

Interruption of Inositol Sphingolipid Synthesis Triggers Stt4p-dependent Protein Kinase C Signaling*[§]

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The protein kinase C (PKC)-MAPK signaling cascade is activated and is essential for viability when cells are starved for the phospholipid precursor inositol. In this study, we report that inhibiting inositol-containing sphingolipid metabolism, either by inositol starvation or treatment with agents that block sphingolipid synthesis, triggers PKC signaling independent of sphingoid base accumulation. Under these same growth conditions, a fluorescent biosensor that detects the necessary PKC signaling intermediate, phosphatidylinositol (PI)-4-phosphate (PI4P), is enriched on the plasma membrane. The appearance of the PI4P biosensor on the plasma membrane correlates with PKC activation and requires the PI 4-kinase Stt4p. Like other mutations in the PKC-MAPK pathway, mutants defective in Stt4p and the PI4P 5-kinase Mss4p, which generates phosphatidylinositol 4,5-bisphosphate, exhibit inositol auxotrophy, yet fully derepress *INO1*, encoding inositol-3-phosphate synthase. These observations suggest that inositol-containing sphingolipid metabolism controls PKC signaling by regulating access of the signaling lipids PI4P and phosphatidylinositol 4,5-bisphosphate to effector proteins on the plasma membrane.

The protein kinase C (PKC) pathway, also known in yeast as the cell wall integrity pathway, is a highly conserved signal transduction pathway that is activated in *Saccharomyces cerevisiae* during periods of polarized cell growth (1, 2) as well as by numerous environmental stresses, including elevated temperature (3), entry into stationary growth phase (4), and treatment with agents that interfere with cell wall biogenesis (5, 6). Signals produced on the cell surface are amplified and relayed by PKC to downstream targets through a three-component MAPK phosphorylation cascade composed of the MEK kinase Bck1p, the redundant MEKs Mkk1p and Mkk2p, and the MAPK Slt2p (Fig. 1A). Pkc1p homologs in mammals are directly regulated by lipids, including diacylglycerol (DAG)²

produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), anionic phospholipids, and sphingolipids (7, 8). Nevertheless, these lipid metabolites do not appear to play identical roles in yeast PKC activation.

In yeast, the phosphorylated derivatives of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI4P) and PI(4,5)P₂, are essential for PKC signaling during heat stress (9). PI4P is produced on the plasma membrane by the PI 4-kinase Stt4p and is subsequently phosphorylated to PI(4,5)P₂ by the PI4P 5-kinase Mss4p. Stt4p was originally identified in a genetic screen for mutants that are hypersensitive to staurosporine, a specific inhibitor of PKC (10). Mutations in both *STT4* and *MSS4* cause cell lysis phenotypes associated with defects in cell wall integrity signaling, and these defects are suppressed by overexpression of *PKC1* (10, 11). Stt4p-dependent pools of PI4P and PI(4,5)P₂ are proposed to regulate PKC signaling by plasma membrane recruitment of the guanine nucleotide exchange factor Rom2p, where it carries out multiple functions. Rom2p activates the Rho1p GTPase, which is needed to stimulate both Pkc1p protein kinase activity (Fig. 1A) (12) and Fks1p, which is involved in the synthesis of 1,3-β-D-glucan, the main structural component of the cell wall (13–15). In addition, Rom2p interacts with the cell surface sensors, Wsc1p and Mid2p (16), which are required for activation of Rom2p. Moreover, all of these components are localized to sites of PKC activation at the growing bud tip, although their recruitment is not dependent upon Stt4p-dependent pools of PI4P and PI(4,5)P₂ (9).

Additional signaling lipids have been implicated in PKC activation in yeast. Levels of the sphingoid bases, dihydrosphingosine (DHS) and phytosphingosine (PHS), are elevated during heat stress (17, 18) and are proposed to activate the redundant kinases Pkh1p and Pkh2p (Fig. 1A), which encode functional counterparts of the mammalian 3-phosphoinositide-dependent protein kinase 1 (PDK1) (19, 20). Both Pkh1p and Pkh2p phosphorylate Pkc1p *in vitro* (21), and both kinases are required for full activation of Pkc1p in response to heat stress *in vivo* (21). In addition to activating PKC, Pkh1p and Pkh2p phosphorylate and activate a pair of functionally overlapping protein kinases, Ypk1p and Ypk2p (Fig. 1A), which are the functional analogs of mammalian serum- and glucocorticoid-induced protein kinase (22, 23). Ypk1p and Ypk2p may act directly in activating Slt2p or may function in a parallel pathway to control cell wall integrity (24). However, the relative contributions of sphingoid bases on PKC activation has not been rigorously tested *in vivo*.

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² The abbreviations used are: DAG, diacylglycerol; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PI, phosphatidylinositol; PI4P, phosphatidylinositol 4-phosphate; DHS, dihydrosphingosine; PHS, phytosphingosine; AbA, aureobasidin A; FLARE, fluorescent lipid-associated reporters PH, pleckstrin homology; ER, endoplasmic reticulum; PI3P, phosphatidylinositol 3-phosphate; IPC, inositol phosphoceramide; MIPC, mannosyl-IPC; M(IP)₂C, mannosyl-di-IPC; PM, plasma membrane.

Previously we reported that lipid metabolism is substantially altered in yeast cells grown in the absence of inositol, including a significant reduction in PI (25). These metabolic changes are accompanied by activation of numerous signaling pathways, including PKC signaling (26–28). Moreover, mutants defective in PKC signaling are inositol auxotrophs (Ino[−] phenotype) (Fig. 1A) (26, 92), a phenotype that is often associated with reduced *INO1* transcription (30). *INO1* encodes inositol-3-phosphate synthase, which catalyzes the rate-limiting step in inositol synthesis, and is required for cell growth in medium lacking inositol. However, the *slt2Δ* mutant exhibits normal regulation of *INO1* and co-regulated UAS_{INO}-containing genes, indicating that misregulation of *INO1* is not responsible for its Ino[−] phenotype. Instead the *slt2Δ* mutant displays severe defects in phospholipid metabolism, suggesting an essential interplay between PKC signaling and lipid homeostasis (26).

In this study, we examined roles that the PI metabolites, PI4P, PI(4,5)P₂, and inositol-containing sphingolipids, play in regulating PKC signaling. We report that like PKC pathway mutants, *stt4^{ts}* and *mss4^{ts}* mutants are inositol auxotrophs. We found that a fluorescent biosensor for PI4P appears on the plasma membrane following inositol starvation in an Stt4p-dependent manner and that this appearance is coincident with the activation of PKC under all conditions tested. In addition, we show that interruption of synthesis of the inositol-containing sphingolipids triggers PKC signaling in a manner that is independent of the buildup of the sphingoid bases DHS and PHS. In agreement with previous reports (31, 32), we show that inositol starvation results in major changes in sphingolipid metabolism, even in wild type cells. Together, these results suggest that inositol-containing sphingolipid metabolism regulates PKC activity by regulating access of protein effectors to signaling lipids on the plasma membrane.

EXPERIMENTAL PROCEDURES

Reagents—Myriocin was purchased from Sigma and stored as a 1 mg/ml stock solution in methanol at 4 °C. Aureobasidin A (AbA) was purchased from Takara Bio Inc. and stored as a 2 mg/ml stock solution in dimethyl sulfoxide at 4 °C. D-Erythrodihydrospingosine and phytosphingosine hydrochloride were purchased from Sigma and stored as 50 mM stock solutions in ethanol at −20 °C. Caffeine was purchased from Fluka Chemika, and calcofluor white (fluorescent brightener 28) was from Sigma. Anti-phospho-44/42 MAPK (Erk1/2) (Thr-202/Tyr-204) (E10) mouse IgG1 monoclonal antibody was purchased from Cell Signaling Technology, Inc. Anti-influenza hemagglutinin (HA) rabbit polyclonal IgG and anti-GFP mouse monoclonal IgG_{2a} were from Santa Cruz Biotechnology. Goat anti-mouse and goat anti-rabbit IgG (H+L)-HRP conjugate antibodies were from Bio-Rad. *myo*-[2-³H]Inositol was purchased from Amersham Biosciences. [³²P]Orthophosphate was purchased from PerkinElmer Life Sciences.

Strains, Plasmids, Media, and Growth Conditions—The strains and plasmids used in this work are listed in Tables 1 and 2. For all double mutant strains constructed in this study,

single mutant strains were mated and sporulated using standard yeast genetic techniques. Tetrads were dissected and spores harboring the appropriate markers were selected. The *pis1^{ts}-1* strain SJY457 was obtained by four consecutive backcrosses of the original parent strain D278-2A (33) into the BY4742 strain background. The *opi1Δ::HIS3MX6* strain SJY90 and *spo14Δ::HIS3* strain YCS388 were constructed by PCR-mediated gene replacement of the entire *OPI1* or *SPO14* open reading frame in BY4742 or SEY6210, respectively, using the template plasmid pFA6a-*HIS3MX6* as described previously (34). Yeast strains were maintained on solid YPD medium (1% yeast extract, 2% bactopectone, 2% glucose) containing 2% agar. Yeast cultures were grown at indicated temperatures in liquid synthetic complete media formulated as described previously (28). Solid medium was the same formulation plus 2% agar. The appropriate amino acids were omitted from synthetic complete medium for selection purposes. Inositol-free medium (I[−]) was synthetic complete medium prepared with yeast nitrogen base lacking inositol as described previously (28). Where indicated, synthetic complete medium was supplemented with 75 μM inositol (I⁺). To starve cells for inositol, the following growth procedure was followed. Cells were pre-grown overnight in selection medium containing 75 μM inositol to late exponential growth phase at 30 °C, and cultures were diluted to A_{600 nm} = 0.1 and grown to A_{600 nm} = 0.4 at 30 °C in synthetic complete medium containing 75 μM inositol. Cells were then collected by filtration, washed with synthetic complete medium lacking inositol, and resuspended in synthetic complete medium containing 0 or 75 μM inositol, and incubated at 30 °C for indicated lengths of time. Temperature-sensitive strains were pre-grown at 25 °C in inositol-containing medium as described above, shifted to synthetic complete medium containing 0 or 75 μM inositol, and incubated at 25 or 37 °C for indicated lengths of time. Myriocin or AbA was added to medium immediately following the shift to 0 or 75 μM inositol at a final concentration of 1 or 2 μg/ml, respectively. In selected experiments, DHS and PHS were added simultaneously with myriocin or AbA at a final concentration of 50 μM.

Analysis of Ino[−] Phenotypes—Yeast strains were grown to mid-logarithmic phase in synthetic complete media containing 75 μM inositol, harvested, washed with water, and resuspended at A_{600 nm} = 1.0. 10-fold serial dilutions were spotted onto plates containing 0 or 75 μM inositol and incubated at the indicated temperature for 2 days. Where indicated, plates contained AbA at a final concentration of 50 ng/ml.

