JBC Papers in Press. Published on June 27, 2011 as Manuscript M111.258798
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M111.258798

Phosphatidate Phosphatase Activity Plays a Key Role in Protection Against Fatty Acid-induced Toxicity in Yeast*

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Running title: PA Phosphatase Protects Against Fatty Acid Toxicity

The *PAH1*-encoded phosphatidate (PA) phosphatase in *Saccharomyces cerevisiae* is a pivotal enzyme that produces diacylglycerol for the synthesis of triacylglycerol (TAG), and simultaneously controls the level of PA used for phospholipid synthesis. Quantitative lipid analysis showed that the *pah1*Δ mutation caused a reduction in TAG mass and an elevation in the mass of phospholipids and free fatty acids; changes that were more pronounced in the stationary phase. The levels of unsaturated fatty acids in the *pah1*Δ mutant were unaltered, although the ratio of palmitoleic acid to oleic acid was increased with a similar change in the fatty acid composition of phospholipids. The *pah1*Δ mutant exhibited classic hallmarks of apoptosis in stationary phase and a marked reduction in the quantity of cytoplasmic lipid droplets. Cells lacking PA phosphatase were sensitive to exogenous fatty acids in the order of toxicity palmitoleic acid > oleic acid > palmitic acid. In contrast, the growth of wild type cells was not inhibited by fatty acid supplementation. In addition, wild type cells supplemented with palmitoleic acid exhibited an induction in PA phosphatase activity and an increase in TAG synthesis. Deletion of the *DGK1*-encoded diacylglycerol kinase, which counteracts PA phosphatase in controlling PA content, suppressed the defect in lipid droplet formation in the *pah1*Δ mutant. However, the sensitivity of the *pah1*Δ mutant to palmitoleic acid was not rescued by the *dgk1*Δ mutation. Overall, these findings indicate a key role of PA phosphatase in TAG synthesis for protection against fatty acid-induced toxicity.

PA[4] phosphatase (EC 3.1.3.4), which was first discovered by Kennedy and coworkers in 1957 (1), catalyzes the dephosphorylation of PA to produce DAG and P_i (1) (Fig. 1). The reaction is dependent on Mg^{2+} ions and is based on a D*X*D*X*(T/V) catalytic motif within a haloacid dehalogenase-like domain in the enzyme (2-4)[5]. The DAG produced by PA phosphatase is used for the synthesis of TAG and for the synthesis of PE and PC via the Kennedy pathway (4-7) (Fig. 1). PA, the enzyme substrate, is utilized for the synthesis of phospholipids via the liponucleotide intermediate CDP-DAG (7) (Fig. 1). Moreover, both PA (e.g., activation of cell growth, membrane proliferation, transcription, and vesicular trafficking) and DAG (e.g., activation of protein kinase C) have lipid signaling functions (8-17), and PA phosphatase plays a role in controlling their cellular concentrations (2, 18). Thus, it is generally recognized that PA phosphatase is a key regulatory enzyme for controlling lipid metabolism and cell physiology (4, 7, 19-21).

The biochemistry and physiological roles of PA phosphatase emanated from studies in the model eukaryote yeast *Saccharomyces cerevisiae* and latterly in mammalian cells (4, 7, 19, 21, 22). PA phosphatase was first purified and characterized from yeast in 1989 (23) and the *PAH1*[6] gene encoding the enzyme was identified in 2006 (2). The discovery that *PAH1*

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encodes PA phosphatase in yeast led to the revelation that the lipodystrophic defect in the fatty liver dystrophy (*fld*) mouse (24, 25) was also a PA phosphatase deficiency arising from mutations in the *lpin1* gene (2, 26). The gene-enzyme relationship for PA phosphatase has since been confirmed in several organisms including humans (2, 27), flies (28, 29), worms (30), and plants (31, 32).

In *S. cerevisiae*, PA phosphatase is a cytosolic enzyme that associates with the nuclear/ER membrane, where its substrate resides, to catalyze the formation of DAG for lipid synthesis (2, 33). The association of PA phosphatase with the membrane is largely governed by the phosphorylation state of the enzyme (33, 34). Phosphorylation promotes a cytosolic location and thus down-regulation of enzymatic activity, whereas dephosphorylation favors membrane association (34). PA phosphatase, a highly phosphorylated enzyme (35), is a target for multiple protein kinases including those encoded by *CDC28* (34, 36), *PHO85* (37, 38), and *DBF2* (39). The phosphorylated form of the enzyme is counter-regulated by the Nem1p-Spo7p protein phosphatase complex located at the nuclear/ER membrane (40, 41). The phosphorylated enzyme in the cytosol is recruited to the nuclear/ER membrane where it is dephosphorylated by the Nem1p-Spo7p complex to be functional *in vivo* (33, 34, 41). This process leads to the anchoring of dephosphorylated enzyme to the membrane via a short N-terminal amphipathic helix (33).

PA phosphatase has a relatively high catalytic efficiency when compared with other enzymes of phospholipid metabolism (23). The overexpression of a dephosphorylated form of the enzyme, which can circumvent the Nem1p-Spo7p requirement for membrane association and dephosphorylation, causes a lethal phenotype (33-35). Thus, mechanisms that attenuate PA phosphatase must exist to regulate its functions in lipid metabolism. Indeed, these mechanisms include phosphorylation (33-35) and a limiting amount of Nem1p relative to Pah1p (34, 42) to control membrane association (33, 34). In addition, the activity of the enzyme is inhibited by nucleotides (43) and sphingoid bases (44). In metazoans, the *PAH1* orthologs act as transcriptional co-activators of several lipogenic targets (19, 45-47). Similarly, chromatin immunoprecipitation analysis indicates that the yeast PA phosphatase is found in the nucleus (41), although the physiological relevance of this location is unclear.

