660}/ml in 20–25 ml of I^+C^-, I^+C^+, I^-C^+, or I^-C^- media and incubated at the indicated temperature for ~2 days. Growth was monitored every 3–4 h using a Klett-Summerson photoelectric colorimeter (Klett MFG Co. Inc., model 800-3). For viability assays, cells were grown in I^+C^- media at 30 °C until ~0.5 OD_{660}/ml. Cells were filtered, washed, resuspended in pre-warmed I^-C^+ medium, and incubated at the indicated temperature. Aliquots were removed every 1.5–3 h. Cells were diluted with water to reach concentrations of 0.3–0.6 OD_{660}/ml and added an equal volume of 0.1% methylene blue solution. The cells were incubated for 10 min at room temperature and visualized under the microscope. A total of 100–150 cells per sample were counted. Cells that were colorless were scored as alive.

DNA Manipulation and Plasmids—Plasmids used in this study are listed in Table 2. Plasmids were isolated from *Escherichia coli* HB101 and DH5α strains using the QIAprep spin miniprep kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's protocol. Yeast genomic DNA was isolated using the Y-DER Yeast DNA extraction reagent kit (Pierce), according to the manufacturer's instructions. Plasmid DNA was introduced into *E. coli* cells using chemically competent cells or by electroporation as described elsewhere (55). Plasmid DNA was introduced into yeast cells using the high efficiency transformation method as described by Gietz and Woods (56).

β-Galactosidase Assays—β-Galactosidase activity was determined by the absorbance (420 nm) of the yellow compound *o*-nitrophenol, a result of the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. The reporter plasmid used in these experiments contains a region of the *INO1* promoter (−359 to −119) with four or more putative UAS_{INO} consensus sequences and the *CYC1* TATA element fused to the bacterial *lacZ* gene (57). It was engineered to carry a *LEU2* nutritional marker for *S. cerevisiae* (50). Transformed cells containing the UAS_{INO}-*lacZ* construct were grown in the selective medium containing inositol (I^+C^- Leu^-) at a specific temperature and harvested at mid-log phase (~0.5–1.0 OD_{660}/ml). Cells were washed, transferred to the media containing 75 μM inositol, and incubated at the same temperature for 3 h. At this point, cells were harvested and tested for β-galactosidase activity using the yeast β-galactosidase assay kit (Pierce) according to the manufacturer's instructions.

RNA Isolation and Microarray Analysis—Strains were grown in I^+C^- medium at 30 °C to mid-logarithmic growth phase. At OD_{600} = 0.5, the cultures were filtered, washed with prewarmed I^-C^+ medium, resuspended in prewarmed I^-C^+, and harvested by filtration at 0 and 3.5 h following the media shift, flash-frozen in dry ice, and stored at −80 °C. Total RNA was isolated from samples using the hot acid phenol method, and mRNA was isolated using oligo(dT) cellulose as described previously (31). Cy5- and Cy3-labeled probes were synthesized from mRNA, mixed, and hybridized to CMT Yeast-S288c gene arrays (version 1.32, Corning Inc.) as described previously (31). Hybridized and washed microarray slides were scanned using a GenePix 4000B array scanner (Molecular Devices, Sunnyvale, CA) and analyzed using the GenePix Pro 4.0 (Molecular Devices). Individual data spots flagged as outliers because of low intensity or poor quality were excluded from further analysis. For the remaining genes, the results were expressed as the $\log_2$ of the sample signal divided by the signal in the reference channel.

Three separate experiments were performed to identify the set of genes regulated by the CWI-MAPK pathway that are sensitive to inositol and choline. Experiment 1 measured the changes in expression in wild type (DL100) cells after a media shift to I^-C^+ for 3.5 h (experimental sample) compared with I^+C^- (0 h, reference). Experiment 2 measured the changes in expression in *mpk1*Δ (DL455) grown exactly as in experiment 1. Experiment 3 compared the overall changes in expression between *mpk1*Δ (experimental) and wild type (reference) cells grown for 3.5 h in I^-C^+ medium. Genes were sorted into the Mpk1p-regulated category if the following three criteria were met: up-regulation in experiment 1 ($\log_2$ expression ratio ≥0.8), no change in expression in experiment 2 ($\log_2$ expression ratio between 0.4 and −0.4), and down-regulation in experiment 3 ($\log_2$ expression ratio ≤ −0.8) provided that each gene showed a p value ≤ 0.05. Genes were sorted in Opi1p- and UPR-regulated categories using criteria described by Ref. 32.

Northern Blot Analysis—For slot blot analysis, 10 μg of total RNA was denatured in denaturing buffer (31) and spotted on BrightStar-Plus nylon membrane using a manifold slot blot apparatus as described (31). For Northern blot analysis, 1 μg of total RNA or 125 ng of mRNA was fractionated on 1% glyoxal-agarose gels and transferred to Nytran SuPerCharge nylon membrane (Whatman, Sanford, ME) in 20× SSC as described (58). To detect specific mRNA transcripts, strand-specific ^{32}P-labeled riboprobes were synthesized from linearized plasmids pJH310-*INO1* (59), pSJ29-*KAR2* (31), pSJ38-*MLP1* (this study), and pSJ38-*YPR078C* (this study) and hybridized to membranes in formamide hybridization buffer as described (31). Quantitation was performed following scanning on a STORM 860 PhosphorImager and analyzed using ImageQuant software (GE Healthcare). pSJ38-*MLP1* was constructed by PCR-amplifying

a portion of the *MLP1* open reading frame using primers MLP1F (ACT CTG CAG ATA CTG TGT GCT CTG AAA T) and MLP1R (CCG CCA TAC TTT CGA TAT GCT CGA ATT) and inserting the resulting 573-bp PstI-EcoRI fragment into pGEM1 through the same restriction sites. pSJ38-*YPR078C* was constructed by PCR-amplifying a portion of the *YPR078C* open reading frame using primers YPR078CF (ATC CTG CAG TAT TAA GCC CCT CAG AGC T) and YPR078CR (GCG AAT TCA ACC TCT GTC TCC AAT CAC G) and inserting the resulting 354-bp PstI-EcoRI fragment into pGEM1 through the same restriction sites.

Immunoblot Analysis—Yeast cells containing plasmids pFL44-SLT2HA and pFL44-SLT2^{K54R}HA (21), a kind gift from C. Mann, were grown at 30 °C on I^+C^- Ura^- media and harvested at ~0.5 OD_{660}/ml. Cells were filtered, washed with pre-warmed I^-C^- media, resuspended in pre-warmed I^-C^+ media, and incubated at 30 °C. Aliquots were taken before filtration (t = 0 h), and 2, 3.5, and 5 h after filtration. Protein was extracted from cells by cell disruption using glass beads as described (60). 100–900 μg of protein were loaded into each well for equal detection of total HA-tagged Mpk1 protein on 10% BisTris NuPAGE precast gels (Invitrogen), subjected to electrophoresis, and transferred overnight to nitrocellulose membranes. Nitrocellulose membranes were blocked for 3 h with 10% bovine serum albumin in 1× PBS at room temperature. Membranes were washed with 5% milk, PBS, 0.1% Tween 20 and incubated with primary antibody in 5% milk, PBS, 0.1% Tween 20 for 3 h at room temperature, washed with 5% milk, PBS, 0.1% Tween 20, and incubated with secondary antibody for 1 h at room temperature. Detection was by enhanced chemiluminescence (Pierce). Phospho-Mpk1p was detected using anti-phospho-44/42 MAPK Thr-202/Tyr-204 mouse IgG1 monoclonal antibody (Cell Signaling Technology Inc, Danvers, CT; 1:1000 dilution) and goat anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad; 1:5000 dilution). Hemagglutinin-tagged Mpk1p was detected using HA probe Y-11 rabbit polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA;1:500 dilution) and goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad; 1:5000 dilution).

Lipid Extraction and Phospholipid Analysis—Yeast cultures (5 ml) were grown until saturation in I^+C^- medium containing 1 μCi/ml [1-^{14}C]acetate-specific activity at 57 μCi/mmol. The culture was diluted back to 0.1 OD_{660}/ml with fresh media of the same composition and grown until saturation to reach steady state labeling. Cells were diluted back to 0.1 OD_{660}/ml in 55 ml of media of the same composition maintaining label at 1 μCi/ml [1-^{14}C]acetate. Cells were incubated at 30 °C until 0.5 OD_{660}/ml, filtered, and switched to I^-C^+ media containing 1 μCi/ml [1-^{14}C]acetate. Samples were harvested both before and 3.5 h after transfer to I^-C^+ medium by collecting 5-ml aliquots in tubes containing 500 μl of 50% trichloroacetic acid. After 15 min of incubation on ice, cells were washed with distilled water and frozen. Lipids were extracted by adding 5 ml of chloroform:methanol (2:1 v/v) and 0.5 ml of 0.6% NaCl solution. After 1 h of incubation at 4 °C, the bottom phase was transferred to a new vial and evaporated under N_2 flow. The extracted lipids were resuspended in 0.5 ml of chloroform:methanol (2:1 v/v), and a fraction of this was used to quantify