Microscopy—Strains carrying pGFP-2XPH^{Osh2} and pGFP-2XPH^{PLCδ1} or pmCherry-2XPH^{Osh2} and pGFP-Sac1ΔC-2XPH^{PLCδ1} (Table 2) were pre-grown to mid-logarithmic growth phase in medium containing 75 μM inositol and shifted to synthetic complete medium containing 0 or 75 μM inositol. In indicated experiments, myriocin, AbA, DHS, and PHS were added immediately following the media shift. After incubation at indicated temperatures and lengths of time, cells were concentrated by centrifugation and examined by deconvolution fluorescence microscopy using a Deltavision RT microscopy system (Applied Precision, LLC). Cells were

TABLE 1

Strains used in this study

Strain	Genotype	Source or Ref.
BY4741	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	82
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	82
SEY6210	<i>MATα leu2-3, 112 ura3-52 hisΔ200 trp-Δ901 lys2-801 suc2-Δ9</i>	83
SEY6210.1	<i>MATα leu2-3, 112 ura3-52 hisΔ200 trp-Δ901 lys2-801 suc2-Δ9</i>	83
W303-1B	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-2</i>	84
AAV608	SEY6210; <i>rho1Δ::HIS3</i> carrying pRS31 <i>rho1</i> ^{E451} (<i>LEU2</i> CEN6 <i>rho1</i> ^{ts})	9
AAV104	SEY6210; <i>pik1Δ::HIS3</i> carrying pRS314 <i>pik1</i> ^{ts} -83 (<i>TRP1</i> CEN6 <i>pik1</i> ^{ts} -83)	56
AAV1191	SEY6210; <i>fen1Δ::HIS3</i>	70
AAV1192	SEY6210; <i>sur4Δ::HIS3</i>	70
AAV1622	SEY6210; <i>slm1Δ::HIS3</i> <i>slm2Δ::HIS3</i> pRS415- <i>slm1</i> ^{ts} -1 (<i>LEU2</i> CEN6 <i>slm1</i> ^{ts} -1)	85
AAV182	SEY6210; <i>sfk1Δ::HIS3</i>	9
AAV2007	SEY6210; <i>tor2Δ::HIS3</i> pRS415- <i>tor2</i> ^{ts} -1 (<i>LEU2</i> CEN6 <i>tor2</i> ^{ts} -1)	70
AAV202	SEY6210; <i>mss4Δ::HIS3</i> carrying YCplac111 <i>mss4</i> ^{ts} -102 (<i>LEU2</i> CEN6 <i>mss4</i> ^{ts} -102)	43
AAV202	SEY6210; <i>stt4Δ::HIS3</i> carrying pRS415 <i>stt4</i> ^{ts} -4 (<i>LEU2</i> CEN6 <i>stt4</i> ^{ts} -4)	56
AAV243	SEY6210; <i>sac7Δ::HIS3</i>	9
AAV246	SEY6210; <i>wsc1Δ::HIS3</i>	9
AAV249	SEY6210; <i>mid2Δ::HIS3</i>	9
AAV252	SEY6210; <i>fks1Δ::HIS3</i>	9
AAV265	SEY6210; <i>rom1Δ::HIS3</i>	9
AAV267	SEY6210; <i>rom2Δ::HIS3</i>	9
AAV313	SEY6210; <i>lsb6Δ::HIS3</i>	86
DL100	<i>MATα leu2-3,12 trp1-1 ura3-52 his4 can1</i> ^r	87
DL376	<i>MATα leu2-3,12 trp1-1 ura3-52 his4 can1</i> ^r <i>pkc1Δ::LEU2</i>	87
DL455	<i>MATα leu2-3,12 trp1-1 ura3-52 his4 can1</i> ^r <i>mpk1Δ(slt2Δ)::TRP1</i>	88
LGY113	<i>MATα his3 leu2 ura3 sec13</i> ^{ts} -1	89
MFY80	SEY6210.1; <i>SAC1-GFP::HIS3-MX6</i>	48
MTY29	SEY6210; <i>phk1Δ::HIS3</i> <i>pkh2Δ::HIS3</i> carrying pRS415- <i>pkh1</i> ^{ts} (<i>LEU2</i> CEN6 <i>pkh1</i> ^{ts})	This study
MTY44	SEY6210; <i>ypk1Δ::HIS3</i> <i>ypk2Δ::HIS3</i> carrying pRS415- <i>ypk1</i> ^{ts} (<i>LEU2</i> CEN6 <i>ypk1</i> ^{ts})	This study
MTY155	SEY6210; <i>aur1Δ::HIS3</i> + pRS415- <i>aur1</i> ^{ts} -19 (<i>LEU2</i> CEN6 <i>aur1</i> ^{ts} -19)	70
PHY102	SEY6210; <i>vps34Δ::TRP1</i>	90
SEY5188	<i>MATα leu2-3, 112 ura3-52 suc2-Δ9 sec18</i> ^{ts} -1	91
SHY625	<i>MATα ura3 his3 leu2 trp1 lys2 sec14</i> ^{ts} -3	29
SHY966	BY4741; <i>sac1Δ::kanMX6</i>	Invitrogen
SHY968	BY4742; <i>cho2Δ::kanMX6</i>	Invitrogen
SJY39	BY4742; <i>scs2Δ::kanMX6</i>	Invitrogen
SJY90	BY4742; <i>opi1Δ::HIS3MX6</i>	This study
SJY103	BY4742; <i>scs2Δ::kanMX6</i> <i>opi1Δ::HIS3MX6</i>	This study
SJY178	BY4742; <i>sac1Δ::kanMX6</i> <i>opi1Δ::HIS3MX6</i>	This study
SJY185	BY4741; <i>plc1Δ::kanMX6</i>	Invitrogen
SJY306	BY4741; <i>erg6Δ::kanMX6</i>	Invitrogen
SJY350	BY4742; <i>scs3Δ::kanMX6</i>	Invitrogen
SJY425	BY4742; <i>ino1Δ::HIS3MX6</i>	26
SJY457	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 pis1</i> ^{ts} -1	This study
SJY477	BY4742; <i>ino1Δ::HIS3MX6</i> <i>opi1Δ::kanMX6</i>	This study
SJY763	BY4742; <i>sur2Δ::kanMX6</i>	Invitrogen
SJY764	BY4742; <i>scs7Δ::kanMX6</i>	Invitrogen
SJY765	BY4742; <i>isc1Δ::kanMX6</i>	Invitrogen
SJY782	BY4742; <i>csg2Δ::kanMX6</i>	Invitrogen
SJY783	BY4742; <i>ipt1Δ::kanMX6</i>	Invitrogen
YCS189	SEY6210; <i>sjl1Δ::HIS3</i> <i>sjl2Δ::HIS3</i> <i>sjl3Δ::TRP1</i> <i>inp54Δ::LEU2</i> carrying pRS415 <i>sjl2</i> ^{ts} -8 (<i>LEU2</i> CEN6 <i>sjl2</i> ^{ts} -4)	43
YCS388	SEY6210; <i>spo14Δ::HIS3</i>	This study

TABLE 2

Plasmids used in this study

Name	Description	Source
pJH310	<i>URA3</i> 2μ <i>UAS</i> _{INO} - <i>lacZ</i>	35
pRS426-GFP-2XPH ^{PLC81}	<i>URA3</i> 2μ <i>GFP-2XPH</i> ^{PLC81}	43
pRS416-GFP-2XPH ^{Osh2}	<i>URA3</i> 2μ <i>GFP-2XPH</i> ^{Osh2}	44
pRS425-mCherry-2XPH ^{Osh2}	<i>LEU2</i> 2μ <i>mCherry-2XPH</i> ^{Osh2}	44
pRS426-GFP-Sac1ΔC-2XPH ^{PLC81}	<i>URA3</i> 2μ <i>GFP-Sac1ΔC-2XPH</i> ^{PLC81}	45
pFL44-SLT2HA	<i>URA3</i> 2μ <i>SLT2-HA</i>	2

viewed using a X71 Olympus microscope equipped with a PlanApo 100× objective (1.35 NA, Olympus), FITC, and rhodamine filters and a Cool Snap HQ digital camera (Photometrics). Acquired images were deconvolved using soft-WoRx 3.5.0 software (Applied Precision, LLC).

β-Galactosidase Assays—Cell extracts, prepared from yeast strains carrying the pJH310 reporter plasmid, were tested for β-galactosidase activity using the yeast β-galactosidase assay kit (Pierce) according to the manufacturer's instructions. β-Galactosidase activity was determined by measuring the

conversion of *O*-nitrophenyl β-D-galactopyranoside to *O*-nitrophenol at $A_{420\text{ nm}}$.

Sac1p Microsome Phosphatase Assay—Sac1p phosphatase activity was determined exactly as described previously.³ Briefly, PI4P-containing liposomes were mixed with microsomes prepared from fractionated cell lysates. Phosphate release was measured by the malachite green assay (Enzo Life Sciences) according to the manufacturer's instructions.

Analysis of MAPK Phosphorylation—Slt2p phosphorylation was detected by immunoblotting using an antibody that recognizes active phosphorylated MAPK. Whole cell extracts were prepared from yeast strains carrying pFL44-SLT2HA that were harvested by centrifugation and washed with 20% TCA. Cell pellets were disrupted using glass beads in the presence of 20% TCA. Suspensions were collected and diluted to 10% TCA with water, and precipitated

³ C. J. Stefan, A. G. Manford, D. Baird, J. Yamada-Hanff, Y. Mao, and S. D. Emr, submitted for publication.

Complex Sphingolipids Regulate PKC Activity

proteins were collected by centrifugation. Pellets were washed with acetone, and proteins were solubilized in 1 M Tris, pH 8.0, and 2× SDS-PAGE loading buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 0.2% bromophenol blue). After 5 min at 95 °C, insoluble material was removed by centrifugation, and the supernatant was analyzed by immunoblotting with appropriate antibodies as described previously (26).