PA phosphatase acts at a pivotal nexus in lipid metabolism; depending on its activity, fatty acids are either channeled towards storage as TAG or to membrane assembly as phospholipids. This is exemplified by the phenotypes exhibited by yeast *pah1* mutants that lack the enzyme. Cells bearing the *pah1*Δ mutation have increased levels of PA and reduced levels of DAG and TAG (2, 3). The elevated PA content in *pah1*Δ mutant cells causes the induction of phospholipid synthesis gene expression[7] and the aberrant expansion of the nuclear/ER membrane (2, 3, 41). The *pah1*Δ mutant also exhibits a respiratory deficiency phenotype and sensitivity to growth at elevated temperature (2, 41).

In this work, we further characterized the *pah1*Δ mutant to gain a greater understanding of the roles PA phosphatase plays in lipid metabolism. An analysis of lipid composition revealed that the *pah1*Δ mutant exhibited a significant increase in the mass of membrane phospholipids and changes in their fatty acyl moieties when compared with wild type cells. In particular, *pah1*Δ mutant cells showed relatively large increases in palmitoleic acid relative to oleic acid. We also discovered that the imbalance in lipid synthesis that characterizes the *pah1*Δ mutant resulted in sensitivity to both saturated and unsaturated fatty acid toxicity. PA phosphatase activity, as opposed to a non-enzymatic function of Pah1p, was essential in protecting cells from this toxicity.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade or better. Growth medium supplies were obtained from Difco Laboratories. New

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England Biolabs was the source of modifying enzymes and restriction endonucleases. DNA gel extraction kit and plasmid DNA purification kit were purchased from Qiagen. Carrier DNA for yeast transformation was from Clontech. Sigma-Aldrich was the source of fatty acids, fatty acid methyl ester standards, Nile red, aprotinin, benzamidine, bovine serum albumin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, and Triton X-100. The fluorescein isothiocyanate annexin V/propidium iodide kit was purchased from Invitrogen. DNA size ladders, electrophoresis reagents, and protein assay reagents were from Bio-Rad. Neutral lipid and phospholipid standards were purchased from Avanti Polar Lipids, and silica gel 60 thin layer chromatography plates were from EM science. Scintillation counting supplies and acrylamide solutions were from National Diagnostics, and radiochemicals were from Perkin-Elmer Life Sciences.

Yeast Strains, Plasmids, and Growth Conditions—The *S. cerevisiae* strains and plasmids used in this work are listed in Table 1. *PAH1* alleles with D398E, D400E, and G80R mutations were subcloned from plasmids pGH312-D398E, pGH312-D400E, and pGH312-G80R (3) to generate the corresponding mutant alleles in plasmid pGH315 (34). Cells were grown at 30°C in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete (SC) medium containing 2% glucose as described previously (48, 49). For selection of yeast cells bearing plasmids, appropriate amino acids were omitted from synthetic complete medium. Fatty acids were added to culture medium supplemented with 0.6% ethanol/tyloxapol (5:1, v/v) from 1% stock solutions made in ethanol (50). Growth of cultures (200 µl) in 96-well plates was monitored with a Thermomax plate reader. Growth parameters were calculated according to the modified Gompertz equation (51). For dry cell weight determination, cells were harvested by centrifugation, washed twice with distilled water, and incubated at 80 °C until a constant weight was obtained.

Analyses of Cytoplasmic Lipid Droplets and Apoptosis by Fluorescence Microscopy—For lipid droplet analysis, cells were stained with 1 µg/ml Nile Red and visualized with a long pass green fluorescent protein filter (excitation 440 nm). For each image 10 confocal frames 0.25 µm apart were captured and displayed as maximum intensity projections. Lipid droplets were counted for 6 frames per strain, with 15-30 cells per frame. Statistics were completed by comparing across frames. Adenine was added to the YEPD growth medium to minimize the autofluorescence of the vacuole that is associated with the *ade2* mutation (50). Apoptosis was assessed using a fluorescein isothiocyanate annexin V/propidium iodide kit; cells were washed with sorbitol buffer (1.2 M sorbitol, 0.5 mM magnesium chloride, 35 mM potassium acetate, pH 6.8), digested with 5.5% glusulase, and 15 U/ml lyticase in sorbitol buffer and resuspended in annexin V and propidium iodide for 20 min (52). Microscopy was performed using a Zeiss Axiovert 200 M using a 63x oil immersion objective, and images were taken with a Hamamatsu Orca-ER camera.

Lipid Extraction—Lipids were extracted from yeast by the method of Folch *et al.* (53) with the modification of Schneiter and Daum (54). In brief, methanol (10 ml) was added to cell pellets derived from 50 ml cultures followed by homogenization with glass beads (0.5 mm diameter). After cell disruption, chloroform (20 ml) was added to the suspension, followed by stirring for 1 h at room temperature. Lipid extracts were collected by filtration, washed once with 2 mM $MgCl_2$ and twice with 2 M KCl in methanol, dried under vacuum, and resuspended in chloroform/methanol (2:1, v/v).