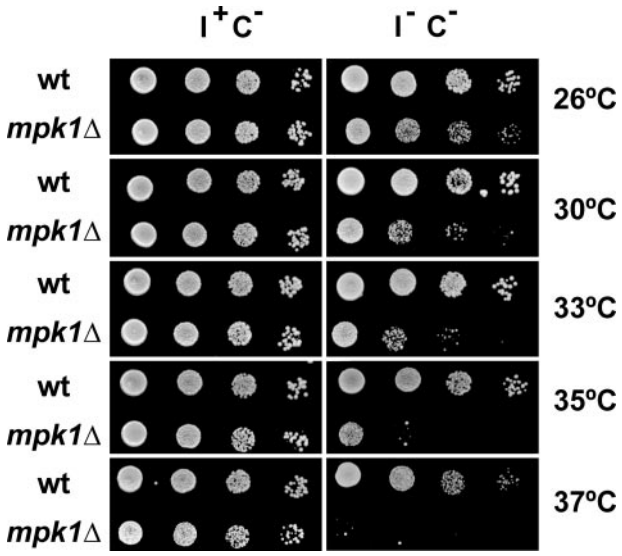

FIGURE 1. **Inositol auxotrophy (Ino$^-$ phenotype) of the *mpk1*Δ mutant at different temperatures.** A suspension of wild type (*wt*) (DL100) and *mpk1*Δ (DL455) cells at a concentration of 1.0 OD_{660}/ml and three subsequent 1:10 serial dilutions of strains were spotted onto plates containing 75 μM inositol but lacking choline (I^+C^-) and in medium lacking both inositol and choline (I^-C^-). Cells were grown for 2 days at the indicated temperature without any osmotic support in media.

counts/min using a scintillation counter. Polar lipids were separated by high performance thin layer chromatography on Whatman Silica Gel 60A plates using chloroform:ethyl acetate:acetone:isopropyl alcohol:ethanol:methanol:water:acetic acid (30:6:6:6:16:28:6:2 v/v) as solvent system. To separate neutral lipids, a mixture of hexane:ethyl ether:formic acid (80:20:2 v/v) was used as a solvent. Quantitation was performed by analysis on a Storm 860 PhosphorImager and analyzed with ImageQuant software. Metabolite identity was based on the mobility of known standards.

[^{14}C]Choline Labeling and Metabolite Analysis—Strains were uniformly labeled by growing to logarithmic phase in I^+C^- medium containing 1 μCi/ml [^{14}C]choline (18 μM). The cells were harvested by filtration and used to inoculate two cultures at equivalent densities in I^-C^- and I^+C^- media. At each time point, an aliquot of cells was removed. The counts associated with the membranous and water-soluble fractions were determined, as described previously (61). Ion-exchange chromatography was employed for the separation of [^{14}C]choline-containing metabolites (62).

RESULTS

Mutants in the CWI-MAPK Pathway Exhibit Ino$^-$ Phenotypes That Are Stronger at Higher Temperatures and When Choline Is Present—Misregulation of phospholipid biosynthetic genes frequently leads to two indicative phenotypes: inositol auxotrophy (Ino$^-$) and overproduction of inositol (Opi$^-$) (54, 63). When mutants defective in several major signaling pathways in yeast were screened for these phenotypes, the *pkc1*Δ, *bck1*Δ, and *mpk1*Δ (*slt2*Δ) single mutants were found to exhibit inositol auxotrophy (50). Factors such as temperature (Fig. 1), addition of choline (Fig. 2), and genetic background (data not shown) enhanced or diminished the stringency of the Ino$^-$ phenotype of strains carrying these mutations. The

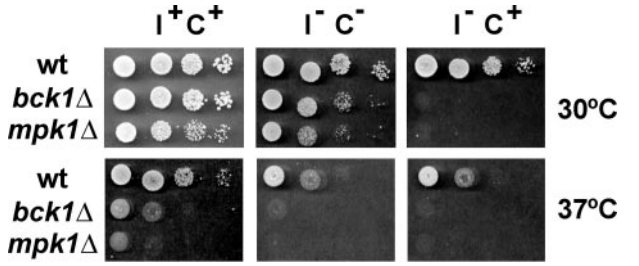

FIGURE 2. **Effect of choline on the Ino⁻ phenotype of *bck1*Δ and *mpk1*Δ mutants.** A suspension of wild type (*wt*) (DL100), *bck1*Δ (DL253), and *mpk1*Δ (DL455) cells at a concentration of 1.0 OD_{660}/ml and three subsequent 1:10 serial dilutions were spotted onto plates containing I^+C^+, I^-C^-, and I^-C^+ medium. Cells were grown for 2 days at 30 and 37 °C without any osmotic support.

*mpk1*Δ mutant was able to grow without inositol (I^-C^- medium) or osmotic support at 26 °C. As the temperature was increased incrementally, its growth was diminished in I^-C^- medium until no growth was observed at 37 °C (Fig. 1). However, when inositol was present (I^+C^- medium), the *mpk1*Δ strain grew without osmotic support even at 37 °C (Fig. 1). The Ino⁻ phenotype of the *bck1*Δ mutant (data not shown) was similar to that of *mpk1*Δ. Choline had little or no effect on growth of *mpk1*Δ or *bck1*Δ cells at 30 °C in the presence of inositol (I^+C^+ medium, Fig. 2). However, when choline was added to medium lacking inositol (I^-C^+ medium), the Ino⁻ phenotype of the *mpk1*Δ and *bck1*Δ strains became quite stringent even at 30 °C (Fig. 2), a temperature at which growth is only slightly diminished when both inositol and choline are both absent (I^-C^-medium, Fig. 1). Moreover, the growth of *mpk1*Δ and *bck1*Δ strains was greatly diminished at 37 °C when choline was present even in the presence of inositol (I^+C^+ medium, Fig. 2).

Tests for temperature and/or osmotic sensitivity of mutants in the CWI-MAPK pathway are often conducted in YPD medium, which contains both inositol and choline derived from yeast extract. In addition, sorbitol is frequently used as an osmotic protectant to the growth medium of mutants in the CWI-MAPK pathway (9). Unlike the *mpk1*Δ and *bck1*Δ mutants, however, *pkc1*Δ mutant strains will not grow at any temperature without osmotic support (9). We have been aware for some time that sorbitol, at the concentrations normally used for osmotic remediation of mutants in the CWI-MAPK signaling pathway, will support some growth of inositol auxotrophs (64). Growth of the *mpk1*Δ strain is comparable with an *ino1*Δ strain in medium supplemented with sorbitol (supplemental Fig. S1). The simplest explanation for these observations is that commercially available preparations of sorbitol contain low levels of inositol as a contaminant. Therefore, to test whether the *pkc1*Δ strain exhibits inositol auxotrophy, it was necessary to use an osmotic support other than sorbitol. In liquid media supplemented with 0.5 M KCl as osmotic support at 25 °C with or without inositol, the *pkc1*Δ strain achieves more sustained growth and reaches a higher cell density when supplemented with inositol (Fig. 3).

*Overexpression of INO1 Relieves the Ino⁻ Phenotype of mpk1*Δ—Deletion of *OPI1*, which encodes the major negative regulator of the phospholipid biosynthetic genes in yeast (52–54), leads to high constitutive overexpression of *INO1* and

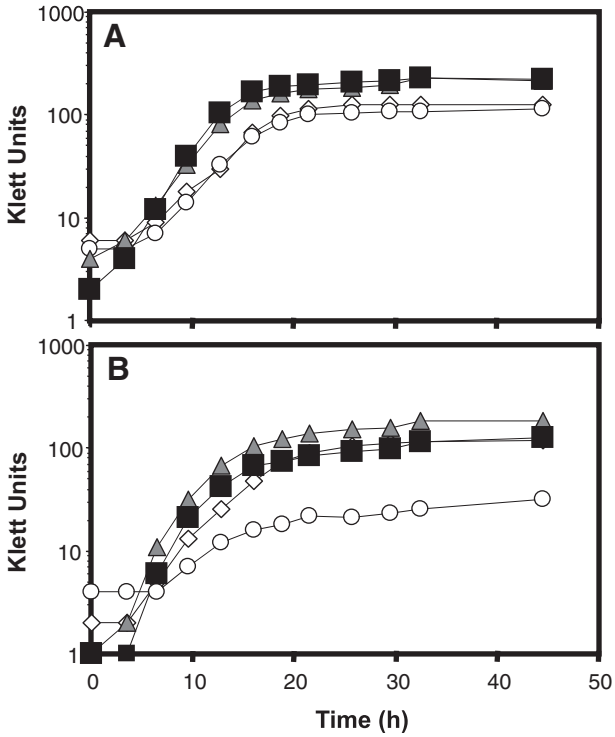

FIGURE 3. **Inositol auxotrophy of *pkc1*Δ mutant.** Cells were grown for 2 days at the permissive temperature of 25 °C in the presence of 0.5 M KCl as osmotic support, in I^+C^- medium (*A*) and I^-C^- medium (*B*). Wild type (*black squares*, DL100), *pkc1*Δ (*open circles*, DL376), *opi1*Δ (*gray triangles*, MRY2062), and *pkc1*Δ *opi1*Δ (*open rhombi*, MRY2065).

other coregulated genes of phospholipid metabolism and to excretion of inositol into the growth media (Opi⁻ phenotype) (52). The *opi1*Δ mutation was introduced into the *pkc1*Δ, *bck1*Δ, and *mpk1*Δ mutant backgrounds and, in each case, eliminated the Ino⁻ phenotype (data not shown). Indeed, on medium lacking inositol, the *bck1*Δ*opi1*Δ and *mpk1*Δ*opi1*Δ strains actually exhibited strong Opi⁻ phenotypes characterized by excess production and excretion of inositol (Fig. 4). In contrast to the *pkc1*Δ mutant, the *pkc1*Δ*opi1*Δ mutant was able to grow in liquid medium lacking inositol at 25 °C, if osmotically buffered with KCl. These cultures reached a cell density equivalent to the growth of the *pkc1*Δ mutant supplemented with inositol (Fig. 3). Moreover, similar to results obtained by deleting *OPI1*, overexpression of the *INO1* gene from a high copy plasmid also relieved the Ino⁻ phenotype in *mpk1*Δ cells (data not shown).