Steady State Phosphoinositide Labeling and Analysis—To label inositol-containing phospholipids to steady state, 15 ml of yeast cultures were maintained in logarithmic growth phase ($A_{600\text{ nm}} = 0.5\text{--}0.7$) for seven generations in synthetic complete medium containing 75 μM inositol in the presence of 100 mCi/ml *myo*-[2- ^3H]inositol at a specific activity of 20 Ci/mmol. Cells were harvested by filtration, washed, and resuspended in synthetic complete medium lacking inositol in the absence of radiolabel and incubated at 30 °C. 5-ml aliquots were removed at 0-, 60-, and 120-min time intervals, and TCA was added to a final concentration of 5%. Total lipids were extracted by the addition of ethanol/ether/water/pyridine/ammonium hydroxide (15:5:15:1:0.018, v/v), as described previously (32), followed by Folch extraction of phospholipids in chloroform/methanol (2:1, v/v) as described previously (36). Labeled phospholipids were deacylated in methylamine reagent (methanol, 40% methylamine, water, 1-butanol, 23:13:8:1.1, v/v) and extracted in 1-butanol/diethyl ether/formic acid ethyl ether (23:4:1, v/v) as described previously (37). Samples were resuspended in 10 mM ammonium phosphate dibasic, pH 3.8, and separated using an ion exchange Partisphere 5 SAX column (Whatman) by HPLC (Waters) equipped with a 600 pump system and an in-line BetaRAM® model 3 Radio-HPLC Detector (INUS Systems, Inc.) as described previously (38) with the following minor modifications. The mobile phase consisted of water (A) and a 1 M ammonium phosphate dibasic buffer solution, pH 3.8 (B). All analyses were performed at a flow rate of 1 ml/min using the following program: 99:1 (A/B) 5 min isocratic, gradient from 99:1 to 80:20 in 40 min, gradient from 80:20 to 0:100 in 10 min, and gradient from 0:100 to 99:1 in 10 min. All data were recorded and processed using Millennium software (Waters).

Analysis of Sphingolipids—Sphingolipids were pulse-labeled with 100 $\mu\text{Ci/ml}$ [^{32}P]orthophosphate at a specific activity of 13.5 mCi/mmol phosphate for 30 min in 10-ml yeast cultures grown to mid-logarithmic growth phase ($A_{600\text{ nm}} = 0.5\text{--}0.7$). TCA was added to cultures at a final concentration of 5%, and total lipids were extracted by addition of 0.5 ml of ethanol/ether/water/pyridine/ammonium hydroxide (15:5:15:1:0.018, v/v) as described previously (32). Lipids were deacylated in chloroform/methanol/water (16:16:5, v/v) containing an equal volume of 0.2 N NaOH in methanol as described previously (39). Mixtures were neutralized by the addition of 1 N acetic acid containing 0.5% EDTA (w/v). Nondeacylated lipids, which contain the labeled sphingolipids, were extracted with chloroform, dried under N_2 , and resuspended in chloroform/methanol/water (16:16:5, v/v). Radiolabeled sphingolipids were separated by two-dimensional TLC as described by Stock *et al.* (39). Quantitation was performed following scan-

ning on a STORM 860 PhosphorImager and analyzed using ImageQuant software (GE Healthcare).

RESULTS

Mutants Defective in the *Stt4p* PI 4-Kinase and *Mss4p* PI4P 5-Kinase Are Inositol Auxotrophs—The inositol auxotrophy (Ino^-) phenotype is classically associated with insufficient *INO1* transcriptional derepression during inositol starvation due to regulatory and signaling defects (40). In contrast to previously studied Ino^- mutants, the *slt2 Δ* mutant, which is defective in PKC signaling (Fig. 1A), expresses wild type levels of *INO1* and other co-regulated phospholipid biosynthetic genes, while exhibiting severe defects in lipid metabolism (26). The pleiotropic phenotypes of the *slt2 Δ* mutant suggest that the essential function of PKC signaling during inositol deprivation is maintenance of lipid homeostasis, rather than regulation of *INO1* and co-regulated lipid metabolic genes. An important remaining question is how lipid metabolism is coordinated with PKC signaling in cells deprived of inositol.

The PI-derived lipids, PI4P and PI(4,5) P_2 , which are known regulators of PKC signaling (9), are ideal candidate signaling lipids for coordinating this response. Production of PI4P and PI(4,5) P_2 on the plasma membrane by the PI 4-kinase *Stt4p* and the PI4P 5-kinase *Mss4p* is proposed to recruit and stimulate effector proteins that are required for the initiation of PKC signaling (Fig. 1A) (9). Accordingly, we tested cells deficient in PI4P or PI(4,5) P_2 production for inositol auxotrophy. Each mutant strain carried a temperature-sensitive conditional allele of the essential genes *STT4*, *MSS4*, or *PIK1*, encoding the other major PI 4-kinase (Fig. 1A). Similar to mutants in the PKC pathway, the *stt4 ts* mutant and the *mss4 ts* mutant exhibited strong inositol auxotrophy at a semi-permissive temperature (Fig. 1B), yet showed normal *INO1* derepression, even at the restrictive growth temperature (Fig. 1C). In contrast, the *pik1 ts* mutant did not display a significant Ino^- phenotype at any temperature tested (data not shown), which implies that the individual PI4P pools produced by *Pik1p* and *Stt4p* function independently in cells grown without inositol. Moreover, these results suggest that distinct pools of PI4P and PI(4,5) P_2 are essential for signaling in response to the lipid metabolic changes that occur during inositol starvation.

Biosensor for PI4P Is Enriched on the Plasma Membrane following Inositol Starvation—To monitor the dynamic changes in the pattern of PI4P and PI(4,5) P_2 distribution during inositol starvation, we used two different fluorescent lipid-associated reporters (FLAREs), GFP-2XPH^{Osh2} and GFP-2XPH^{PLC δ 1}, as *in vivo* probes for each phosphoinositide. Both FLAREs contain GFP and tandem pleckstrin homology domains of yeast Osh2p or mammalian PLC δ 1, which bind to PI4P or PI(4,5) P_2 , respectively (41, 42). As reported previously, PI(4,5) P_2 was detected on the plasma membrane (Fig. 2A, left and middle panels) (43). However, comparison of wild type cells grown in the presence or absence of inositol showed no change in localization or distribution pattern of PI(4,5) P_2 (Fig. 2A). In a subset of cells (~5%), PI(4,5) P_2 was detected in structures resembling en-

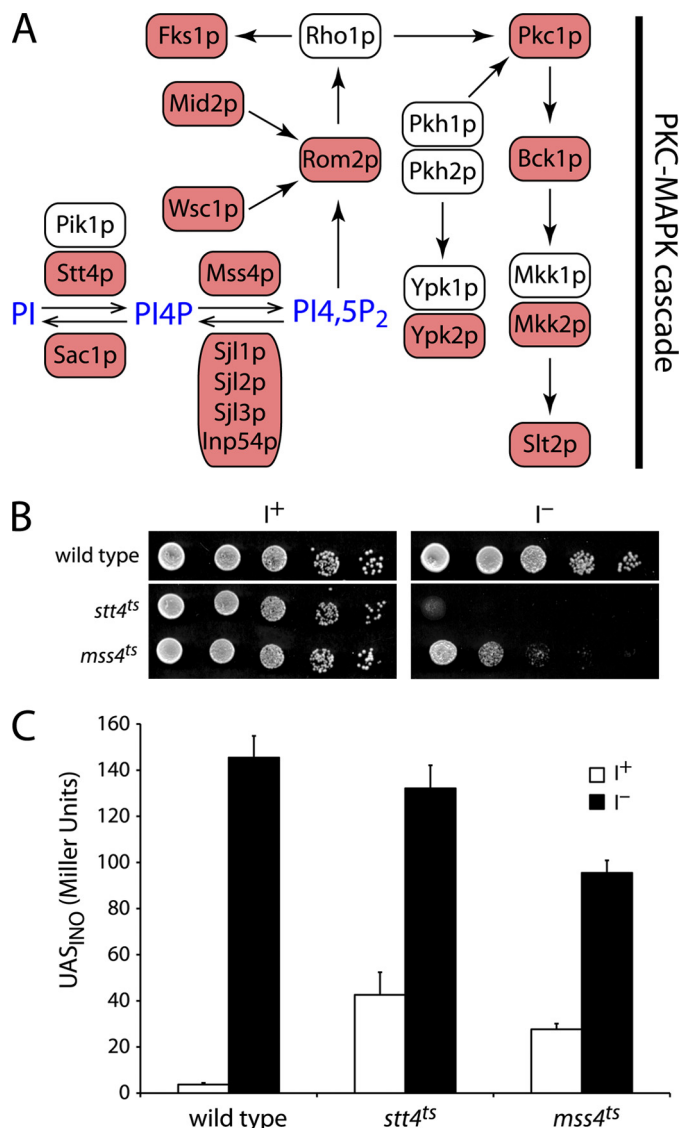


FIGURE 1. Plasma membrane pools of PI4P and PI(4,5)P₂ are required for growth in the absence of inositol. *A*, schematic diagram illustrating the integration of PI4P and PI(4,5)P₂ metabolism with the PKC signaling pathway in yeast. PI kinases and PI phosphatases that catalyze the interconversion of PI-derived lipids (blue) are shown above the open arrows. Interactions (closed arrows) among components (boxes) in the pathway are indicated. Colored boxes denote mutant gene products that exhibit Ino⁻ phenotypes (26)³ (this study). *B*, serial 10-fold dilutions of wild type cells (SEY6210) and isogenic mutant strains (*stt4^{ts}* and *mss4^{ts}*) were spotted on synthetic media containing or lacking 75 μ M inositol. Plates were incubated at 30 °C for 2 days. *C*, wild type (SEY6210) and isogenic mutant strains (*stt4^{ts}* and *mss4^{ts}*), expressing the UAS_{INO}-lacZ reporter from pJH310, were pre-grown at 25 °C in synthetic media containing 75 μ M inositol. At mid-logarithmic growth phase, cells were pelleted, washed, and resuspended in media with (I⁺) or without (I⁻) inositol. Following the medium shift, cells were incubated at 37 °C for 2.5 h. β -Galactosidase activity was measured as described under "Experimental Procedures." The average values and standard deviation of three independent experiments are shown.

dosomes following inositol starvation (Fig. 2A, right panel). Because accumulation of PI(4,5)P₂ in these structures has been correlated with overproduction of PI(4,5)P₂ (43), this observation suggests that PI(4,5)P₂ levels may increase following inositol starvation.