Lipid Analysis—Neutral lipids were analyzed by thin-layer chromatography using the solvent system hexane/diethyl ether/glacial acetic acid (40:10:1, v/v/v). The identity of the lipids on the plates was confirmed by comparison with standards after spraying the plates with a 0.05% solution of primulin in acetone/water (80:20). Fatty acids were analyzed by gas-liquid chromatography. Margaric acid (C17:0) was added to the lipids before transmethylation, which was performed by heating at 70 °C with a mixture of 0.5 N HCl/methanol (3 ml) and toluene (0.5 ml) for 2

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h. Fatty acid methyl esters were analyzed with a Hewlett Packard 5890 gas chromatograph equipped with a 30 m x 0.32 mm Supelco MDN-55 column and a flame ionization detector; helium was the carrier gas (10 psi). The column temperature was programmed as follows: 100 °C for 10 min and then increased to 300 °C at 10 °C/min. The injector temperature was 250 °C and the detector temperature was 325°C. Fatty acid methyl esters were identified by reference to authentic standards. High performance liquid chromatography was performed with a Waters 2695 Alliance system. A 4.6 x 100 mm Waters Spherisorb column coupled to a SEDEX 55 evaporative light scattering detector was used. The analysis was run at 45 °C using a tertiary solvent gradient (55). Standard curves were constructed and used for the quantitation of both neutral lipids and phospholipids.

For lipid analysis by liquid chromatography-mass spectrometry, lipid extracts were evaporated to dryness under a nitrogen stream and redissolved in isooctane/tetrahydrofuran (9:1, v/v). The lipids were analyzed in a single chromatographic method using a Dionex UltiMate 3000 LC system coupled to an Applied Biosystems 4000 Q Trap mass spectrometer with an electrospray ionization source. The high performance liquid chromatography column was a Waters Spherisorb® S5W 4.6 x 100 mm silica cartridge, 5 μm particle size, with a Waters Spherisorb® S5W 4.6 x 10 mm guard cartridge. The solvent gradient was modified from that of Homan and Anderson (55). The mass spectrometer program consisted of three periods. The first period, from 0 to 17 minutes, included 41 multiple reaction monitoring ion pairs for the measurement of individual TAGs. The second period, from 17 to 29 minutes, included four experiments in parallel: a neutral loss of 141 scan in positive mode (for PE); a neutral loss of 87 scan in negative mode (for PS); a precursor of 153 scan in negative mode (for PA); and a precursor of 241 scan in negative mode (for PI). The third period, from 29 to 50 minutes, included a precursor of 184 scan in positive mode for PC. The data were analyzed with Analyst 1.4.2 software (Applied Biosystems). TAG amounts were adjusted for the internal standard and estimated from a standard curve containing seven TAG standards (triolein, tritridecanoic acid, tripalmitolein, trilinolein, tripalmitin, tristearin, and trimyristin). The standard curve mixture used for quantitation of phospholipids included at least one compound from each class. The compounds used were: dioleoyl-PC, dimyristoyl-PC, dieicosenoyl-PC, dioleoyl-PE, dioleoyl-PI, dioleoyl-PS, and dioleoyl-PA. For PI, a mixture extracted from liver was used for the standard curve.

Preparation of Cell Extracts—Cell extracts were prepared by disruption of yeast cells with glass beads (0.5 mm diameter) using a BioSpec Products Mini-BeadBeater-16 at 4 °C (56). The cell disruption buffer contained 50 mM Tris-HCl (pH 7.5), 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. Protein concentration was estimated by the method of Bradford (57) using bovine serum albumin as the standard.

PA Phosphatase Activity—PA phosphatase activity was measured by following the release of water-soluble $^{32}P_i$ from chloroform-soluble [^{32}P]PA (10,000 cpm/nmol) (56). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 1 mM $MgCl_2$, 0.2 mM PA, 2 mM Triton X-100, and enzyme protein in a total volume of 0.1 ml. In some experiments, the $MgCl_2$ was substituted by 2 mM EDTA to inhibit Mg^{2+}-dependent PA phosphatase activity. All enzyme assays were conducted in triplicate at 30 °C. The average standard deviation of the assays was ± 5%. The reactions were linear with time and protein concentration. A unit of PA phosphatase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product per minute.

Analysis of Data—Statistical analysis of the data was performed with SigmaPlot software. P values < 0.05 were taken as a significant difference.

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RESULTS

The pah1Δ Mutation Modulates Phospholipid, TAG, and Free Fatty Acid Levels as a Function of Growth Stage—Radiolabeling studies have indicated that the loss of *PAH1* has major effects on the relative amounts of phospholipids, TAG, and free fatty acids in both the exponential and stationary phases of growth (2). However, information on the effects of the *pah1*Δ mutation on the mass of these lipids, as well as the molecular species that comprise the lipids has been lacking. In this work, we used a combination of standard analytical methods to further characterize the roles of PA phosphatase in lipid metabolism. In exponential phase cells, the *pah1*Δ mutant exhibited increases in the mass of total phospholipids (24%) and free fatty acids (86%), and a decrease in the mass of TAG (40%) when compared with the wild type control (Fig. 2A). With respect to the individual phospholipids (Fig. 2B), mass increases were shown for PA (250%), PE (48%), PI (33%), and PC (28%), whereas there was a mass decrease for PS (35%). Because of the defect in PA phosphatase and the lack of choline or ethanolamine supplementation, the synthesis of phospholipids in the *pah1*Δ would primarily occur through the CDP-DAG pathway (58). Thus, the decreased abundance of PS in the mutant might be attributed to its utilization for the synthesis of PE and PC via the CDP-DAG pathway (59).