Both *mpk1*Δ and *mpk1*Δ*opi1*Δ failed to grow on YPD medium in the presence of 5 mM caffeine, another phenotype reported for mutants in this pathway (9, 10), unless sorbitol was present as osmotic support (supplemental Fig. S2). Wild type and *opi1*Δ cells were unaffected by caffeine under either growth condition. Thus, *opi1*Δ does not suppress all of the growth phenotypes associated with defective PKC signaling but instead appears to be specific to the Ino⁻ phenotype.

Expression of INO1 Does Not Require Active PKC Signaling—Because the Ino⁻ phenotype is often associated with failure to express the *INO1* gene (54, 63), *INO1* expression was examined in the *mpk1*Δ strain and compared with wild type, using an *INO1-LacZ* reporter construct, as described under "Experi-

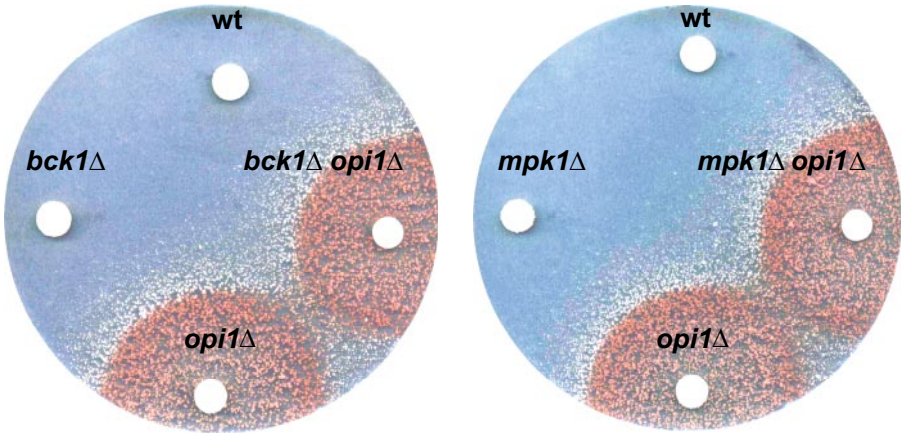

FIGURE 4. **Overproduction of inositol (Opi$^-$) phenotype of *opi1*Δ, *bck1*Δ*opi1*Δ, and *mpk1*Δ*opi1*Δ strains.** Wild type (*wt*) (DL100), *bck1*Δ (DL253), *mpk1*Δ (DL455), *opi1*Δ (MRY2062), *bck1*Δ *opi1*Δ (MRY2061), and *mpk1*Δ *opi1*Δ (LN106) cells were spotted on plates containing I$^-$C$^-$ medium and incubated for 2 days at 25 °C. A cell suspension of AID indicator strain (SHY227), which grows only in the presence of inositol, was sprayed on the plates and incubated for a further 2 days at 30 °C. Strains excreting inositol are visible as *red halos* around the strain being tested.

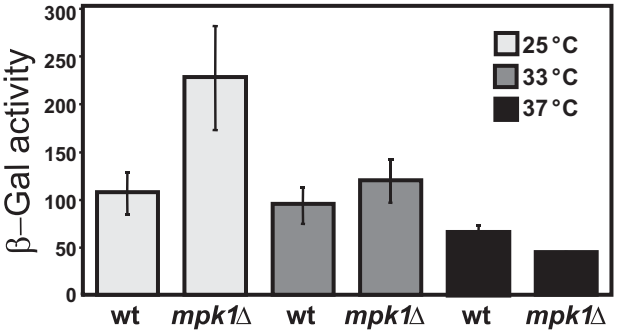

FIGURE 5. **Expression levels of the reporter gene of the wild type strain and *mpk1*Δ mutant at different temperatures.** Wild type (*wt*) (DL100) and *mpk1*Δ (DL455) cells transformed with pMR1036 were grown in I$^+$C$^-$ Leu$^-$ medium at 25, 33, and 37 °C and harvested at 0.5–1.0 OD_{660}/ml. Cells were pelleted, washed, and resuspended in I$^-$C$^-$ Leu$^-$ medium and incubated at 25, 33, and 37 °C for 3 h. β-Galactosidase activity was measured in cells following the medium shift. The average values and standard deviation of three independent experiments are shown.

mental Procedures." Cells were grown in I$^+$C$^-$ medium at 25 °C, conditions that are fully repressing for the *INO1* gene (59) and permissive for *mpk1*Δ growth (Fig. 1). When transferred to I$^-$C$^-$ medium at 25 °C for 3 h, the extent of derepression of the *INO1 lacZ* reporter construct in the *mpk1*Δ strain was ~2-fold higher than in wild type (Fig. 5). When the cells were transferred to I$^-$C$^-$ medium at 33 °C, a condition where *mpk1*Δ shows diminished growth compared with wild type on plates (Fig. 1), the extent of derepression of the reporter gene in *mpk1*Δ was comparable with that of wild type (Fig. 5). Even at 37 °C in I$^-$C$^-$ medium, a growth condition in which the Ino$^-$ phenotype of *mpk1*Δ is quite stringent (Fig. 1), the extent of *INO1* derepression was only 25–30% lower in *mpk1*Δ than in wild type cells (Fig. 5).

Given that no evidence of elevated expression of CWI-MAPK target genes was detected in previous microarray experiments comparing wild type cells grown in the absence of inositol with or without choline (31), we suspected that PKC signaling following a shift to inositol-free medium might be transient. Therefore, *INO1* mRNA levels were measured by quantitative slot blot analysis over an 11-h time course, following a shift from I$^+$C$^-$ to I$^-$C$^+$ medium at 30 °C. Throughout the time course of the experiment, the cells were maintained in mid-logarithmic growth phase by sequential dilution with fresh I$^-$C$^+$ medium. *mpk1*Δ cultures were observed to increase in optical density at a rate comparable with wild type for ~3.5 h after transfer to I$^-$C$^+$ medium at 30 °C (data not shown). After 3.5 h, growth slowed, and by 4 h, growth had ceased altogether (data not shown), and the cells had begun to lose viability (supplemental Fig. S3). Quantitative slot blot analysis revealed that *INO1* mRNA levels in the *mpk1*Δ strain, normalized to *ACT1*, were comparable with levels in wild type cells (data not shown) up to and including the time when *mpk1*Δ cells stopped growing and began to lose viability between 3.5 and 4 h. Thus, *INO1* expression levels that are adequate to support growth of wild type cells in medium lacking inositol do not support growth of *mpk1*Δ cells, especially at high temperatures or in the presence of choline. This observation was subsequently confirmed by microarray analysis and quantitative Northern blot analysis, described below.

Mpk1p Is Activated within 3.5 h following a Shift to I$^-$C$^+$ Medium at 30 °C—To determine whether active Mpk1p is required for growth in I$^-$C$^+$ medium, *mpk1*Δ cells were transformed with a plasmid carrying HA-tagged wild type *MPK1* or kinase-dead mutant allele, *mpk1*^{K54R}, which is unable to transmit a signal to downstream targets (21). *mpk1*Δ cells transformed with *MPK1*-HA exhibited growth comparable with wild type. Conversely, *mpk1*Δ cells transformed with the *mpk1*^{K54R} mutant construct exhibited an Ino$^-$ choline-sensitive phenotype comparable with *mpk1*Δ cells (data not shown), demonstrating that downstream signaling by Mpk1p is required for growth under these conditions.