In contrast to PI(4,5)P₂, a striking change in the pattern of PI4P distribution was observed in cells grown in the pres-

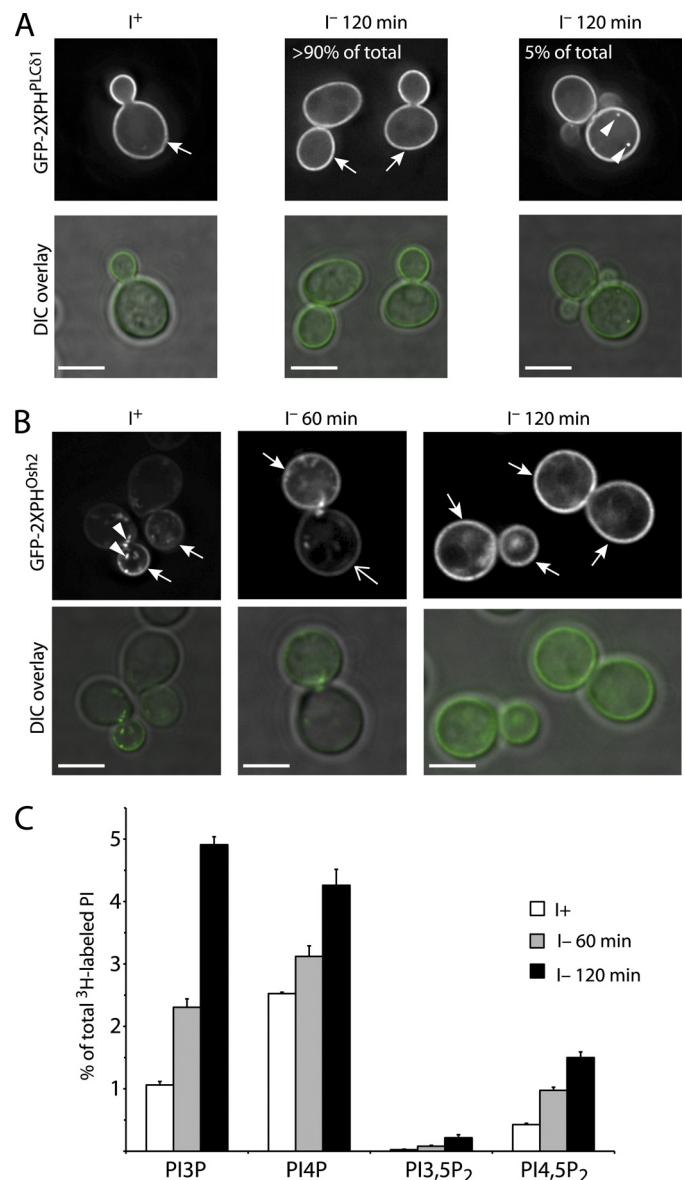


FIGURE 2. PI4P biosensor is enriched on the plasma membrane following inositol starvation. *A*, wild type (BY4742) cells, expressing a PI(4,5)P₂-binding probe (GFP-2XPH^{PLCδ1}) from the constitutive PRC1 promoter carried on a 2 μ plasmid (pRS426), were pre-grown at 30 °C in synthetic media containing 75 μ M inositol. At mid-logarithmic growth phase, cells were harvested by filtration, washed, and resuspended in medium with (I⁺) or without (I⁻) inositol. Cells were subsequently incubated for 120 min at 30 °C and examined by fluorescence microscopy. Images in the left and middle panels represent >90% of cells observed. Closed arrows indicate plasma membrane. The image in the right panel represents ~5% of cells growing in the absence of inositol. Arrowheads point to endosomes. Overlay with differential interference contrast (DIC) image is shown. Scale bar, 5 μ m. *B*, wild type cells (BY4742), constitutively expressing a PI4P-binding probe (GFP-2XPH^{Osh2}) from pRS426-PRC1, were pre-grown, harvested, washed, and resuspended in I⁺ or I⁻ media as described in A. Following the medium shift, cells were incubated at 30 °C and examined by fluorescence microscopy at the indicated times. Images are representative of >95% of cells observed. Arrowheads indicate Golgi. Closed arrows show polarized plasma membrane distribution in bud tips (I⁺ and I⁻ 60 min) and uniform plasma membrane distribution in mother and daughter cells (I⁻ 120 min). Open arrow shows early appearance in plasma membrane. Scale bar, 5 μ m. *C*, wild type (BY4742) cells were labeled to steady state with myo-[³H]inositol in the presence of 75 μ M inositol. One-third of the culture (I⁺) was collected, and lipids were extracted, deacylated, and separated by HPLC. The remaining two-thirds of the culture was harvested by filtration, washed, resuspended in medium lacking inositol (I⁻) without label, and incubated at 30 °C. At 60 and 120 min, cells were harvested and processed as for the I⁺ sample.

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ence *versus* the absence of inositol. In cells grown in the presence of inositol, the PI4P FLARE was localized primarily to Golgi puncta, whereas a small amount of the PI4P probe was detected on the plasma membrane in the growing bud tip (Fig. 2B, I^+), consistent with the previously reported the pattern of PI4P localization (41, 44). Conversely, in cells grown in the absence of inositol, the PI4P FLARE was highly enriched in the plasma membrane in both mother and daughter cells but was less apparent at Golgi compartments (Fig. 2B, I^- 120 min), a pattern hereafter referred to as PM PI4P. Enrichment of PM PI4P was detected ~60 min following transfer of cells to inositol-free medium (Fig. 2B, I^- 60 min) and was more pronounced by 120 min (Fig. 2B, I^- 120 min). We observed this pattern of PM PI4P enrichment following inositol starvation in wild type cells of four different strain backgrounds (Table 3). In cells maintained in exponential growth phase in the absence of inositol (12–15 generations), PM PI4P was detected, albeit to a lesser extent than after 2–3 h of inositol starvation, suggesting that the PM PI4P FLARE may reflect transient signaling during inositol starvation (supplemental Fig. S1). Consistent with this idea, PM PI4P was diminished within 10–15 min following addition of inositol (supplemental Fig. S1), which correlates with the rapid burst in PI synthesis that is induced under these conditions (25). Together, these results suggest that the PM PI4P pool is dynamic and sensitive to changes in PI metabolism.

To confirm that the appearance of the PI4P FLARE on the plasma membrane is due to the detection of PI4P by the GFP-PH^{Osh2} biosensor, we utilized a plasma membrane-targeted PI-4-phosphatase fusion construct that selectively degrades PI4P on the plasma membrane (45). The GFP-Sac1ΔC-2XPH^{PLCδ1} chimera contains GFP, the Sac1 phosphatase domain of yeast Sac1p, and pleckstrin homology domain of mammalian PLCδ1, which targets to PI(4,5)P₂ on the plasma membrane. In cells co-expressing GFP-Sac1ΔC-2XPH^{PLCδ1} and mCherry-2XPH^{Osh2}, the PI4P FLARE did not localize to the plasma membrane during inositol starvation but instead localized primarily to the Golgi compartment (supplemental Fig. S2). These data suggest a pool of PI4P on the plasma membrane increases in accessibility to the PI4P biosensor during inositol starvation.

Plasma Membrane Enrichment of the PI4P FLARE Requires Stt4p—To determine whether the appearance of the PI4P FLARE on the plasma membrane following inositol starvation is due to PI4P synthesized directly on the plasma membrane or by trafficking of PI4P from the Golgi to the plasma membrane, we examined the distribution of PI4P in strains carrying temperature-sensitive alleles encoding the two essential PI 4-kinases, Stt4p and Pik1p. Although Stt4p produces PI4P from PI in the plasma membrane (9), Pik1p produces PI4P at the Golgi that is required for Golgi-to-plasma membrane vesicle-mediated transport (46). When starved for inositol at the permissive temperature, PM PI4P was detected in both mutants (Fig. 3A). However, PM PI4P was not detected in the *stt4^{ts}* mutant at the restrictive temperature in inositol-starved cells, whereas PM PI4P in the *pik1^{ts}* mutant was similar to wild type (Fig. 3B). These results indicate that the PM PI4P

TABLE 3

Plasma membrane enrichment of PI4P FLARE in selected mutants

Mutation(s)	Growth Medium					
	I^+			I^-		
Wild type						
BY4742	+/a	+/b	+++ ^c	+++ ^a	+++ ^b	+++ ^c
SEY6210	+/a	+/b	+++ ^c	+++ ^a	+++ ^b	+++ ^c
DL100		+/b			+++ ^b	
W303		+/b			+++ ^b	
PKC Pathway						
<i>fks1Δ</i>		+/b			+++ ^b	
<i>mid2Δ</i>		+/b			+++ ^b	
<i>pkc1Δ</i>	+/a [*]	+/b [*]	+++ ^c		N.T.	
<i>pkh1^{ts} pkh2Δ</i>	+/b	+++ ^c		+++ ^b	+++ ^c	
<i>rho1^{ts}</i>	+/a	+++ ^c		+++ ^a	+++ ^c	
<i>rom1Δ</i>		+/b			+++ ^b	
<i>rom2Δ</i>		+/b			+++ ^b	
<i>sac7Δ</i>		+/b			+++ ^b	
<i>slt2Δ</i>		+/b			+++ ^b	
<i>wsc1Δ</i>		+/b			+++ ^b	
<i>ypk1^{ts} ypk2Δ</i>	+/b	+++ ^c		+++ ^b	+++ ^c	
Phosphoinositide Metabolism						
<i>lsb6Δ</i>		+/b			+++ ^b	
<i>mss4^{ts}</i>	+/a	– ^c		+++ ^a	– ^c	
<i>pik1^{ts}</i>	+/a	+++ ^c		+++ ^a	+++ ^c	
<i>sac1Δ</i>		+++ ^b			+++ ^b	
<i>sac1Δ opi1Δ</i>		+++ ^b			+++ ^b	
<i>sjf1Δ</i>		+/b			+++ ^b	
<i>sjl1Δ sjl2^{ts} sjl3Δ sjl4Δ</i>	+/a	+/b	+++ ^c	+/a	+++ ^b	+++ ^c
<i>stt4^{ts}</i>	+/a	– ^c		+/a	– ^c	
<i>vps34Δ</i>		+/b			+/b	
Phospholipid Metabolism						
<i>cho2Δ</i>		+/b			+/b	
<i>ino1Δ</i>		+/b			+++ ^b	
<i>opi1Δ</i>		+/b			+/b	
<i>opi1Δ ino1Δ</i>		+/b			+++ ^b	
<i>ple1Δ</i>		+/b			+++ ^b	
<i>pis1^{ts}</i>		+++ ^b			+++ ^b	
<i>scs2Δ</i>		+++ ^b			+++ ^b	
<i>scs2Δ opi1Δ</i>		+/b			+/b	
<i>spo14Δ</i>		+/b			+++ ^b	
Sphingolipid Metabolism						
<i>aur1^{ts}</i>	+/a	+++ ^b	+++ ^c	+++ ^a	+++ ^b	+++ ^c
<i>csg2Δ</i>		+/b			+++ ^b	
<i>fen1Δ</i>		+/b			+++ ^b	
<i>ipt1Δ</i>		+/b			+++ ^b	
<i>isc1Δ</i>		+/b			+++ ^b	
<i>scs7Δ</i>		+/b			+++ ^b	
<i>sur2Δ</i>		+/b			+++ ^b	
<i>sur4Δ</i>		+/b			+++ ^b	
Secretory Pathway						
<i>sec13^{ts}</i>	+/a	+++ ^c		+/a	+++ ^c	
<i>sec14^{ts}</i>	+/a	+++ ^c		+++ ^a	+++ ^c	
<i>sec18^{ts}</i>	+/a	+++ ^c		+++ ^a	+++ ^c	
Other						
<i>erg6Δ</i>		+/b			+++ ^b	
<i>scs3Δ</i>		+/b			+++ ^b	
<i>slm1^{ts} slm2Δ</i>	+/a	+++ ^c		+++ ^a	+++ ^c	
<i>tor2^{ts}</i>	+/a	+++ ^c		+++ ^a	+++ ^c	

^a Growth was at 25 °C for 120 min.