The effects of the *pah1*Δ mutation on the masses of lipids were more striking at the stationary phase of growth. The masses of total phospholipids and free fatty acids were elevated by 96% and 77%, respectively, whereas the amount of TAG was reduced by 80% when compared with the wild type control (Fig. 2A). The large change in the phospholipid mass of stationary phase mutant cells was attributed to increased amounts of PC (84%), PE (154), PI (88%), PS (52%), and PA (450%) (Fig. 2B). Overall, the total lipid masses of *pah1*Δ mutant cells in the exponential (36.3 ± 4.2 μg/mg cell dry weight) and stationary (47.5 ± 2.1 μg/mg cell dry weight) phases of growth were 47% and 60% greater, respectively, when compared with those found in the exponential (24.7 ± 1.5 μg/mg cell dry weight) and stationary (29.6 ± 1.4 μg/mg cell dry weight) phases of growth for wild type cells.

The pah1Δ Mutation Affects the Fatty Acid Composition of Phospholipids and TAG—We examined the effects of the *pah1*Δ mutation on the fatty acid composition of total lipids. For this experiment, the total lipid fractions of exponential and stationary phase cells were isolated, transmethylated, and subjected to gas-liquid chromatography. As described previously (60, 61), the major fatty acids in wild type cells were palmitic, palmitoleic, stearic, and oleic acids with palmitoleic and oleic acids being most abundant (Table 2). Myristic acid, which was observed in the phospholipid fraction (see below), was a minor constituent of the total lipids. While the *pah1*Δ mutation did not impact the proportions of saturated fatty acids, it had a major effect on the relative proportions of palmitoleic and oleic acids. In both exponential and stationary phase cells, the loss of *PAH1* caused an increase (22-27%) in palmitoleic acid and a decrease (17-21%) in oleic acid.

The phospholipid molecular species were determined by liquid chromatography-mass spectrometry (Table 3). The 16:0-16:1, 16:1-16:1, 16:0-18:1, and 16:1-18:1 species were most abundant in phospholipids of wild type and *pah1*Δ mutant cells. The 16:0-18:0 species was only present in PA, whereas the 18:0-18:1 species was present in PI and PA of both wild type and *pah1*Δ mutant cells. The 14:0-14:0 and 14:0-16:1 species were present in PI and PE, respectively, in *pah1*Δ mutant cells, but they were absent in wild type cells. This analysis indicated that the increase in palmitoleic acid of *pah1*Δ mutant cells was primarily attributed to the 16:0-16:1 molecular species in PE, PI, and PA and the 16:1-16:1 molecular species in PC, PE, PS, and PA. The decrease in oleic acid of the *pah1*Δ mutant was primarily attributed to the 16:0-18:1 species of PE, PI, and PA, the 16:1-18:1 species of PC, PE, PI, PS, and PA, and the 18:1-18:1 species of PC, PE, PI, and PA. The *pah1*Δ mutation also caused an increase in the 18:0-18:1 species of PA.

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Similarly, we examined the TAG molecular species from exponential and stationary phase cells (Table 4). We observed a wide distribution of fatty acid species with 16:1-16:1-16:1 and 16:1-16:1-18:1 being the most abundant. The phase of growth had an effect on the presence and absence of some of the molecular species. The 16:0-18:0-18:0 and 18:0-18:0-18:0 species were present only in stationary phase cells, whereas 16:0-18:1-18:1 and 18:0-18:1-18:1 were present only in exponential phase cells. In exponential phase cells, the *pah1*Δ mutation caused decreases in the relative amounts of 16:1-16:1-18:1 (22%), 16:1-18:1-18:1 (50%), and 18:0-18:1-18:1 (40%), and an increase in 18:1-18:1-18:1 (260%). The other species were not affected greatly by the *pah1*Δ mutation in exponential phase cells. In stationary phase cells, the mutation caused decreases in the amounts of 16:0-18:0-18:0 (82%) and 18:0-18:0-18:0 (82%) and increases in the amounts of 16:0-16:1-16:1 (42%) and 18:1-18:1-18:1 (213%). Interestingly, the trend of increased abundance of palmitoleic acid in the phospholipids of *pah1*Δ mutant cells was not observed for the fatty acids of TAG.

The pah1Δ Mutation Causes Hallmark Apoptotic Phenotypes in Stationary Phase Cells—Previous studies have shown that an excess of fatty acids due to a block in TAG synthesis results in the induction of the unfolded protein response (50), apoptosis (50), and necrosis (62). The *pah1*Δ mutant had a significant increase in free fatty acid content and a great reduction in TAG content in stationary phase. Moreover, it has been shown that the *pah1*Δ mutant exhibits a constitutive induction of the unfolded protein response (63). Accordingly, we questioned whether the mutant displayed some of the hallmark characteristics of apoptosis. This was addressed by staining stationary phase cells with annexin V (for PS externalization on the plasma membrane) and with propidium iodide (for nuclear DNA of permeabilized cells) (52, 64). Treatment of cells of any genotype with the apoptosis inducer valproic acid (25 mM) was used to validate the assay (not shown). The numbers of *pah1*Δ mutant cells that exhibited annexin V and propidium iodide staining were 82% and 73% greater, respectively, when compared with wild type cells (Fig. 3). That *pah1*Δ cells showed increased staining with the combination of both reagents was an indication of late stage apoptosis (52), although it is likely that some of these cells were also necrotic (64). In contrast to the stationary phase cells, the exponential phase cells did not exhibit these apoptotic/necrotic phenotypes.