To determine the kinetics of Mpk1p activation following transfer to I$^-$C$^+$ medium, *mpk1*Δ cells transformed with *MPK1*-HA or *mpk1*^{K54R}-HA were transferred from I$^+$C$^-$ to I$^-$C$^+$ medium for 0.2, 3.5, and 5 h and subjected to immunoblotting using an antibody that recognizes active, phosphorylated Mpk1p. In extracts from cells transformed with *MPK1*-HA, a faint signal from phosphorylated Mpk1p was detected transiently at 3.5 h (Fig. 6). These results suggest that a signal activating Mpk1p occurs in wild type cells within 3.5 h after introduction into I$^-$C$^+$ medium. However, this signal is transient, presumably due to restoration of cellular homeostasis resulting from downstream signaling. In cells transformed with the kinase-dead allele, strong phosphorylation of Mpk1p^{K54R} was observed at 3.5 h and continued at 5 h (Fig. 6), demonstrating that in *mpk1*^{K54R}-HA, the activating signal is also received by 3.5 h following the shift to I$^-$C$^+$ medium. Presumably, because Mpk1p^{K54R} cannot phosphorylate its downstream tar-

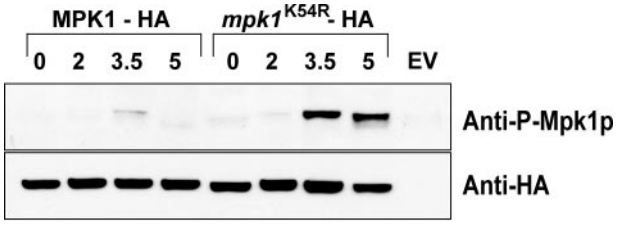

FIGURE 6. **Immunoblot analysis reveals activation of Mpk1p following transfer to I^-C^+ medium.** *mpk1*Δ (DL455) strain was transformed with HA-tagged wild type *MPK1* (*MPK1*-HA), HA-tagged kinase-dead *mpk1*^{K54R} allele (*mpk1*^{K54R}-HA), or empty vector (*EV*), grown at 30 °C in I^+C^- Ura^- medium and harvested at 0.5 OD_{660}/ml. Cells were filtered, washed, and resuspended in pre-warmed I^-C^+ Ura^- medium and incubated at 30 °C. Aliquots were taken before filtration ($t = 0$ h) and 2, 3.5, and 5 h after filtration. Cell extracts from each time point were fractionated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-phospho-44/42 MAPK Thr-202/Tyr-204 mouse monoclonal antibody (*upper panel*) or rabbit anti-HA polyclonal antibody (*lower panel*).

gets, signaling continues and is intensified compared with wild type.

CWI-MAPK-regulated Genes Are Activated in the Absence of Inositol When Choline Is Present—Given that Mpk1p is activated and is required for viability in cells grown in I^-C^+ medium, we asked whether there was any effect on the expression levels of known inositol- and choline-sensitive genes (31, 32). To identify the set of genes regulated by Mpk1p-dependent signaling stimulated by the shift from I^+C^- to I^-C^+ medium, the genome-wide expression levels in wild type and *mpk1*Δ strains were measured in three experiments, as described under "Experimental Procedures." Relative transcript abundance for all genes was assessed in wild type and *mpk1*Δ cells grown in I^+C^- medium and shifted to I^-C^+ medium for 3.5 h, the time at which Mpk1p shows maximal activation under these growth conditions (Fig. 6).

A subset of genes that showed a statistically significant change in expression were subsequently grouped in three clusters, Opi1p-, UPR-, and Mpk1p-regulated (Fig. 7), according to criteria described below. Both Opi1p- and UPR-regulated genes are highly enriched in the data set. These two categories of genes have been shown to respond to distinct membrane-associated signals (32), and thus, were grouped into two separate clusters (Fig. 7).

Opi1p-regulated genes, defined as those bound by Ino2p-Ino4p (65) and negatively regulated by Opi1p (31, 32), were significantly enriched in our dataset, as expected (Fig. 7). These included phospholipid biosynthetic genes, such as *INO1*, *OPI3*, *CKI1*, and *CHO2*, and the inositol transporter *ITR1*, and transcriptional regulators *INO2* and *OPI1*, as well as other Opi1-regulated genes identified in previous studies by our laboratory (31, 32). The 29 genes in this cluster were up-regulated after the shift from I^+C^- to I^-C^+ in both wild type and *mpk1*Δ strains indicating that the CWI-MAPK signaling pathway does not control the expression of these genes. Consequently, the failure of *INO1* derepression does not underlie the Ino^- phenotypes of PKC pathway mutants.

Similarly, UPR targets (66), including such canonical UPR targets as ER chaperones and oxireductases, were also up-regulated in both wild type and *mpk1*Δ strains (Fig. 7), suggesting that the CWI-MAPK cascade plays no role in the activation of

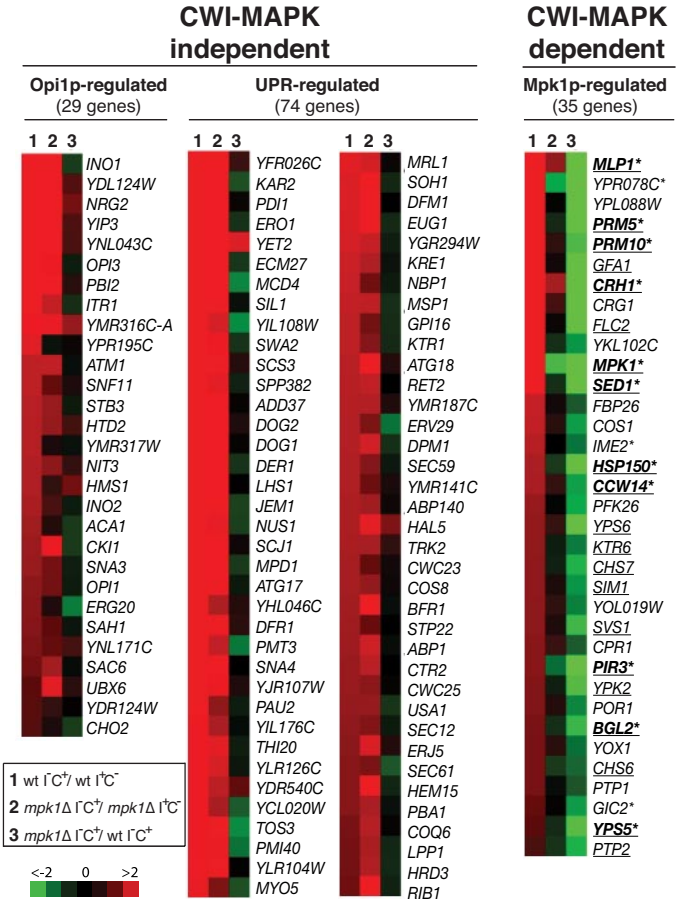

FIGURE 7. **Expression of CWI-MAPK-independent and -dependent genes regulated by inositol and choline.** mRNA from wild type (DL100) and *mpk1*Δ (DL455) cells grown in I^+C^- medium (0 h) and shifted to I^-C^+ medium for 3.5 h was analyzed by microarray gene expression profiling in three separate experiments in triplicate as described under "Experimental Procedures." The $\log_2$ expression ratios of 138 genes grouped into Opi1p-, UPR-, or Mpk1p-regulated categories are shown. Genes were sorted in Opi1p- and UPR-regulated categories using criteria described by Jesch *et al.* (32). Genes were sorted into Mpk1p-regulated category if the following three criteria were met: up-regulation in experiment 1 (*wt* I^-C^+/*wt* I^+C^-), no change in expression in experiment 2 (*mpk1*Δ I^-C^+/*mpk1*Δ I^+C^-), and down-regulation in experiment 3 (*mpk1*Δ I^-C^+/*wt* I^-C^+). *Asterisks* indicate genes regulated by the transcription factor Rlm1p (11, 65). Genes involved in cell wall organization and biogenesis or MAP kinase function are *underlined* (*Saccharomyces* Genome Data Base). Those genes present in both categories are shown in *boldface*.

UPR genes in response to the absence of inositol. This observation is consistent with other studies showing that Mpk1p activation under ER stress is UPR-independent (24). Thus, our results confirm that the CWI-MAPK pathway signaling does not influence the expression of UPR target genes.

Rlm1p Transcription Factor Controls the Expression of Many CWI-MAPK-regulated Genes in the Absence of Inositol—Genes that are controlled by Mpk1p upon shift to I^-C^+ medium were defined as described under "Experimental Procedures." In all, 35 genes (Fig. 7) were identified that met these stringent criteria. This group was significantly enriched for cell wall genes as well as genes previously shown to be regulated by Mpk1p. Significantly, approximately one-half of these genes were previously shown to be regulated by the Rlm1p transcription factor following phosphorylation by Mpk1p (11, 14), including *MLP1*/

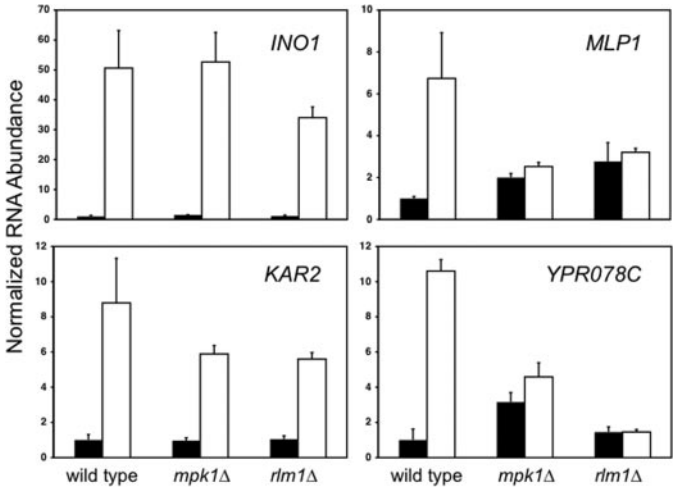

FIGURE 8. **Quantitative Northern blot analysis of Mpk1p target genes regulated by inositol and choline.** RNA from wild type (DL100), *mpk1*Δ (DL455), and *rlm1*Δ (SHY933) cells grown in I^+C^- medium (*white bars*) and shifted to I^-C^+ medium for 3.5 h (*black bars*) was analyzed in triplicate by quantitative Northern blot as described under "Experimental Procedures" using the indicated probes. Fold expression for each transcript are normalized to the total counts/min for *ACT1* and expressed as the fraction of the amount of mRNA from cells grown in the absence of inositol and choline. The standard deviation for individual transcripts at each growth condition is indicated.