^b Growth was at 30 °C for 120 min.

^c Growth was at 37 °C for 120 min.

^d Growth media were supplemented with 1 M sorbitol.

observed in cells starved for inositol is directly produced on the plasma membrane rather than delivered to the plasma membrane by vesicle-mediated transport from the Golgi.

Given that Stt4p-generated PI4P is the immediate precursor of PI(4,5)P₂ on the plasma membrane (9, 47), we considered whether the appearance of PM PI4P is due to increased turnover via dephosphorylation of PI(4,5)P₂ during inositol starvation. To examine the consequence of blocking PI(4,5)P₂ turnover on PM PI4P, we examined PI4P distribution in a quadruple mutant, *sjl1Δ sjl2^{ts} sjl3Δ inp54Δ*, which lacks all known PI 5-phosphatase activity (43). In cells starved for ino-

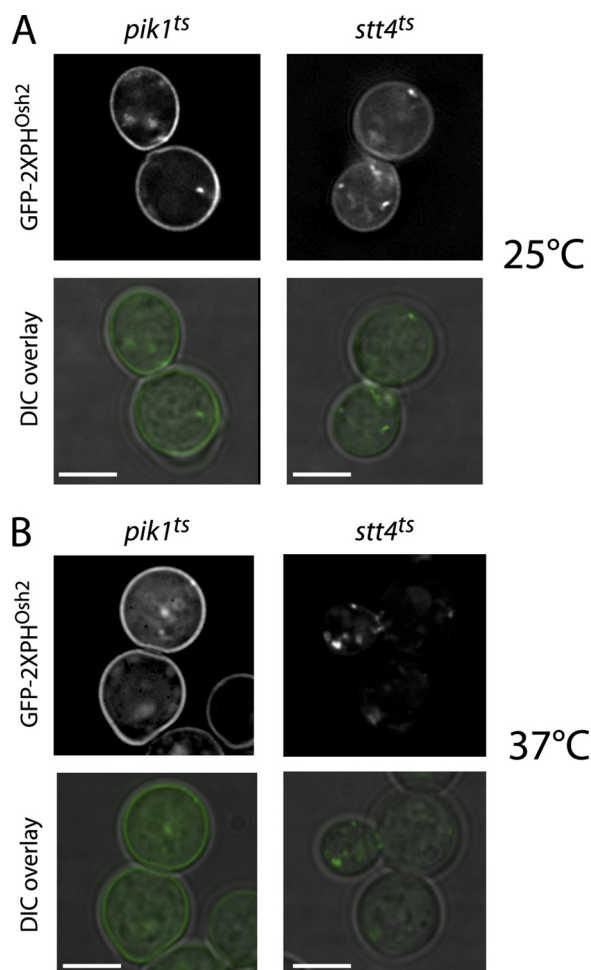


FIGURE 3. Stt4p is required for enrichment of the PI4P biosensor on the plasma membrane. *A*, isogenic mutant strains (*pik1^{ts}* and *stt4^{ts}*) expressing a PI4P-binding probe (GFP-2XPH^{Osh2}) from a constitutive promoter were pre-grown at 25 °C in synthetic media containing 75 μ M inositol. At mid-logarithmic growth phase, cells were harvested by filtration, washed, and resuspended in medium with (I^+) or without (I^-) inositol. Cells were subsequently incubated for 120 min at 25 °C and examined by fluorescence microscopy. Scale bar, 5 μ m. *B*, same as *A*, except cells were shifted to 37 °C for 15 min prior to harvesting. Following media shift, cells were incubated at 37 °C for 120 min and examined by fluorescence microscopy. DIC, differential interference contrast.

sitol, PM PI4P was detected at the restrictive temperature in the quadruple 5-phosphatase mutant (Table 3), suggesting that PI(4,5)P₂ turnover is not required for generating PM PI4P during inositol starvation.

We noted that the enrichment of PM PI4P following inositol starvation phenocopies the plasma membrane accumulation of PI4P that is observed in loss-of-function *sac1* mutants (41).⁴ Because the bulk of Stt4p-produced plasma membrane PI4P is turned over by the PI 4-phosphatase Sac1p (48), we asked if regulation of Sac1p function might account for appearance of the PM PI4P FLARE observed during inositol starvation. However, we did not detect any change in overall PI 4-phosphatase activity in membranes isolated from wild type cells grown in the absence or presence of inositol (data not shown). Next, we asked if Sac1p localization changes following inositol starvation. Endoplasmic reticulum (ER)-localized Sac1p is proposed to control levels of PI4P on the plasma membrane in *trans* through membrane contact sites between

the ER and plasma membrane (49).⁴ Given that Sac1p is known to traffic from the ER to the Golgi following glucose deprivation (50, 51), we hypothesized that relocalization of Sac1p from the ER-plasma membrane contact sites might lead to a buildup of PM PI4P. In cells grown in the presence of inositol, Sac1p-GFP was localized to the ER and Golgi (supplemental Fig. S3A), as reported previously (48, 50–52). Following inositol starvation, GFP-Sac1p was present in the ER and Golgi as well as the vacuole, suggesting that a portion of Sac1p traffics to the vacuole following inositol starvation. Because GFP is not degraded by vacuolar hydrolases (53), quantitation of free GFP released from a chimera following delivery to the vacuole is a convenient method for estimating the amount of a given protein that enters the vacuole. We estimate that ~10% of total Sac1p-GFP is delivered to the vacuole following inositol starvation (supplemental Fig. S3B), suggesting that only a minor portion of Sac1p may traffic from the ER, away from ER-plasma membrane contact sites, during inositol starvation. In addition, the PM PI4P FLARE was detected following inositol starvation in *sec13^{ts}* and *sec18^{ts}* mutants blocked in ER export (Table 3), indicating that membrane trafficking was not necessary for the appearance of the PM PI4P biosensor. Although regulation of the PI4P pool in the plasma membrane requires Sac1p (41, 44),⁴ additional mechanisms appear to be necessary for the appearance of the PM PI4P FLARE following inositol starvation.

Relative Levels of PI4P and PI(4,5)P₂ Increase following Inositol Starvation—Next we measured phosphoinositide levels in wild type cells following inositol starvation. Cells were labeled to steady state with *myo*-[³H]inositol, shifted to media lacking inositol, and labeled lipids were analyzed by HPLC at selected time points. PI4P levels were slightly elevated by 1.2-fold at 60 min and increased to 1.7-fold at 120 min, in agreement with the observed enrichment of the PM PI4P reporter (Fig. 2C). Similarly, PI(4,5)P₂ levels were increased by 2.3-fold at 60 min and 3.5-fold at 120 min (Fig. 2C). Together, these results are consistent with the observation that elevation of PI4P and PI(4,5)P₂ levels contribute to activation of PKC signaling during inositol starvation (see below). We noted that levels of PI3P and phosphatidylinositol 3,5-bisphosphate were also elevated following inositol starvation (Fig. 2C). However, we did not detect any change in the subcellular distribution of either PI3P or phosphatidylinositol 3,5-bisphosphate in wild type cells grown in the presence or absence of inositol utilizing FLAREs for either phosphoinositide (data not shown). Likewise, a previous study reported that accumulation of PI3P in a mutant lacking three PI 3-phosphatases leads to hyperactivation of PKC (54), implicating PI3P buildup in PKC pathway regulation. Nonetheless, *vps34* Δ mutant cells, which lack the sole PI 3-kinase and do not synthesize PI3P and phosphatidylinositol 3,5-bisphosphate (55), are inositol prototrophs (data not shown) and exhibit the PM PI4P FLARE during inositol starvation (Table 3).

Enrichment of PM PI4P Correlates with PKC Activation—The magnitude of PI4P and PI(4,5)P₂ increases that we observed following inositol starvation (Fig. 2C) is similar to the reported increase in both phosphoinositides following heat shock (9, 56), a growth condition also known to induce PKC

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signaling (3, 9). Activation of PKC signaling initiates a phosphorylation cascade that culminates in the phosphorylation of the MAPK Slt2p and subsequent up-regulation of cell wall gene expression (24). This signaling requires the PI 4-kinase Stt4p (Fig. 1A) (9). Because enrichment of the PI4P FLARE on the plasma membrane also requires Stt4p (Fig. 3), we examined the requirement for PI4P and PI(4,5)P₂ production during PKC-MAPK activation initiated in response to inositol starvation by monitoring phosphorylation Slt2p in wild type and *stt4^{ts}* cells. Whereas phosphorylation of Slt2p was not detected in wild type and *stt4^{ts}* cells grown in the presence of inositol, shifting wild type and *stt4^{ts}* cells to media lacking inositol for 2 h at 25 °C resulted in robust phosphorylation of Slt2p (Fig. 4A). As expected, Slt2p phosphorylation was not detected in the *stt4^{ts}* mutant grown at the restrictive temperature in the presence or absence of inositol (Fig. 4B), confirming that production of both PI4P and PI(4,5)P₂ on the plasma membrane is necessary for PKC activation during inositol starvation, similar to other conditions that induce PKC signaling (9). Consistent with previous reports (3, 9), robust Slt2p phosphorylation was observed at 37 °C, even in cells grown in the presence of inositol (Fig. 4B).

Because the PI4P biosensor is enriched on the plasma membrane following inositol starvation, we asked if other reported conditions that activate PKC signaling also lead to the accumulation of the PI4P reporter on the plasma membrane. Consistent with this notion, we also observed enrichment of PM PI4P on the plasma membrane in the presence of inositol under other conditions that induce PKC signaling including, heat stress at 37 °C (Fig. 4C) (3, 9), growth to stationary phase (Fig. 4C) (4), and treatment with caffeine (supplemental Fig. S4) (6), and calcofluor white (supplemental Fig. S4) (5). Moreover, in untreated cells growing the presence of inositol, we detected PM PI4P predominantly in the growing bud (Fig. 2B, I⁺), which is the major site of PKC activation in exponentially growing cells (1, 2). Therefore, we hypothesized that enrichment of PI4P FLARE on the plasma membrane is a marker for cells undergoing PKC activation.