Fatty Acids are Toxic to pah1Δ Mutant Cells—We examined the effects of fatty acid supplementation on *pah1*Δ mutant cells. The rationale for this experiment was based on previous observations that unsaturated fatty acids are toxic to cells lacking the *DGA1*- and *LRO1*-encoded acyltransferase enzymes that convert DAG to TAG (50, 65, 66), and that the *pah1*Δ mutant had a much reduced amount of TAG. In addition, we were interested in examining the effects of palmitoleic and oleic acids on *pah1*Δ growth because the mutant exhibited changes in the relative proportions of these fatty acids in membrane phospholipids. At the concentrations used in this work, the fatty acids solubilized in detergent are readily taken up by yeast cells (50, 60, 65, 67). Control experiments indicated that the detergent vehicle for the solubilization of the fatty acids was not inhibitory to growth. Consistent with previous results (50, 60, 65, 68), neither saturated nor monounsaturated fatty acids inhibited the growth of wild type cells (Fig. 4A). In fact, the fatty acid supplementations resulted in slightly higher cell densities without having significant effects on the growth rates of wild type cells (Fig. 4A). In striking contrast, the fatty acid supplementations had major effects on the growth of *pah1*Δ mutant cells (Fig. 4B). Of the two saturated fatty acids, only palmitic acid inhibited the growth of *pah1*Δ mutant cells. At the highest concentration used (i.e., 0.5 mM), palmitic acid caused an 8 h lag in growth, a 46% decrease in growth rate, and a decrease in maximum cell number when compared with cells without fatty acid supplementation. Both palmitoleic and oleic acids caused dose-dependent inhibitions of *pah1*Δ mutant growth

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with palmitoleic acid being more toxic (Fig. 4B). At the concentration of 0.1 mM, palmitoleic acid caused changes in the growth parameters of *pah1*Δ that were similar (e.g., an increased lag time and decreases in growth rate and maximum cell number) to those caused by 0.5 mM palmitic acid (Fig. 4B). Higher concentrations of palmitoleic acid inhibited *pah1*Δ growth more potently. For example, the 0.25 mM concentration caused a 15 h increase in lag time, a 94% decrease in growth rate, and a 73% decrease in maximum cell number when compared with the wild type control (Fig. 4B). At the same concentration, oleic acid caused a 5 h increase in lag time and a 50% decrease in growth rate, but only a 4% decrease in maximum cell number (Fig. 4B).

Deletion of DGK1 Does Not Protect pah1Δ Mutant Cells from Fatty Acid Toxicity—*DGK1* encodes a DAG kinase enzyme that catalyzes the formation of PA from DAG and CTP (69, 70) (Fig. 1). This enzyme counterbalances the *PAH1*-encoded PA phosphatase in controlling the cellular levels of PA (69). Owing to the fact that loss of DAG kinase activity suppresses phenotypes (e.g., elevated PA content, abnormal nuclear/ER membrane expansion, and induction of phospholipid synthesis genes) caused by the loss of PA phosphatase activity, we questioned whether loss of *DGK1* would protect *pah1*Δ mutant cells from the toxicity caused by palmitoleic acid. Whereas sublethal (0.1 mM) and lethal (0.25 mM) concentrations did not affect the growth of *dgk1*Δ mutant cells, the toxic effects of palmitoleic acid on the growth of the *pah1*Δ mutant was not suppressed by the *dgk1*Δ mutation (Fig. 5). In fact, the *dgk1*Δ *pah1*Δ double mutant was more sensitive to 0.1 mM palmitoleic acid when compared with the *pah1*Δ mutant. We also noticed that the *pah1*Δ mutant in the RS453 background (Fig. 5) was less sensitive to 0.1 mM palmitoleic acid when compared with the *pah1*Δ mutant in the W303-1A background (Fig. 4). However, the *pah1*Δ mutants from both genetic backgrounds were acutely sensitive to 0.25 mM palmitoleic acid.

The pah1Δ Mutation Markedly Impairs Cytoplasmic Lipid Droplet Formation—TAG, which is derived from PA via the PA phosphatase reaction (2), accumulates in cytoplasmic lipid droplets (71-73). Accordingly, we questioned what effect the *pah1*Δ mutation would have on the number of lipid droplets. Stationary phase cells were stained with the lipophilic dye Nile Red to visualize lipid droplets by fluorescence microscopy (50, 65). The number of lipid droplets in *pah1*Δ mutant cells was reduced to 20% of that shown by wild type cells (Fig. 6). However, the number of lipid droplets in *dgk1*Δ *pah1*Δ mutant cells was not significantly different from that observed in wild type cells, as well as from *dgk1*Δ mutant cells (Fig. 6). Similar observations have recently been reported for exponential phase cells by Adeyo et al. (74).

PA Phosphatase Activity Is Required for Resistance to Fatty Acid-induced Toxicity—We questioned if the fatty acid toxicity of the *pah1*Δ mutant was specifically due to the loss of PA phosphatase activity or due to the loss of a non-enzymatic function of Pah1p. To address this question, we utilized *pah1*Δ cells expressing mutant alleles (G80R, D398E, D400E) of *PAH1* that encode catalytically inactive forms of PA phosphatase (3). The loss-of-growth phenotype caused by 0.25 mM palmitoleic acid was complemented by the wild type *PAH1* allele, but not by the G80R, D398E and D400E alleles (Fig. 7). These data indicated that the specific loss of PA phosphatase activity was responsible for the fatty acid-induced toxicity.