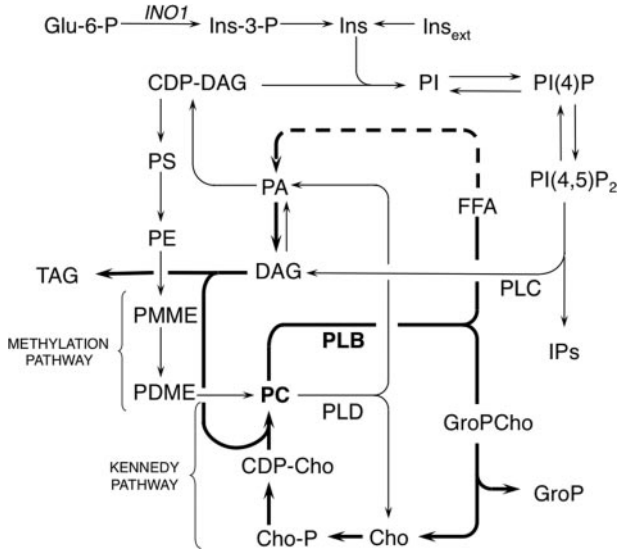

FIGURE 9. **Schematic representation of phospholipid metabolism in *S. cerevisiae*.** *Solid arrows* indicate direct enzymatic conversions. *Dashed arrows* indicate conversions that require more than one enzymatic step. The metabolic pathway of PC synthesis and turnover influenced by CWI-MAPK signaling is shown in *boldface*. The phospholipases catalyzing phospholipid turnover are shown adjacent to the *arrows*: *PLB*, phospholipase B; *PLC*, phospholipase C, *PLD*, phospholipase D. Soluble phospholipid precursors and turnover products are as follows: *Cho*, choline; *Cho-P*, choline phosphate; *CDP-Cho*, cytidinediphosphate choline; *Glu-6-P*, glucose 6-phosphate; *Gro-P*, glycerol phosphate; *GroPCho*; glycerophosphocholine; *Ins*, inositol; Ins-3-P, inositol 3-phosphate; Ins_{ext}, inositol externally added; *IPs*, inositol polyphosphates. Phospholipids used are as follows: *CDP-DAG*, cytidinediphosphate diacylglycerol; *PA*, phosphatidic acid; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; *PI(4)P*, phosphatidylinositol 4-phosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; *PDME*, phosphatidyldimethylethanolamine; *PMME*, phosphatidylmonomethylethanolamine; *PS*, phosphatidylserine. Neutral lipids used are as follows: *DAG*, diacylglycerol; *FFA*, free fatty acid; *TAG*, triacylglycerol.

YKL161C, PRM5, PRM10, CRH1, MPK1, SED1, IME2, HSP150, CCW14, PIR3, BGL2, GIC2, and *YPS5.*

Unexpectedly, we observed down-regulation of *MPK1* in experiment 2, which measured the differential gene expression of *mpk1*Δ cells grown in I^-C^+ compared with *mpk1*Δ cells grown in I^+C^- medium (Fig. 7). This result can be explained by the method whereby the *MPK1* open reading frame was disrupted in the DL455 *mpk1*Δ strain (6) used in our study. The *TRP1* gene replaces approximately three-quarters of the 3′ end of the *MPK1* open reading frame, while leaving its remaining 5′ end and upstream regulatory sequences intact. Thus, the truncated *MPK1* gene is transcribed in the *mpk1*Δ strain and can efficiently hybridize with the *MPK1* cDNA printed on the microarray slides used in our study.

In addition, nine Mpk1p-regulated genes were identified that function in cell wall organization and biogenesis but were not previously shown to be Rlm1p target genes, including *GFA1*, *FLC2*, *YPS6*, *KTR6*, *CHS7*, *SIM1*, *SVS1*, *YPK2*, and *CHS6*. Together, these results strongly indicate that a consequence of Mpk1p activation in the absence of inositol is activation of cell wall component gene expression.

To define whether Rlm1p plays a functional role in the activation of Mpk1p-regulated genes in cells grown in I^-C^+ medium, two genes that showed the strongest Mpk1p-dependent regulation in our genome-wide dataset (Fig. 7) were assessed in wild type, *mpk1*Δ and *rlm1*Δ strains. The expression of *MLP1*/*YKL161C* (11, 14) and *YPR078C* was measured using quantitative Northern blot analysis. In agreement with our microarray data, these genes were up-regulated by 6- and 5-fold, respectively, in wild type cells but did not show significant differences in expression in *mpk1*Δ when shifted to I^-C^+ medium (Fig. 8). Importantly, under these same conditions, these genes also failed to show a significant change in expression in the *rlm1*Δ strain (Fig. 8), suggesting that the up-regulation of these genes in response to inositol deprivation through the CWI-MAPK pathway occurs through the Rlm1p transcription factor. To our knowledge, this is the first report of Rlm1p-dependent regulation of *YPR078C*, which encodes a gene of unknown function. In addition, the expression of representative Opi1p-regulated (*INO1*) and UPR-regulated (*KAR2*) genes was monitored in wild type, *mpk1*Δ, and *rlm1*Δ strains. Both *INO1* and *KAR2* were normally activated in all three strains in response to inositol deprivation, showing that their expression is not CWI-MAPK-dependent. Taken together, these results suggest that activation of the CWI-MAPK pathway by shifting to I^-C^+ medium activates the expression of genes, including cell wall genes that are controlled by the Rlm1p transcription factor.

*Levels of PC, DAG, and Triacylglycerol in mpk1*Δ *Cells Significantly Exceed Wild Type Levels following Transfer to I^-C^+ Medium*—Both inositol and choline, which enter lipid metabolic pathways at distinct positions (Fig. 9), dramatically alter the abundance and the pattern of synthesis and turnover of phospholipids (47). Because the transcriptional control of Opi1p- and UPR-regulated genes is CWI-MAPK-independent (Figs. 5, 7 and 8), we speculated that the choline-sensitive Ino^- phenotype of CWI-MAPK mutants is due to defect(s) in lipid metabolism. As no previous studies of lipid metabolism or composition for *mpk1* mutants have been reported, we compared

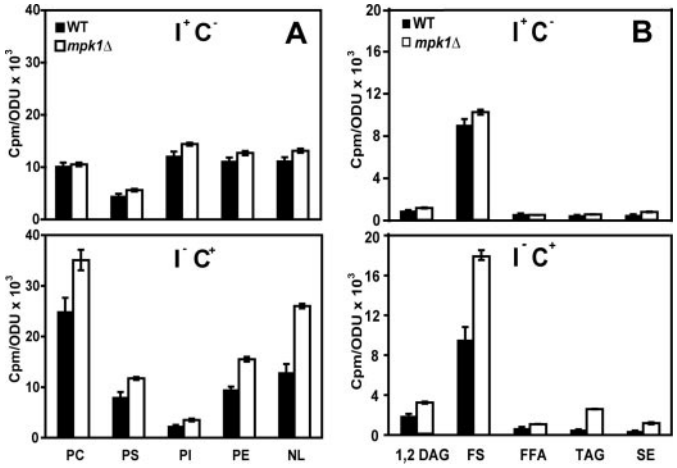

FIGURE 10. **Changes in the pattern of phospholipid and neutral lipid classes in the *mpk1*Δ mutant following the transfer to I^-C^+ medium.** Wild type (*WT*) and *mpk1*Δ strains were grown at 30 °C in I^+C^- medium in the presence of 1 μCi/ml [1-^{14}C]acetate until mid-logarithmic phase. One-half of each culture was harvested for lipid analysis, and the other half was filtered, washed, and resuspended in warm I^-C^+ medium containing 1 μCi/ml [1-^{14}C]acetate and incubated at 30 °C. Samples were harvested at 3.5 h. Lipids were extracted and analyzed as described under "Experimental Procedures." *A,* phospholipid composition: *PC,* phosphatidylcholine; *PS*, phosphatidylserine; *PI,* phosphatidylinositol; *PE*, phosphatidylethanolamine; *NL*, total neutral lipids. *B,* neutral lipid composition: *1,2 DAG*, 1,2-diacylglycerol; *FS*, free sterols; *FFA*, free fatty acids; *TAG*, triacylglycerol; *SE*, sterol esters. Data represent mean ± S.E. of two independent experiments performed in duplicate.