Next, we asked if the signal that leads to the appearance of PM PI4P during inositol starvation is dependent upon components of the PKC signaling pathway (Fig. 1A). Previous studies have shown that a regulator of the pathway, the guanine nucleotide exchange factor Rom2p, binds to PI4P and PI(4,5)P₂ (9). Similar to the PM PI4P FLARE, Rom2p distribution is polarized to yeast cell bud tips but redistributes throughout the cell periphery in mother and daughter cells upon activation of the PKC signaling by heat stress (9). Likewise, localization of Rom2p is dependent upon Stt4p and Mss4p kinase functions (9). Given the similarities between Rom2p relocalization and the distribution of the PM PI4P FLARE, we asked if Rom2p is required for the appearance of the PI4P biosensor on the plasma membrane following inositol starvation. However, the *rom2Δ* mutant did not affect the appearance of PM PI4P during inositol starvation (Table 3). We also tested mutants in PKC pathway components that function downstream of Rom2p (Fig. 1A), including *rho1^{ts}*, *pkc1Δ*, and *slt2Δ* mutants. As in the *rom2Δ* mutant, the appearance of the PI4P FLARE on the

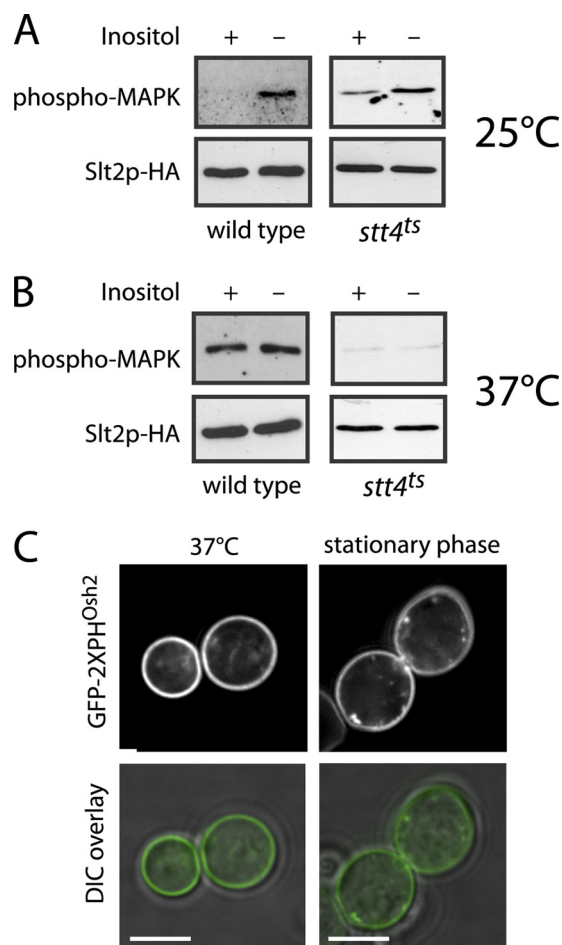


FIGURE 4. Stt4p-dependent enrichment of a PI4P biosensor correlates with protein kinase C activation. A, wild type (SEY6210) and *stt4^{ts}* mutant cells, constitutively expressing HA-tagged SLT2 from a 2 μ vector (pFL44-SLT2HA), were pre-grown at 25 °C in Ura⁻ selective media containing 75 μ M inositol. At mid-logarithmic growth phase, cells were harvested by filtration, washed, and resuspended in Ura⁺ medium with (I⁺) or without (I⁻) inositol. Cells were subsequently incubated for 120 min at 25 °C. Cell extracts 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). B, same as A, except cells were shifted to 37 °C for 15 min prior to media shift. Following media shift, cells were incubated at 37 °C for 120 min, and cell extracts were examined by Western blot as in A. C, wild type (SEY6210) cells, constitutively expressing a PI4P-binding probe (GFP-2XPH^{Osh2}), were grown at 30 °C in synthetic medium containing 75 μ M inositol. Cells shown in the left panel (37 °C) were harvested, shifted to fresh inositol media, and shifted to 37 °C during exponential growth phase. Following incubation for an additional 120 min, cells were examined by fluorescence microscopy. Cells shown in the right panel (stationary phase) were grown at 30 °C until early stationary growth phase and examined by fluorescence microscopy. Scale bar, 5 μ m.

plasma membrane following inositol starvation (Table 3) was identical to wild type cells. Additionally, examination of PKC pathway mutants that function upstream of Rom2p (Fig. 1A), including *mid2Δ* and *wsc1Δ*, revealed no effect on PM PI4P enrichment following inositol starvation (Table 3). We also tested mutants in the functionally redundant PDK1 homologs, Pkh1p and Pkh2p, which phosphorylate Pkc1p (4), and mutants of the functionally redundant serum- and glucocorticoid-induced protein kinase homologs, Ypk1p and Ypk2p. However, like mutants in the PKC signaling pathway, neither *pkh1^{ts} pkh2Δ* nor *ypk1^{ts}*

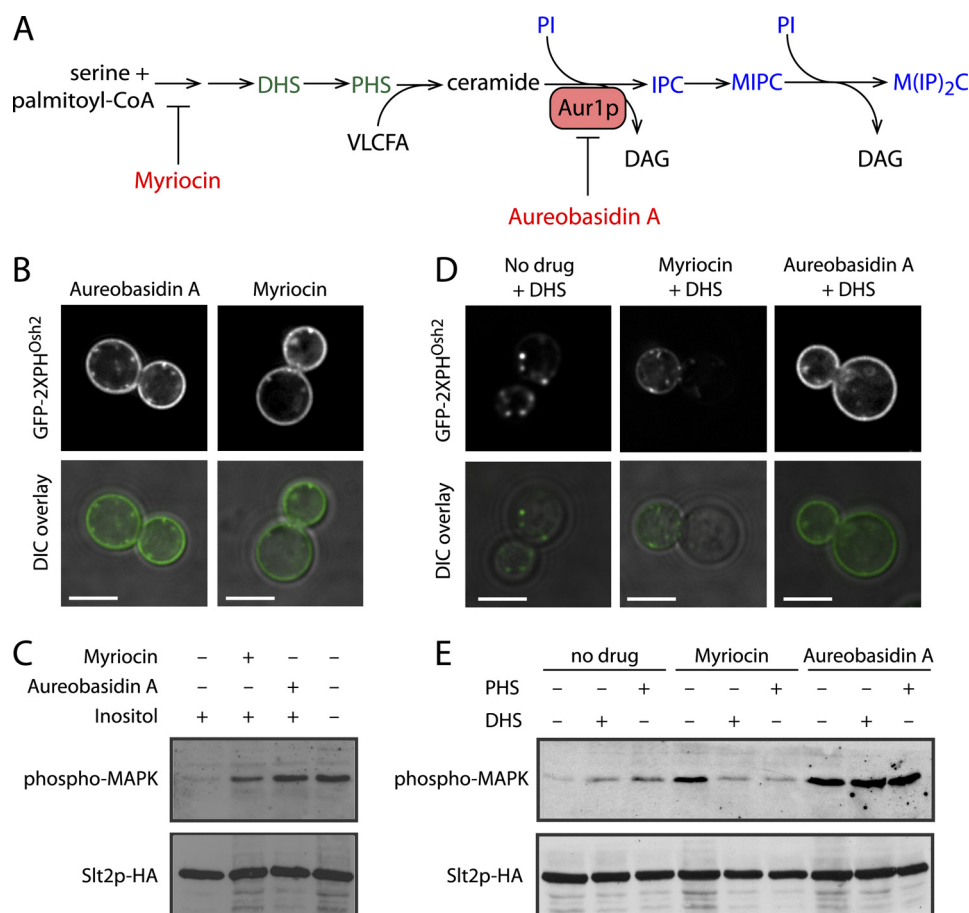


FIGURE 5. Inhibiting complex sphingolipid synthesis induces PKC activation. *A*, schematic representation of the sphingolipid metabolic pathway. Metabolic steps inhibited by myriocin and AbA are indicated. Positions of relevant sphingolipids, DHS and PHS (green) and complex sphingolipids (blue), are indicated. VLCFA, very long chain fatty acid. *B*, wild type (SEY6210) cells, constitutively expressing a PI4P-binding probe (GFP-2XPH^{Osh2}), were pre-grown at 30 °C in synthetic media containing 75 μ M inositol. At mid-logarithmic growth phase, cells were harvested by filtration, washed, and resuspended in inositol-containing medium. Myriocin or AbA was added to final concentrations of 1 and 2 μ g/ml, respectively, incubated at 30 °C for 120 min, and examined by fluorescence microscopy. Scale bar, 5 μ m. *C*, wild type cells (SEY6210), expressing HA-tagged SLT2, pre-grown at 30 °C in synthetic media containing 75 μ M inositol. At mid-logarithmic growth phase, cells were harvested by filtration, washed, and resuspended in medium with or without inositol. Myriocin or AbA was added at concentrations indicated in *A*. Cells were incubated at 30 °C for 120 min, and cell extracts were examined as described in Fig. 4 legend. *D*, same as *B*, except DHS was added to a final concentration of 50 μ M simultaneously with the drug. Scale bar, 5 μ m. *E*, same as *C*, except DHS or PHS was added simultaneously with the indicated drug at a final concentration of 50 μ M.

ypk2 Δ double mutants had any effect on the enrichment of the PI4P biosensor on the plasma membrane during inositol starvation (Table 3).

In all, 44 mutant strains were examined for defects in the appearance of the PM PI4P biosensor following inositol starvation (Table 3). These included mutants that function in the PKC pathway, phosphoinositide metabolism, phospholipid metabolism, sphingolipid metabolism, and the secretory pathway. Although many of the examined mutants exhibit an Ino⁻ phenotype,³ only the *stt4*^{ts} and *mss4*^{ts} mutants showed a total absence of the PM PI4P FLARE following inositol starvation (Table 3). Together, these results suggest that the signal for producing PM PI4P enrichment lies upstream of Stt4p and Mss4p, rather than a positive feedback mechanism mediated by the PKC/Slt2p MAPK signaling cascade.

Inhibiting Sphingolipid Metabolism Activates PKC Signaling—Because inositol starvation triggers the appearance of PM PI4P and PKC activation, we hypothesized that the upstream signal arises from inositol-dependent lipid metabolism. Complex sphingolipids, which are important structural

components of the plasma membrane and play important roles as signaling intermediates (57), are ideal candidates for this signaling. In yeast, complex sphingolipids contain inositol phosphate derived from PI (Fig. 5A). The first steps in sphingolipid synthesis involve the formation of the sphingolipid secondary messengers, DHS and PHS from palmitoyl-CoA and serine (Fig. 5A). PHS is subsequently converted to ceramide, the precursor of complex sphingolipids. Yeast synthesize three complex sphingolipids containing an inositol phosphate moiety derived from PI, including inositol phosphoceramide (IPC), mannosyl-IPC (MIPC), and mannosyl-di-IPC (M(IP)₂C) (Fig. 5A), which collectively account for ~30% of the lipids present in the plasma membrane (58–61). We asked if inhibiting complex sphingolipid synthesis mimics the effects of inositol starvation by treating cells with AbA (62), a potent and specific inhibitor of the phosphatidylinositol:ceramide phosphoinositol transferase Aur1p (Fig. 5A) (63, 64), which catalyzes the first step in complex sphingolipid synthesis. Consistent with our hypothesis, AbA treatment led to the appearance of PM PI4P in >90% of cells, even when grown in

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the presence of inositol (Fig. 5B). Similar to the effect of inositol starvation, enrichment of PM PI4P was detected following treatment with AbA for 60 min (data not shown) and strengthened to the maximum observed intensity by 120 min (Fig. 5B). Moreover, PM PI4P was not observed following AbA treatment of *slt4^{ts}* mutant cells grown in the absence or presence of inositol at the restrictive temperature. Importantly, Slt2p was strongly phosphorylated following treatment with AbA in wild type cells to a level that was similar to inositol-starved cells (Fig. 5C), indicating that PKC is activated following an inhibition of complex sphingolipid synthesis. Together these results show that a signal is produced from sphingolipid metabolism that activates PKC signaling.