PA Phosphatase Activity Is Induced in Response to Palmitoleic Acid Supplementation with a Concomitant Increase in TAG Synthesis—Because PA phosphatase activity was required for the prevention of fatty acid-induced toxicity, we questioned if the enzyme activity was regulated in response to palmitoleic acid supplementation. For these experiments, wild type cells were first grown to mid-exponential phase in YEPD medium without fatty acid supplementation. Palmitoleic acid (0.25 mM) was then added to the cultures, and then cell extracts were prepared and assayed for PA phosphatase activity. The fatty acid supplementation resulted in a time-dependent increase (5-fold by 60 min) in the level of PA

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phosphatase activity (Fig. 8). This stimulation of activity was not observed when palmitoleic acid was not supplemented to the growth medium. The fatty acid-mediated induction of PA phosphatase activity was not attributed to the Mg^{2+}-independent lipid phosphate phosphatase activities encoded by the *DPP1*- and *LPP1*-encoded enzymes that also utilize PA as a substrate (75, 76). The induction in activity was abolished by the substitution of EDTA for $MgCl_2$ in the assay mixture (Fig. 8). Indeed, the *PAH1*-encoded PA phosphatase is absolutely dependent on Mg^{2+} ions for its activity (2, 23). The *pah1*Δ mutant still exhibits a Mg^{2+}-dependent PA phosphatase activity whose gene has yet to be identified (2). To rule out the possibility that this enzyme was induced upon palmitoleic acid supplementation, the same experiment was performed with *pah1*Δ mutant cells. Under the conditions of this experiment, the *pah1*Δ mutant continued to grow over the 90-min time period. When the standard PA phosphatase assay (i.e., with $MgCl_2$) was performed with cell extracts derived from the *pah1*Δ mutant, there was no induction of PA phosphatase activity (Fig. 8). These experiments further confirmed that *PAH1*-encoded PA phosphatase activity was induced by the palmitoleic acid supplementation.

The synthesis of lipids was also examined over the 90-min time course of this experiment. The addition of palmitoleic acid to the growth medium of wild type cells resulted in a time-dependent increase (5.6-fold by 60 min) in TAG content (Fig. 9A). With the exception of a small increase in ergosterol esters at 30 min, the fatty acid supplementation did not have a significant effect on the synthesis of other lipids (Fig. 9A). In contrast to wild type cells, the addition of palmitoleic acid to the growth medium of *pah1*Δ mutant cells did not result in an increased synthesis of TAG (Fig. 9B). However by 30 min, there was an increase in the amounts of PC (47%), PE (53%), PI (50%), and PS (130%) (Fig. 9B).

DISCUSSION

PA phosphatase catalyzes the penultimate step in the *de novo* pathway of TAG synthesis (2, 7). Since its discovery (1), researchers have asserted that PA phosphatase must play an important regulatory role in lipid metabolism because the enzyme is located at the branch point where PA is partitioned between the synthesis of TAG and membrane phospholipids (4, 7, 19-21). This critical juncture determines the fate of unesterified fatty acids that in conditions of excess abundance become toxic. The importance of the PA phosphatase was not fully demonstrated until the *PAH1* gene encoding the enzyme had been identified in yeast (2) and distinct phenotypes of *pah1* mutants lacking a functional enzyme had been scored (2, 3, 33-35, 41, 74). Pronounced phenotypes include the aberrant expansion of the nuclear/ER membrane and the derepression of phospholipid synthesis gene expression (3, 41). That these phenotypes have been ascribed to an elevated PA content (2, 3) indicated the importance of PA phosphatase in controlling the cellular amounts of PA. Similarly the loss of metazoan PA phosphatase causes lipodystrophy, peripheral neuropathy, and insulin resistance as evidenced in the fatty liver dystrophy (*fld*) mouse model (18, 24, 25, 77, 78). In the current study of yeast cells lacking *PAH1*, the analysis of lipid mass confirmed that the *pah1*Δ mutation caused a marked accumulation of PA and the major membrane phospholipids. These changes in lipid composition were more pronounced in the stationary phase in which the TAG levels were reduced by 80%. Collectively, this and previously published work (2, 3) substantiated the conclusion that PA phosphatase plays a major role in partitioning PA between the synthesis of phospholipids and TAG.

The *pah1*Δ mutation also caused an increase (~ 80%) in the mass of free fatty acids and a change in the molecular species of the accumulated membrane phospholipids. The increase in free fatty acid mass is most likely due to the decreased capacity of mutant cells to incorporate fatty acids into TAG. This phenotype indicated the importance of PA phosphatase in providing the DAG for the *DGA1*-encoded acyltransferase enzyme that uses fatty acids for TAG synthesis (Fig. 1). In addition, the elevated PA content in the *pah1*Δ mutant might be expected to cause the

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derepression of *FAS1*, *FAS2*, and *ACC1*, genes that encode fatty acid synthesis enzymes (79-82). The expression of these genes is subject to the same PA-mediated regulation that governs the expression of several phospholipid synthesis genes (81, 83) (Fig. 1). Thus, the increased PA content in the *pah1*Δ mutant favors both the synthesis of fatty acids and phospholipids, and thus further exacerbates the impact of lipid overloading.