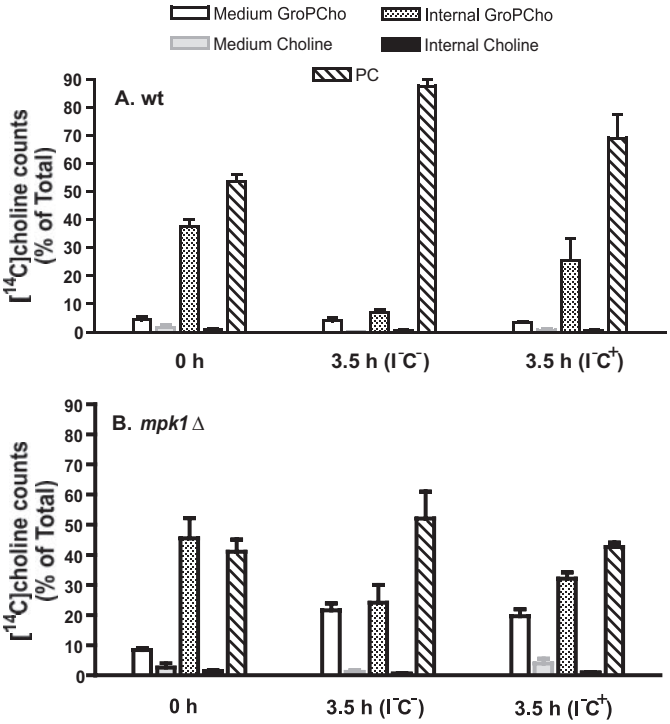

FIGURE 11. **Turnover of PC in wild type and *mpk1*Δ cells.** Wild type (*wt*) (DL100) and *mpk1*Δ (DL455) cells were grown to uniform labeling in medium containing and 18 μM [^{14}C]choline and 75 μM inositol. At time 0, cells were harvested by filtration and inoculated into fresh I^-C^- or I^-C^+. Counts associated with cell membranes, the trichloroacetic acid extract fractions of the cell, or the growth medium were determined as described under "Experimental Procedures." Data represent mean ± S.E. of two independent experiments performed in duplicate.

the phospholipid and neutral lipid composition of *mpk1*Δ cells to wild type before and after a shift to I^-C^+ medium.

Phospholipid and neutral lipid compositions of *mpk1*Δ and wild type cells grown were indistinguishable when grown in I^+C^- medium (Fig. 10). Following the shift to I^-C^+ medium, both wild type and *mpk1*Δ cells showed a decrease in PI and increase in PC levels. The change in wild type phospholipid composition is consistent with the report by Gaspar *et al.* (47). However, the changes in lipid composition observed in *mpk1*Δ cells were more extreme than those in wild type cells. PC content increased more than 3-fold, compared with 2-fold in wild type cells (Fig. 10*A*). Total neutral lipid levels in the *mpk1*Δ strain also increased by ~2-fold by 3.5 h after the shift to I^-C^+ medium (Fig. 10) compared with a slight increase in total neutral lipids in wild type. DAG levels increased at least 3-fold in *mpk1*Δ cells following the shift to I^-C^+ medium, reaching levels close to double those seen in wild type, whereas TAG reached levels 5-fold higher in *mpk1*Δ cells compared with wild type cells following the shift to I^-C^+ medium. The levels of free sterols and sterol esters were approximately doubled in *mpk1*Δ cells shifted to I^-C^+ medium (Fig. 10*B*). The levels of free sterols increased 2-fold, and the levels of sterol esters increased 3-fold in *mpk1*Δ cells compared with wild type cells following the shift to I^-C^+ medium.

*Pattern of PC Turnover Is Altered in mpk1*Δ *Cells*—One explanation for the higher levels of PC and DAG observed in *mpk1*Δ cells after the media shift to I^-C^+ is inappropriate turnover of PC. To examine this possibility, PC and its turnover products, choline and glycerophosphocholine (GroPCho), were quantitatively measured as described under "Experimental Procedures." Wild type and *mpk1*Δ cells were labeled with [^{14}C]choline to steady state in I^+C^- medium and subsequently transferred to unlabeled medium lacking inositol (I^-C^- or I^-C^+). Fig. 11 shows the distribution of internal and external [^{14}C]choline label in wild type and *mpk1*Δ cultures immediately following transfer (0 h) and 3.5 h after transfer to unlabeled medium.

In wild type cells, immediately after transfer to unlabeled I^-C^- or I^-C^+ medium (0 h), [^{14}C]choline was mainly intracellular, with label partitioned slightly over 50% to PC and nearly 40% GroPCho, the turnover product produced by the deacylation of PC (see Fig. 9 for pathways). After 3.5 h in unlabeled I^-C^- medium, about 90% of the label in wild type cells had chased into PC. Small amounts of GroPCho were detected both internally and externally in the growth medium. In contrast, when shifted to I^-C^+ medium for 3.5 h, 25–30% of total label in wild type cells was detected in intracellular GroPCho, and only about 70% of label was associated with PC, indicating a higher rate of PC turnover when choline is present. These results are consistent with the previous report by Dowd *et al.* (61), who showed that yeast cells exposed to exogenous choline or shifted to 37 °C exhibited higher rates of PC turnover, primarily via a phospholipase B (PLB)-mediated deacylation mechanism. Zaccheo *et al.* (67) subsequently showed that deacylation of PC under these conditions is mediated by Nte1p (see Fig. 9 for the reaction catalyzed by PLB).

In *mpk1*Δ cells, immediately after transfer to unlabeled medium (I^-C^+ or I^-C^-), the ^{14}C label associated with GroPCho represented close to 50% of total label, and label associ-

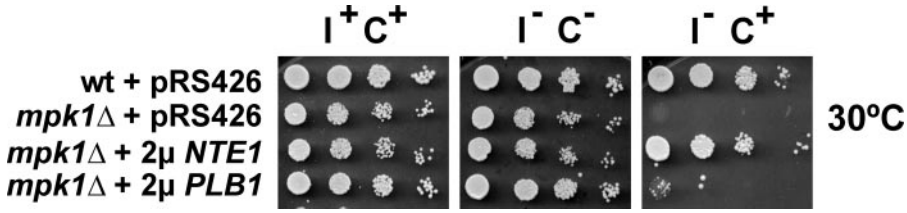

FIGURE 12. **NTE1 overexpression suppresses the choline-sensitive Ino⁻ phenotype of the *mpk1*Δ and the *nte1*Δ and *rlm1*Δ mutants exhibit Ino⁻ phenotypes at 37 °C.** Wild type (*wt*) and *mpk1*Δ cells transformed with pNTE1, pPLB1, or pRS426 were pre-cultured to mid-logarithmic phase of growth (OD_{600} = 0.5) at 30 °C in I^+C^- Ura^- medium, spotted as a 10-fold dilution series on I^+C^+ Ura^- and I^-C^+ Ura^- medium, and incubated at 30 °C for 2 days.

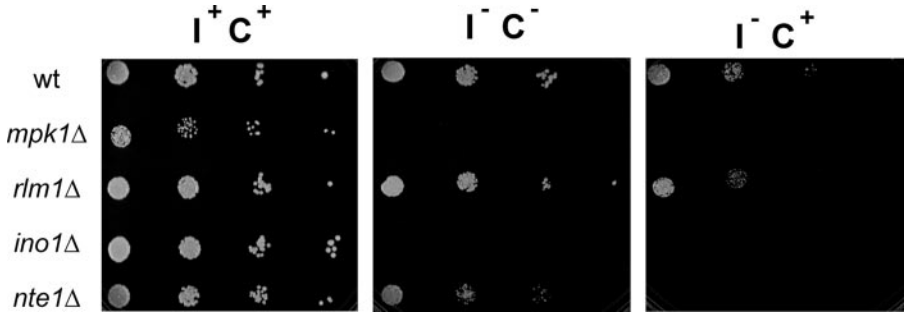

FIGURE 13. **Ino⁻ phenotype of the *rlm1*Δ and *nte1*Δ mutants at 37 °C.** Wild type (*wt*) (BY4742), *mpk1*Δ (SHY932), *rlm1*Δ (SHY933), *ino1*Δ (SYJ425), and *nte1*Δ (SHY934) cells were pre-cultured to mid-logarithmic phase of growth (OD_{600} = 0.5) at 30 °C. Cell suspensions were subsequently spotted as 10-fold dilution series at an initial concentration of 0.5 OD_{660}/ml on I^+C^+, I^-C^-, and I^-C^+ medium and grown for 2 days at 37 °C.

ated with PC represented less than 50%. In other words, in contrast to wild type, label associated with PC was lower, and label associated with GroPCho was higher. After incubation in unlabeled I^-C^- medium for 3.5 h, the proportion of external GroPCho in *mpk1*Δ cells represented more than 20% of total label, compared with about 5% in wild type. Label associated with internal GroPCho represented slightly more than 20%, compared with less than 10% in wild type, whereas label associated with PC increased to only slightly more than 50% of the total label, compared with 90% in wild type cells. Following transfer to I^-C^+ medium for 3.5 h, the proportion of label in PC in *mpk1*Δ cells was changed very little, representing less than 50% of total label compared with 70% in wild type cells. However, internal GroPCho decreased to about 30%, compared with less than 10% in wild type, whereas external GroPCho represented about 20% of total label compared with less than 5% in wild type cultures. Under both growth regimes, one of the most striking differences between *mpk1*Δ and wild type cells was the substantial accumulation of external GroPCho in *mpk1*Δ cultures, a topic that will be taken up under "Discussion."

*Expression of NTE1 from a High Copy Plasmid Suppresses the Ino⁻ Phenotype of mpk1*Δ—Because *mpk1*Δ cells exhibited increased accumulation of PC and an abnormal pattern of PC turnover compared with wild type cells, we explored the effect of overexpression of several genes encoding phospholipases. The *NTE1* gene encodes an ER-localized PLB, known to be responsible for turnover of PC when exogenous choline is present (67). Turnover of PC catalyzed by Nte1p results in the production of free fatty acids and internal GroPCho (Fig. 9). *PLB1* encodes a plasma membrane-localized PLB responsible for production of external GroPCho (68, 69). As shown in Fig. 12, *mpk1*Δ cells carrying *NTE1* on a high copy plasmid are able to grow at 30 °C on I^-C^+ medium, whereas *PLB1* overexpression had minimal, if any, effect. Overexpression of *NTE1* partially rescued *mpk1*Δ growth on I^-C^+ medium even at 37 °C (data not shown and see Ref. 70). Several other genes encoding phospholipases, including *PLB2*, *PLD1* (*SPO14*) ,and *PLC1*, were also tested for their ability to suppress the *mpk1*Δ Ino⁻ choline-sensitive phenotype. None of these genes served as a high copy suppressor of the Ino⁻ phenotype of *mpk1*Δ (data not shown and see Ref. 70).