To determine whether the buildup of complex sphingolipid precursors during AbA treatment triggers PKC activation, we asked if blocking sphingolipid synthesis altogether results in enrichment of PM PI4P and Slt2p phosphorylation. Cells were treated with myriocin, an inhibitor of serine palmitoyl-transferase, the first committed step in sphingolipid synthesis (Fig. 5A) (65). Similar to AbA treatment, cells incubated with myriocin for 120 min in the presence of inositol led to the appearance of PM PI4P (Fig. 5B) and robust Slt2p phosphorylation (Fig. 5C). Because cells treated with myriocin do not synthesize the sphingoid bases DHS or PHS (66), these results suggest that accumulation of endogenous DHS or PHS following inhibition of complex sphingolipid synthesis does not stimulate PKC signaling.

We asked if addition of exogenous DHS or PHS to cells blocked in sphingolipid synthesis by myriocin treatment alleviated the plasma membrane enrichment of the PI4P FLARE and activation of PKC. Exogenous DHS or PHS rescues the growth inhibition caused by myriocin (65) and restores normal complex sphingolipid levels (67). We observed that addition of DHS to cells treated with myriocin resulted in a pattern of PI4P FLARE distribution that was identical to untreated cells grown in the presence of inositol (Fig. 5D). Conversely, addition of DHS to cells treated with AbA had no effect on the pattern of PM PI4P (Fig. 5D). Consistent with these results, PM PI4P was not enriched when DHS was added to untreated cells grown in the presence of inositol (Fig. 5D). Together, these findings suggest that accumulation of DHS and/or PHS does not lead to the enrichment of PM PI4P. Instead reduced levels of inositol-containing sphingolipids themselves appear to stimulate PM PI4P enrichment and PKC signaling.

In contrast to our results presented above, previous studies have indicated that DHS and PHS lead to PKC activation (19, 20), although this proposal had not been rigorously tested. To directly ascertain if excess DHS or PHS induces PKC activation, we added DHS or PHS to cells growing in the presence of inositol and measured Slt2p phosphorylation. In agreement with results obtained with the PI4P biosensor (Fig. 5D), addition of exogenous DHS or PHS to cells growing in the presence of inositol did not lead to increased Slt2p phosphorylation (Fig. 5E). Moreover, Slt2p phosphorylation was negligible in cells incubated with myriocin in the presence of DHS or PHS (Fig. 5E), whereas Slt2p phosphorylation was unaffected by exogenous DHS or PHS in cells treated with AbA (Fig. 5E).

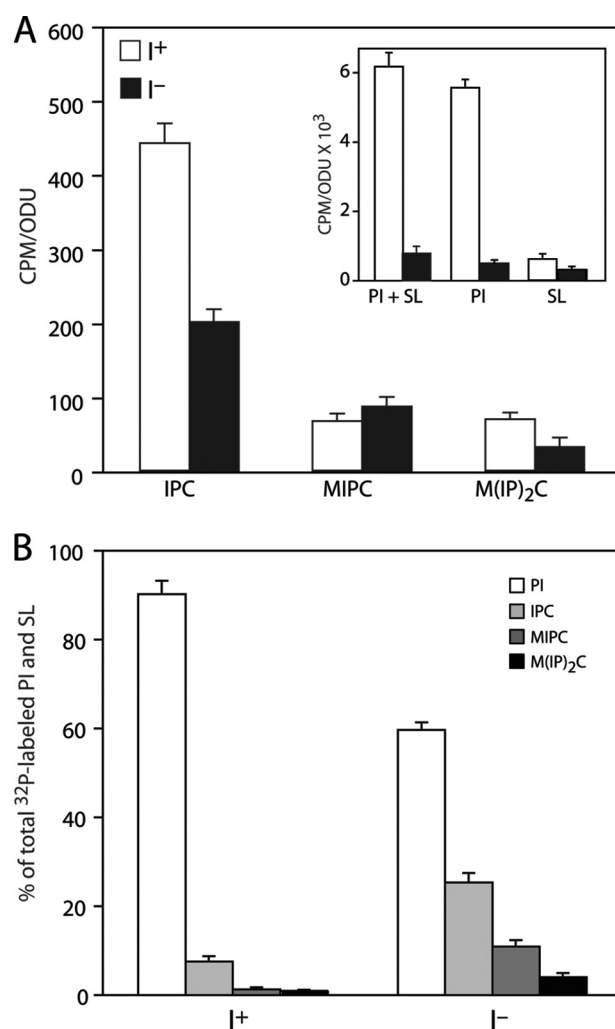


FIGURE 6. Inositol-containing sphingolipid synthesis is reduced during inositol starvation. *A*, wild type cells (BY4742) were pre-grown at 30 °C in synthetic media containing 75 μ M inositol. At mid-logarithmic growth phase, cells were harvested by filtration, washed, resuspended in medium with (I⁺) or without (I⁻) inositol, and incubated for an additional 2 h at 30 °C. After 2 h, cells were pulse-labeled with [³²P]orthophosphate for 30 min. Immediately following labeling, lipids were extracted, deacylated, and separated by two-dimensional TLC. Amounts of ³²P-labeled sphingolipids were quantitated by PhosphorImager analysis and normalized to cell culture density. Data are plotted to illustrate absolute levels of ³²P-containing sphingolipids. *Inset* shows total ³²P label incorporation into PI and sphingolipids. The rate of ³²P incorporation into PI was measured from lipid extracts, prior to the deacylation step, that were prepared from pulse-labeled parallel cultures as described above. *B*, data from *A*, *inset*, are plotted as a percentage of ³²P label derived from PI to illustrate relative levels of sphingolipids.

Together, these results indicate that inhibiting complex sphingolipid synthesis, independent of DHS and PHS accumulation, produces a signal that leads to PKC activation.

Complex Sphingolipid Levels Are Reduced during Inositol Starvation—In cells growing in the absence of inositol, PI levels dramatically decrease (25, 68, 69) (Fig. 6A, *inset*). This reduction in PI synthesis is predicted to significantly affect levels of downstream metabolites, particularly the inositol-containing sphingolipids, which derive their inositol phosphate moiety from PI (Fig. 5A). To assess complex sphingolipid synthesis during inositol starvation in wild type cells, we employed a ³²P pulse-labeling protocol. After 0 or 120 min of

growth in the absence of inositol, cells were pulsed with [32 P]orthophosphate for 30 min to label newly synthesized phospholipids. Labeled sphingolipids were subsequently separated by two-dimensional TLC and quantitated by normalizing total labeled sphingolipids to culture density. We observed an approximate 2.2-fold reduction in synthesis of both IPC and M(IP) $_2$ P (Fig. 6A), whereas MIPC synthesis was not significantly affected (Fig. 6A), presumably because PI is not utilized in this step (Fig. 5A). These data show that the rate of complex sphingolipid synthesis is reduced by $\sim$ 50% when cells are starved for inositol (Fig. 6A, *inset*). These results are consistent with data previously reported by Lester and co-workers (31, 32).

Next, we determined the fraction of newly synthesized PI that is utilized for inositol-containing sphingolipid synthesis in cells grown in the presence and absence of inositol. First, we calculated the relative rate of *de novo* PI synthesis in both growth conditions. Because labeled phosphate enters PI from cytidine diphosphate diacylglycerol and is sequentially transferred to IPC and M(IP) $_2$ C through inositol phosphate derived from PI (Fig. 5A), the total amount of PI that is actually synthesized in the 30-min pulse was calculated by summing the 32 P label remaining in PI itself plus the 32 P label transferred from PI to IPC, MIPC, and M(IP) $_2$ C. This analysis revealed that the total amount of PI that is synthesized during the 30-min pulse is decreased by 8-fold over this time interval in cells starved for inositol (Fig. 6A, *inset*, PI + SL). However, the 32 P label remaining in PI is decreased by 12-fold in cells starved for inositol (Fig. 6A, *inset*, PI), which indicates that a larger proportion of newly synthesized PI is utilized to synthesize sphingolipids. Therefore, this analysis reveals that a greater fraction of PI is used for sphingolipid production in cells grown without inositol than in cells supplemented with inositol. Consistent with this idea, less than 10% of the total 32 P label from newly synthesized PI is incorporated into complex sphingolipids during the 30-min pulse in cells grown in the presence of inositol, whereas almost 40% of the total 32 P label was recovered in inositol-containing sphingolipids following 120 min of inositol starvation (Fig. 6B). Although wild type cells starved for inositol synthesize fewer complex sphingolipids, these results suggest that wild type cells adapt to inositol starvation by channeling a larger fraction of newly synthesized PI into sphingolipids.