An unexpected finding of this study was that the *pah1*Δ mutant exhibited an increase in the ratio of palmitoleic acid to oleic acid in the phospholipid molecular species. While the reason for this change is unknown, it is reminiscent of the effects that are caused by the overexpression of the *OLE1*-encoded Δ9 desaturase (84), the enzyme responsible for introducing the double bond between carbons 9 and 10 of palmitic and stearic acids (85), and the enzyme might have a preference for palmitic acid. *OLE1* expression is not subject to the PA-mediated regulatory circuit that controls the expression of fatty acid and phospholipid synthesis genes (85). Thus, if the enzyme is overexpressed in the *pah1*Δ mutant, another mechanism would be involved. Additional studies are needed to address whether the Δ9 desaturase was responsible for the changes in the molecular species of the phospholipids in *pah1*Δ mutant cells. Nonetheless, the increase in palmitoleic acid would increase the fluidity of the membrane bilayer, and it is known that increased fluidity compromises membrane integrity that can lead to cell death (86). In this regard, stationary phase *pah1*Δ mutant cells exhibited the classic hallmarks of apoptosis (52). Moreover, it is known that free fatty acids alone or through their stimulatory effects on the synthesis of bioactive lipids (e.g., sphingolipids) cause so-called lipotoxicity (50, 87-89). Thus, excess free fatty acids and the changes in phospholipid molecular species may contribute to the apoptotic phenotype exhibited by *pah1*Δ mutant cells. In addition, the recent work of Jonikes *et al.* (63) has shown that the *pah1*Δ mutant exhibits a constitutive induction of the unfolded protein response. That the loss of *PAH1*-encoded PA phosphatase was involved in the initiation of cell death in yeast is supported by the work of Fuentes *et al.* (90), which showed that inhibition of PA phosphatase activity results in the onset of apoptosis in a variety of mammalian cells.

The most striking phenotype of the *pah1*Δ mutant discovered in this work was the acute sensitivity to unsaturated fatty acids with palmitoleic acid being the most toxic. Saturated fatty acids in general are not toxic to yeast cells (50, 65). However, in the case of the *pah1*Δ mutant, palmitic acid also caused a decrease in cell growth, although to a lesser extent when compared with palmitoleic acid and oleic acid. We surmise that excess free fatty acids, in combination with the higher amounts of palmitoleic acid esterified to the accumulated membrane phospholipids, contributed to this phenotype. The data also indicated that the loss-of-growth phenotype in response to palmitoleic acid supplementation was attributed to the defect in TAG synthesis in the *pah1*Δ mutant. Indeed, an important physiological function of TAG synthesis is to buffer the toxicity caused by free fatty acids (50, 65, 87, 91). In fact, Petschnigg *et al.* (65) and Garbarino *et al.* (50) originally showed that unsaturated fatty acids have toxic effects on yeast growth when the conversion of DAG to TAG is blocked by the double deletion of the *DGA1* and *LRO1* genes. However, there are significant differences in the consequences on growth and lipid metabolism because of the *pah1*Δ mutation when compared with the *dga1*Δ *lro1*Δ double mutation. As discussed in this paper, *pah1*Δ mutant cells exhibit several phenotypes indicating the critical role of PA phosphatase activity in lipid metabolism and cell physiology, whereas *dga1*Δ *lro1*Δ double mutants only show a dramatic growth defect when challenged with unsaturated fatty acids (50, 65, 92).

As observed in previous studies (50, 60, 65), exogenous palmitoleic acid did not impact the growth of wild type cells, but it was associated with an increase in TAG synthesis. This result reinforced the notion that the synthesis of TAG buffers the toxic effects of fatty acids. In contrast, the palmitoleic acid supplementation had only a small effect on the synthesis of

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ergosterol esters. Connerth *et al.* (66) have shown that oleic acid inhibits ergosterol ester synthesis by a mechanism that includes a direct effect on the activity of the *ARE2*-encoded ergosterol acyltransferase enzyme. Perhaps this enzyme was also inhibited by palmitoleic acid. Coincident with the increased synthesis of TAG was the induction of PA phosphatase activity. This regulation was not observed in *pah1*Δ mutant cells that lack the major PA phosphatase enzyme in yeast (2). We also showed that Mg^{2+}-independent PA phosphatase activity, which is attributed to the *DPP1* and *LPP1* genes (75, 76), was not induced by palmitoleic acid. Moreover, the loss-of-growth phenotype of *pah1*Δ mutant cells in response to palmitoleic acid supplementation was specifically attributed to the loss of PA phosphatase activity; cells that expressed catalytically inactive forms of the enzyme had the same loss-of-growth phenotype as the *pah1*Δ mutant. Preliminary studies indicated that the regulation of PA phosphatase activity in response to palmitoleic acid supplementation occurs on multiple levels (e.g., genetic and biochemical). The elucidation of this complex regulation will require additional studies.

In yeast, the synthesis of TAG occurs during logarithmic growth primarily through the activity of the *LRO1*-encoded enzyme that uses membrane phospholipids as acyl donors (93). At stationary phase, the *DGA1*-encoded acyltransferase reaction predominates (94), resulting in the donation of acyl groups from acyl CoA, and ultimately the accumulation of TAG (as well as ergosterol esters) in cytoplasmic lipid droplets (50, 65, 71-73, 95). Our studies and those of Adeyo *et al.* (74) showed that the *pah1*Δ mutation causes a decrease in the number of lipid droplets, but this phenotype was suppressed by the *dgk1*Δ mutation. This finding is reminiscent of the effect of the *dgk1*Δ mutation on the suppression of the aberrant nuclear/ER membrane expansion and derepression of phospholipid synthesis genes caused by the *pah1*Δ mutation (3, 41). Genetic and biochemical evidence has shown that the *DGK1*-encoded DAG kinase activity counteracts PA phosphatase activity with respect to controlling the cellular levels of PA (69, 70). This conclusion is supported by the fact that the overexpression of *DGK1*, like the *pah1*Δ mutant, results in an increased PA content, the aberrant expansion of the nuclear/ER membrane, and the derepression of phospholipid synthesis genes (69). Thus, the massive elevation of PA, in conjunction with the expansion of the nuclear/ER membrane might be the basis for the defect in cytoplasmic lipid droplet formation in the *pah1*Δ mutant. An alternative explanation for the defect in lipid droplet formation is a reduced amount of DAG due to the loss of PA phosphatase activity (74).