*nte1*Δ *and rlm1*Δ *Mutants Exhibit Choline-sensitive Inositol Auxotrophy*—Because overexpression of *NTE1* suppressed the Ino⁻ phenotype of *mpk1*Δ, we examined the phenotype of an *nte1*Δ strain. The *nte1*Δ mutant failed to grow at 37 °C on I^-C^+ medium (Fig 13), but it was able to grow on I^-C^- medium at 37 °C and on I^-C^+ medium at 30 °C (data not shown). In contrast, *mpk1*Δ failed to grow at 30 °C on I^-C^+ medium (Fig. 2) or on I^-C^- medium at 37 °C (Fig. 1). Thus, failure to activate Nte1p-mediated turnover cannot fully explain the *mpk1*Δ phenotype.

The *rlm1*Δ mutant also exhibited somewhat reduced growth as compared with wild type on I^-C^+ medium at 37 °C, but it grew normally on I^-C^- medium at 37 °C. This phenotype is not unexpected given that activation of Rlm1p by Mpk1p is necessary for activation of cell wall genes. The phenotype of *rlm1*Δ is less severe than either *nte1*Δ or *mpk1*Δ (Fig. 13), indicating that functions not under Rlm1p control contribute to the choline-sensitive Ino⁻ phenotype of *mpk1*Δ cells.

DISCUSSION

In this study, we demonstrate that PKC signaling is activated and essential for viability in cells grown in medium lacking inositol and containing choline, a growth condition that results in major changes in lipid metabolism (Figs. 10 and 11) (47) Wild type cells grown under these conditions activate the expression of Mpk1p-dependent genes controlled by the Rlm1p transcription factor. Importantly, we provide clear evidence for a novel role for the CWI-MAPK pathway in maintaining lipid homeostasis in yeast. We have shown that cells lacking intact PKC signaling through the Mpk1p kinase exhibit an abnormal pattern of PC turnover and accumulation. *mpk1*Δ cells exhibit abnormally high levels of the PC turnover product GroPCho, both internally and externally and retain a lower proportion of label from choline in PC than wild type cells, both before and after a shift to I^-C^+ medium (Fig. 11). Despite this apparent elevation in PC turnover, *mpk1*Δ cells shifted from I^+C^- medium to I^-C^+ medium at 30 °C accumulate higher overall levels of PC compared with wild type cells and exhibit elevated levels of DAG (Fig. 10), which serves as a precursor to PC in the

CDP-choline (Kennedy) pathway (Fig. 9). These observations suggest that both the synthesis and turnover of PC are perturbed in the absence of PKC signaling. However, in contrast to its effects on cell wall integrity, the mechanism of control of lipid homeostasis by the CWI-MAPK pathway in response to phospholipid precursors is unlikely to be transcriptional, because the regulation of genes involved in lipid metabolism is normal in *mpk1*Δ cells (Figs. 5, 7, and 8).

Ino⁻ Phenotype of Cell Integrity Pathway Mutants Is Not Due to Failure to Derepress INO1—The Ino⁻ phenotype is commonly associated with the failure to derepress the *INO1* gene encoding inositol-3-phosphate synthase (54, 63). Ino1p catalyzes the conversion of glucose 6-phosphate to inositol 3-phosphate, the rate-limiting step in the synthesis of inositol (Fig. 9). *INO1* is the most highly regulated of a set of coregulated genes of lipid metabolism, which are activated by the Ino2p and Ino4p transcription factors (57, 71) and repressed by Opi1p (31, 32, 53, 72). The mechanism of repression of *INO1* and coregulated genes by inositol involves the direct sensing of metabolic flux through the pathway of PI synthesis by Opi1p. In cells growing in the absence of inositol, Opi1p is anchored in the perinuclear ER through a direct interaction with the VAP homolog, Scs2p, via its FFAT motif and phosphatidic acid (PA) (72–74). Both interactions are required for proper ER targeting of Opi1p (72). Upon introduction of inositol into the growth medium, PA is rapidly consumed in the production of CDP-DAG (47), the immediate precursor of PI (Fig. 9), which leads to the translocation of Opi1p from the ER to the nucleus (72) and transcriptional repression of target genes, including *INO1* (31, 32).

Rivas *et al.* (75) reported that *sac1* mutants, which accumulate elevated levels of phosphatidylinositol 4-phosphate, are Ino⁻ despite having normal *INO1* expression. Nevertheless, we were surprised to find that *INO1* derepression of an *INO1-lacZ* reporter gene in the *mpk1*Δ strain was not compromised at 30 °C in I^-C^- medium (Fig. 5). At 25 °C, a temperature at which no impairment of growth was detected in the absence of inositol, *mpk1*Δ cells expressed higher levels of an *INO1* reporter construct than wild type cells (Fig. 5) after being shifted to I^-C^- medium. Microarray experiments (Fig. 7) comparing gene expression in *mpk1*Δ and wild type cells shifted from I^+C^- medium and grown for a further 3.5 h in I^-C^+ medium at 30 °C confirmed that expression of *INO1* and the entire repertoire of Opi1p-regulated genes undergoes normal derepression in *mpk1*Δ cells shifted to I^-C^+ medium. Thus, we conclude that the failure to derepress *INO1* and other Opi1p-regulated genes is not the explanation for the Ino⁻ phenotype of *mpk1*Δ cells. Our results also clearly indicate that CWI-MAPK signaling via Mpk1p is not directly involved in the regulation of Opi1p-regulated genes. Moreover, the microarray experiments depicted in Fig. 7 also clearly show that genes under the control of the UPR response are activated in both wild type and *mpk1*Δ cells when they are shifted to I^-C^+ medium.

Suppression of Ino⁻ phenotypes by *opi1*Δ or overexpression of *INO1* from a high copy plasmid is often interpreted as evidence that the mutants in question have reduced *INO1* expression (54, 63). However, it is important to emphasize that expression of *INO1* at levels achieved by wild type strains grown in the absence of inositol does not support the level of PI synthesis achieved in the same cells grown in the presence exogenous inositol. Wild type cells grown in the presence of inositol have steady state levels of PI that are 5–6-fold higher than PI levels in cells grown in the absence of inositol, whereas levels of PA are reduced overall by at least 50%, and CDP-DAG levels are reduced by 65%. At the same time, overall levels of other phospholipids, including PS and PC, are reduced when inositol is available, whether or not choline is present (47) (Fig. 10). In contrast, *INO1* is constitutively expressed at levels at least 2-fold higher than wild type in *opi1*Δ cells. *INO1* overexpression leads to overproduction and excretion of inositol into the growth medium (52) (Fig. 4). This excessive production of inositol and elevated production of PI in *opi1*Δ strains (76) mimics the provision of exogenous inositol and leads to elevated PI levels. We propose that the alterations in phospholipid metabolism that occurs when cells are grown in the absence of inositol are inherently stressful and that cells require active PKC signaling to survive under these circumstances.

Growth in the Absence of Inositol Activates CWI-MAPK Pathway Signaling—In wild type cells grown for 3.5 h at 30 °C in I^-C^+ medium, transient phosphorylation of Mpk1p was observed (Fig. 6). In *mpk1*Δ cells carrying a kinase-dead *mpk1* allele, this signaling was intensified and protracted (Fig. 6). The subsequent failure to grow and loss of viability by *mpk1*Δ cells (supplemental Fig. S3) indicates that the signal transmitted by Mpk1p to downstream targets is essential for survival when cells are grown in I^-C^+ medium. Presumably, phosphorylation of Mpk1p in *mpk1*Δ cells carrying the wild type *MPK1* gene is transient (Fig. 6) because the signaling results in the maintenance or restoration of cellular homeostasis via activation of Mpk1p targets. Consistent with activation of CWI-MAPK signaling in wild type cells at 3.5 h following transfer to I^-C^+ medium, microarray analysis revealed up-regulation of a number of genes known to respond to PKC signaling via the Mpk1p target Rlm1p (11, 14) (Fig. 8). These results explain why two previous microarray experiments from our laboratory (31, 32) did not detect a change in expression of genes controlled by the CWI-MAPK pathway in response to inositol or choline. Neither of these microarray experiments were carried out in a manner that could have detected transcriptional responses to a signal occurring transiently at 3.5 h following a shift to I^-C^+ medium. The first study (31) examined steady state differences in transcript abundance in wild type cells in response to inositol and choline, whereas the second study (32) examined the kinetics of changes in genome-wide expression for an interval of 2 h following the introduction of inositol to cells acclimated to growth in I^-C^+ medium.