Inhibiting Inositol-containing Sphingolipid Synthesis Produces Inositol Auxotrophy—The findings presented above indicate that cells undergoing inositol starvation synthesize less complex sphingolipids compared with cells grown in the presence of inositol. Therefore, we asked if a further inhibition of complex sphingolipid synthesis would reveal a growth phenotype in cells grown in the absence of inositol. Indeed, like the *stt4^{ts}* mutant, the *aur1^{ts}* mutant displayed a strong Ino $^-$ phenotype at 30 °C (Fig. 7A). Moreover, wild type cells grown in the presence of AbA, which inhibits Aur1p function, also displayed an Ino $^-$ phenotype when grown in the presence of a sublethal dose of AbA (Fig. 7B). We also found that the *stt4^{ts}* mutant is hypersensitive to AbA (Fig. 7B), consistent with the reported synthetic lethal genetic interaction between the *stt4^{ts}* mutant and *aur1^{ts}* mutant (70). In addition, lack of

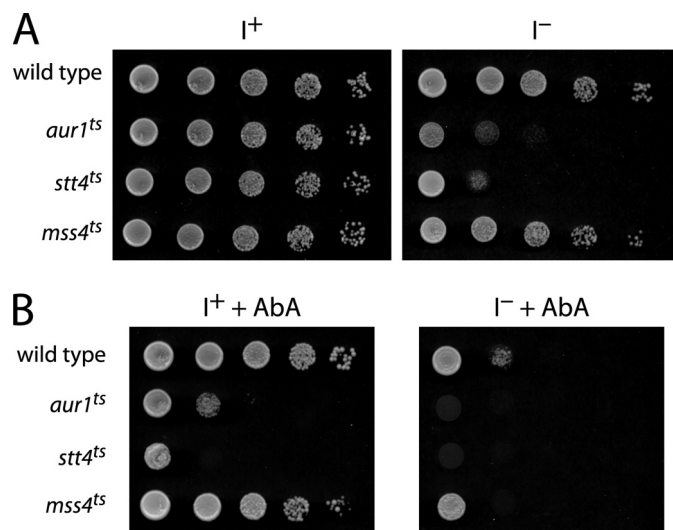


FIGURE 7. Partial interruption of inositol-containing sphingolipid synthesis induces inositol auxotrophy. A, serial 10-fold dilutions of wild type cells (SEY6210) and isogenic mutant strains (*aur1^{ts}*, *stt4^{ts}*, and *mss4^{ts}*) were spotted on synthetic media containing or lacking 75 μ M inositol. Plates were incubated at 30 °C for 2 days. B, serial 10-fold dilutions of wild type cells (SEY6210) and isogenic mutant strains (*aur1^{ts}*, *stt4^{ts}*, and *mss4^{ts}*) were spotted on synthetic media containing 50 ng/ml AbA with (I $^+$) or without (I $^-$) 75 μ M inositol. Plates were incubated at 30 °C for 2 days.

inositol exacerbates the hypersensitivity of the *aur1^{ts}* mutant to AbA (Fig. 7B), underscoring the need for complex sphingolipid synthesis during inositol starvation. Likewise, AbA hypersensitivity of the *stt4^{ts}* mutant and the *mss4^{ts}* mutant is intensified in the absence of inositol. These results indicate a functional relationship between sphingolipid and phosphoinositide metabolism.

DISCUSSION

In this study, we demonstrate that inositol-containing sphingolipid metabolism generates a signal that regulates PKC activity during inositol starvation. Similar to heat stress, activation of PKC signaling during inositol starvation requires the activity of Stt4p and Mss4p, which produce PI4P and PI(4,5)P $_2$ on the plasma membrane. Like other mutants defective in PKC signaling (26), *stt4^{ts}* and *mss4^{ts}* mutants exhibit inositol auxotrophy, yet fully derepress *INO1*. Consistent with the established roles of Stt4p and Mss4p in PKC signaling, the intensity of the PI4P biosensor profoundly increases on the plasma membrane under all conditions tested in which PKC is activated. However, components of the PKC pathway that function downstream of PI4P and PI(4,5)P $_2$ are not necessary for the appearance of the PM PI4P FLARE. Instead, the appearance of PM PI4P is triggered by the interruption of inositol-containing sphingolipid synthesis. Inhibiting complex sphingolipid synthesis with AbA or myriocin results in both enhancement of the PM PI4P signal and PKC activation. Moreover, the rate of inositol-containing sphingolipid synthesis is decreased in cells grown in the absence of inositol, yet a greater fraction of newly synthesized PI is utilized for complex sphingolipid synthesis under this growth condition. Inhibiting inositol-containing sphingolipid synthesis with AbA or in the *aur1^{ts}* mutant results in inositol auxotrophy, which

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underscores the importance of inositol-containing sphingolipid synthesis during inositol starvation.

Role of Sphingolipids in PKC Signaling—Previous studies have implicated sphingolipid metabolism in regulating PKC signaling in yeast, although the mechanism by which they do so has remained obscure. One model involves direct activation of the PDK1 redundant homologs Pkh1p and Pkh2p by sphingoid bases. Similar to PDK1 in mammals, Pkh1p and Pkh2p phosphorylate Pck1p as well as the redundant serum- and glucocorticoid-induced protein kinase homologs Ypk1p and Ypk2p, all of which are required for full activation of PKC signaling (21, 71). In addition, *pkh1^{ts} pkh2Δ* and *ypk1^{ts} ypk2Δ* mutants exhibit phenotypes similar to mutants defective in PKC signaling (21, 71). Interestingly, high copy *PKH1* and *YPK1* bypass the growth inhibition caused by myriocin treatment (72), and Pkh1p and Pkh2p kinase activity is stimulated by DHS and PHS *in vitro* (19), suggesting that yeast use these sphingoid bases as lipid mediators in PKC signaling. Moreover, levels of both DHS and PHS increase following heat stress (17, 18), a growth condition that activates PKC signaling (3, 9). However, in direct contrast to the model outlined above, a recent study found no evidence for a requirement of sphingoid bases in activating Pkh1p and Pkh2p *in vivo* (67).

We observed robust PKC activation in cells treated with AbA, a condition that leads to increased DHS and PHS levels (73). In addition, slowing of complex sphingolipid synthesis during inositol starvation is expected to increase levels of sphingoid bases, although this has not been measured. These observations tend to support the model whereby DHS and PHS activate PKC signaling. However, we showed that treatment of cells with myriocin, which abolishes DHS and PHS synthesis, also leads to PKC activation. Moreover, we observed that PKC activation by myriocin was significantly attenuated by supplementation of DHS and PHS, yet sphingoid base supplementation had no effect on cells treated with AbA. Moreover, supplementation of untreated wild type cells with exogenous DHS or PHS did not lead to activation of PKC signaling. Together, these results strongly suggest that activation of PKC signaling by inhibiting sphingolipid synthesis is due to the reduced synthesis of inositol-containing sphingolipids and not increased levels of sphingoid bases.

The sphingolipid metabolites, DAG and ceramide, are proposed to play opposing roles in regulating PKC activity. DAG, which is generated during the synthesis of IPC and M(IP)₂C as well as during the synthesis of sphingomyelin, the mammalian equivalent of IPC, is a potent activator of PKC in mammalian cells (7). Moreover, DAG produced by complex sphingolipid metabolism has been shown to activate PKC in the pathogenic fungi *Cryptococcus neoformans* (74). Conversely, ceramides, which are immediate precursors and turnover products of complex sphingolipid metabolism, inhibit PKC activation in mammals (75, 76). However, because DAG levels are expected to decrease and ceramide levels are expected to increase when complex sphingolipid synthesis is inhibited during inositol starvation, neither DAG nor ceramides are likely to significantly contribute to PKC activity under this growth condition. Thus, levels of inositol-containing sphingo-

lipids themselves appear to generate the signal for activating PKC during inositol starvation.

Coordination of Sphingolipid and Phosphatidylinositol Metabolism—Yeast cells grown in the absence of inositol contain 5–6-fold lower levels of PI compared with wild type cells grown in the presence of inositol (25). Pioneering work by Lester and co-workers (31, 32) showed that inositol-containing sphingolipid synthesis is reduced during inositol starvation, which agrees with the work reported here. Furthermore, addition of inositol to wild type cells following inositol starvation leads to a decrease in ceramide levels, mostly likely due to increased synthesis of IPC (77). Clearly, overall levels of PI contribute to the capacity for synthesis of inositol-containing sphingolipids.

Competition between phosphoinositide and sphingolipid metabolism for the common precursor PI may contribute to metabolic regulation of complex sphingolipid levels. Brice *et al.* (73) reported that the *sac1Δ* PI4P phosphatase mutant, which is deficient in turnover of PI4P to PI, contains significantly lower PI and inositol-containing sphingolipid levels compared with wild type. Conversely, treatment of wild type cells with wortmannin, an inhibitor of Stt4p, causes an increase in PI and complex sphingolipids (73). In contrast, Tabuchi *et al.* (70) found that overall levels of complex sphingolipids are reduced in *stt4^{ts}* and *mss4^{ts}* mutant strains compared with wild type. We observed a 2-fold decrease in the overall rate of complex sphingolipid synthesis and a corresponding increase in the relative levels of all four phosphoinositides following inositol starvation for 120 min. However, the degree by which metabolic channeling of PI into phosphoinositides contributes to sphingolipid levels is unresolved and remains to be further investigated. Nevertheless, the observation that the *stt4^{ts}* mutant is hypersensitive to AbA and the *stt4^{ts}* mutation is synthetically lethal with the *aur1^{ts}* mutation (70) argues for a functional relationship between phosphoinositide and complex sphingolipid metabolism.

Sphingolipid Levels May Control Plasma Membrane Access of Effector Proteins Required for PKC Activation—Current models for initiation of PKC signaling in yeast suggest that production of restricted pools of PI4P and PI(4,5)P₂ on the plasma membrane recruit signaling effectors that are required for PKC activation (24). Audhya and Emr (9) proposed that the Rho-GEF Rom2p is recruited to the plasma membrane through its interaction with PI(4,5)P₂, thereby activating Rho1p-dependent PKC signaling. Our observation that inhibition of sphingolipid synthesis leads to Stt4p-dependent PKC signaling is consistent with this model. Furthermore, our findings indicate that the signal generated from complex sphingolipid metabolism is relayed through PI4P and PI(4,5)P₂. We propose that the amount of inositol-containing sphingolipids in the plasma membrane provides an architecture that regulates signaling through PI4P and PI(4,5)P₂ by controlling the activities of Stt4p and Mss4p lipid kinases and/or by coordinating access of effector proteins to these signaling lipids.

Several lines of evidence suggest that lipid microdomains on the cell surface, which are composed of dynamic assemblies of sphingolipids and sterols, contribute to activation of signal transduction cascades (78). First, in yeast, sphingolipids

and sterols are sorted together at the trans-Golgi network into transport carriers that are delivered to the cell surface (79). Second, filipin, a sterol ligand, preferentially interacts with sterols in regions of the plasma membrane where sphingolipids are absent (80). Finally, sphingolipid composition in cells is modified in response to changes in sterol composition (81), suggesting that both classes of lipids functionally interact. Indeed, besides *aur1^{ts}*, several other nonessential mutants in sphingolipid and sterol metabolism exhibit an Ino[−] phenotype,³ suggesting that a proper balance between inositol-containing sphingolipids and sterols may be necessary for signaling.

Sphingolipid-sterol-rich domains may control signaling through PI4P and PI(4,5)P₂ by the inclusion or exclusion of regulatory complexes at these sites. For example, localization of Stt4p and Mss4p into restricted regions in the plasma membrane called PIK patches is necessary for their lipid kinase activity (9, 44). Changes in complex sphingolipid metabolism may regulate PI4P and PI(4,5)P₂ levels by controlling the localization of lipid kinases and/or regulatory factors to PIK patches on the plasma membrane. Alternatively, changes in inositol-containing sphingolipid levels may control access of protein effectors that bind to PI4P and PI(4,5)P₂ buried in the plasma membrane. Metabolic regulation of complex sphingolipid levels could provide a sensitive mechanism for controlling the organization of signaling complexes in the plasma membrane. Future studies will be necessary to uncover the links between sphingolipid and phosphoinositide metabolism in cell signaling.

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