Many of the Mpk1p target genes that are activated in wild type cells following the shift to I^-C^+ medium are targets of Rlm1p, including *MLP1/YKL161C*, *CCW14*, *PRM5*, *PRM10*, *CRH1*, *MPK1*, *SED1*, *IME2*, *HSP150*, *CCW14*, *PIR3*, *BGL2*, *GIC2*, and *YPS5* (11), most of which are involved in cell organization and biogenesis. We also demonstrate for the first time that *YPR078C* is regulated in an Mpk1p- and Rlm1p-dependent manner (Fig. 8). Interestingly, a number of Mpk1p-regulated genes activated in I^-C^+ medium are involved in MAP kinase function, suggesting that this pathway may be regulated tran-

scriptionally under these growth conditions. These genes include *MPK1* itself, *PTP2*, a phosphotyrosine-specific phosphatase known to negatively regulate Mpk1p (77), and *MLP1*, a catalytically inactive paralog of Mpk1p (13, 78). Moreover, a recent report by Levin and co-workers (78) showed that Mpk1p and Mlp1p independently function as transcriptional coactivators of *FKS2* expression during cell wall stress through a non-catalytic mechanism. The *FKS2* gene encodes the catalytic subunit of 1,3-β-glucan synthase, which synthesizes the major component of the yeast cell wall. Although *FKS2* is not regulated by Rlm1p (11), nor was it detected as an Mpk1p target gene in this study, it was highly up-regulated following the shift to I^-C^+ medium in both wild type and *mpk1*Δ cells (data not shown). This finding suggests that up-regulation of either *MPK1* or *MPL1* expression under these growth conditions may activate the transcription of additional cell wall genes that are not under the control of Rlm1p and may explain why the Ino^- phenotype of *rlm1*Δ mutants is less severe than *mpk1*Δ mutants (Fig. 13).

Earlier studies of the effect of inositol starvation in *ino1* mutants, which are completely unable to synthesize inositol *de novo*, revealed defects in the synthesis of β-glucans and mannans involved in cell wall biosynthesis (79, 80). Thus, we considered the possibility that the growth defects of *mpk1*Δ cells in the absence of inositol were due to a slowing of synthesis of cell wall components, because of the dependence of these processes on PI synthesis. Consistent with this idea, PI synthesis is reduced even in wild type cells forced to rely on endogenous inositol biosynthesis (Fig. 10) (47). According to this hypothesis, cells grown in the absence of exogenous inositol require active PKC signaling through Mpk1p and Rlm1p to up-regulate cell wall target genes, which restores cellular homeostasis under conditions of reduced PI synthesis. However, the Ino^- phenotype of the *rlm1*Δ mutant is less severe than that of the *mpk1*Δ mutant (Fig. 13). This indicates that failure to activate cell wall genes in the absence of inositol most likely contributes to the Ino^- phenotype of *mpk1*Δ cells, but it is not the entire explanation. The fact that cell wall biosynthesis is defective in cells that are incapable of synthesizing inositol is consistent with the transient activation of PKC signaling observed in wild type cells deprived of inositol.

Active CWI-MAPK Signaling via Mpk1p Is Essential for Normal PC Turnover—In addition to defects in activation of cell wall genes, the *mpk1*Δ mutant exhibits elevated turnover of PC, which we detected as external GroPCho. The levels of external GroPCho observed in *mpk1*Δ cultures following the shift to unlabeled medium exceeded the levels seen in wild type cultures by 4–5-fold in cells grown in I^-C^- or I^-C^+ medium (Fig. 11). Although internal GroPCho is the breakdown product produced when PC is deacylated by Nte1p in the ER (67), external GroPCho is primarily produced via the action of Plb1p, which is localized to the plasma membrane and periplasmic space (68, 69). Thus, the presence of elevated levels of external GroPCho suggests that elevated turnover of PC is occurring at the plasma membrane. Yet, of the phospholipase genes overexpressed and tested for suppression of the choline-sensitive Ino^- phenotype of *mpk1*Δ cells, *NTE1* was the strongest high copy suppressor. *PLB1* overexpression caused a minimal improvement in growth (Fig. 12), and *PLD1*, *PLB2*, and *PLC1* overexpression had no effect (data not shown and see Ref. 70). These data suggest that increased PC turnover in *mpk1*Δ cells most effectively mitigates the toxic effects of excess PC in the ER, the site of PC synthesis via the Kennedy pathway, which is the predominant route of PC synthesis in the presence of exogenous choline (Fig. 9). Furthermore, the *nte1*Δ strain is unable to grow at 37 °C in I^- medium when choline is present (Fig. 13) indicating that Nte1p is essential under these growth conditions, which are also the conditions under which *mpk1*Δ cells fail to grow.

Because overexpression of *NTE1* suppresses the *mpk1*Δ phenotype, it is tempting to speculate that the failure to sufficiently activate Nte1p is at the root of at least some of the pleiotropic changes in lipid metabolism observed in *mpk1*Δ cells. However, *mpk1*Δ cells showed aberrant PC turnover, even under conditions where Nte1p is not essential in wild type cells, such as at 30 °C in I^+ medium (Fig. 11). Under these growth conditions, less label from choline was retained in PC in *mpk1*Δ cells, and abnormally high levels of both external and internal GroPCho were detected (Fig 11*B*, 0-time point, compare with wild type, Fig. 10*A*). These results suggest increased turnover of PC is associated with signaling via Mpk1p. Recently, Chen *et al.* (81) reported that PKC activation in human neuroblastoma cells using phorbol 12-myristate 13-acetate leads to down-regulation of Nte1p protein levels, enzymatic activity, and *NTE1* mRNA levels. The regulation reported by Chen *et al.* (81) therefore should lead to decreased PC turnover catalyzed by Nte1p in response to PKC signaling. The apparent increase in PC turnover observed in *mpk1*Δ cells would be consistent with a similar mechanism in yeast. However, the suppression of the choline-sensitive Ino^- phenotype of the *mpk1*Δ mutant by high copy expression of *NTE1* is not consistent with this model. Moreover, *NTE1* does not appear to be a target of transcriptional regulation mediated by the CWI-MAPK pathway signaling in yeast, because no change in its expression was detected in our microarray experiments (Fig. 7). We suggest that overexpression of *NTE1* suppresses the Ino^- phenotype of the *mpk1*Δ mutant by counteracting the toxic buildup of PC that occurs in the ER of *mpk1*Δ grown in the absence of inositol, especially if choline is present.

PKC Signaling via Mpk1p Is Essential to Lipid Homeostasis When Inositol Is Limiting, Especially When Choline Is Present—Despite elevated turnover of PC in comparison with wild type cells, *mpk1*Δ cells shifted to I^-C^+ medium showed a significantly greater overall increase in the level of PC, indicating that both synthesis and turnover of PC are elevated in *mpk1*Δ cells. Moreover, DAG levels were almost double, and TAG levels were 5-fold higher than wild type (Fig. 10). These observations indicate that CWI-MAPK signaling is essential for maintaining the relative flow of lipid intermediates at crucial branch points in lipid biosynthesis (Fig. 9). One of these branch points is the flow of PA into CDP-DAG, the immediate precursor of both PI and PS. PS then serves as a precursor to both phosphatidylethanolamine and PC (Fig. 9). Several previous studies have shown that the presence of exogenous inositol causes a major increase in PI synthesis, depleting both PA and CDP-DAG pools (46, 47, 72), resulting in elevated levels of PI and decreased levels of PC (47). In contrast, when inositol is absent, especially when cho-

line is present, PI levels are low and PC levels are elevated (47). Under these conditions, DAG levels are also elevated, even in wild type cells, suggesting that excess PA that is not used in PI or PS synthesis is dephosphorylated to form DAG, which in turn can drive PC or TAG synthesis or both. When choline is present it also stimulates increased PC turnover (61) mediated by Nte1p in the ER (67). Elevated Nte1p-mediated PC turnover also occurs in cells grown at 37 °C (61, 67).

What do these observations concerning lipid metabolism tell us about the root cause of the choline-sensitive Ino^- phenotype of *mpk1*Δ? In both wild type and *mpk1*Δ cells, inositol offsets the accumulation of PC by drawing PA via CDP-DAG into increased PI synthesis, reducing the availability of PA to serve as a precursor to DAG, and subsequently PC and TAG (Fig. 9). However, in I^-C^+ medium the presence of choline is expected to drive higher rates of PC synthesis via the Kennedy pathway, whereas the absence of inositol leads to high PA levels, leading to a higher rate of synthesis of PC through the methylation pathway (Fig. 9). Under these conditions, elevated PC synthesis in *mpk1*Δ cells may overwhelm the ability of the cell to eliminate excess PC, a condition that can be balanced by overexpression of Nte1p.

Our observations also suggest that excess PC is toxic to the cell. Indeed, Xie *et al.* (82) presented evidence that build up of PC is toxic to Golgi function. We propose that when the capacity of the cell to synthesize and eliminate excess PC is exceeded, the CWI-MAPK pathway is activated and required for cell viability. Although the precise targets of CWI-MAPK signaling via Mpk1p that control the delicate balance among divergent pathways using PA and DAG as precursors have not been identified, the mechanisms by which CWI-MAPK signaling might drive this process does not involve transcriptional activation of genes of lipid metabolism (Fig. 7). Clearly the lack of inositol in yeast leads to activation of PKC signaling via Mpk1p through the transcription factor Rlm1p, which leads to up-regulation of cell wall genes. Future work will be directed to elucidating the Mpk1p targets essential to this lipid-mediated signaling.

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