Although the *dgk1*Δ mutation suppressed the defect in lipid droplet formation in *pah1*Δ mutant cells, it did not suppress the toxicity caused by the supplementation of palmitoleic acid. As discussed above, the data indicated that the basis for the fatty acid-induced toxicity was due to the defect in TAG synthesis. Indeed, the *dgk1*Δ *pah1*Δ double mutant is defective in the synthesis of TAG, although not as severe as that observed for the *pah1*Δ mutant (69, 96), and the major neutral lipid found in these mutants is ergosterol esters (2, 69). Thus, the synthesis of ergosterol esters and normal lipid droplet formation in the *dgk1*Δ *pah1*Δ double mutant are not sufficient to buffer cells from the toxic effects of fatty acids. This notion is also supported by the loss-of-growth phenotype caused by unsaturated fatty acid supplementation to *dga1*Δ *lro1*Δ double mutant cells that also form ergosterol ester-containing lipid droplets (65). Indeed, it is the type of lipid (i.e., TAG) synthesized and stored in lipid droplets that is important for buffering fatty acid-induced toxicity. Thus, it seems plausible that a lipotoxic state that manifests as lipodystrophy persists in the fatty liver dystrophy (*fld*) mouse lacking PA phosphatase.

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REFERENCES

1. Smith, S. W., Weiss, S. B., and Kennedy, E. P. (1957) *J.Biol.Chem.* **228,** 915-922

2. Han, G.-S., Wu, W.-I., and Carman, G. M. (2006) *J Biol.Chem.* **281,** 9210-9218

3. Han, G. S., Siniossoglou, S., and Carman, G. M. (2007) *J.Biol.Chem.* **282,** 37026-37035

4. Carman, G. M. and Han, G. S. (2006) *Trends Biochem Sci* **31,** 694-699

5. Brindley, D. N. (1984) *Prog.Lipid Res.* **23,** 115-133

6. Nanjundan, M. and Possmayer, F. (2003) *Am.J Physiol Lung Cell Mol.Physiol* **284,** L1-23

7. Carman, G. M. and Han, G.-S. (2009) *J.Biol.Chem.* **284,** 2593-2597

8. Waggoner, D. W., Xu, J., Singh, I., Jasinska, R., Zhang, Q. X., and Brindley, D. N. (1999) *Biochim.Biophys.Acta* **1439,** 299-316

9. Sciorra, V. A. and Morris, A. J. (2002) *Biochim.Biophys.Acta* **1582,** 45-51

10. Testerink, C. and Munnik, T. (2005) *Trends Plant Sci* **10,** 368-375

11. Wang, X., Devaiah, S. P., Zhang, W., and Welti, R. (2006) *Prog.Lipid Res.* **45,** 250-278

12. Brindley, D. N. (2004) *J Cell Biochem* **92,** 900-912

13. Howe, A. G. and McMaster, C. R. (2006) *Can.J Physiol Pharmacol.* **84,** 29-38

14. Foster, D. A. (2007) *Cancer Res.* **67,** 1-4

15. Bishop, W. R., Ganong, B. R., and Bell, R. M. (1986) *J.Biol.Chem.* **261,** 6993-7000

16. Kearns, B. G., McGee, T. P., Mayinger, P., Gedvilaite, A., Phillips, S. E., Kagiwada, S., and Bankaitis, V. A. (1997) *Nature* **387,** 101-105

17. Carrasco, S. and Merida, I. (2007) *Trends Biochem Sci* **32,** 27-36

18. Nadra, K., De Preux Charles, A.-S., Medard, J.-J., Hendriks, W. T., Han, G.-S., Gres, S., Carman, G. M., Saulnier-Blache, J.-S., Verheijen, M. H. G., and Chrast, R. (2008) *Genes Dev.* **22,** 1647-1661

19. Csaki, L. S. and Reue, K. (2010) *Annu.Rev.Nutr.* **30,** 257-272

20. Reue, K. and Dwyer, J. R. (2009) *J.Lipid Res.* **50 Suppl,** S109-S114

21. Reue, K. and Brindley, D. N. (2008) *J.Lipid Res.* **49,** 2493-2503

22. Harris, T. E. and Finck, B. N. (2011) *Trends Endocrinol.Metab*

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23. Lin, Y.-P. and Carman, G. M. (1989) *J.Biol.Chem.* **264,** 8641-8645

24. Peterfy, M., Phan, J., Xu, P., and Reue, K. (2001) *Nat.Genet.* **27,** 121-124

25. Phan, J. and Reue, K. (2005) *Cell Metab* **1,** 73-83

26. Donkor, J., Sariahmetoglu, M., Dewald, J., Brindley, D. N., and Reue, K. (2007) *J Biol.Chem.* **282,** 3450-3457

27. Han, G.-S. and Carman, G. M. (2010) *J.Biol.Chem.* **285,** 14628-14638

28. Valente, V., Maia, R. M., Vianna, M. C., and Paco-Larson, M. L. (2010) *FEBS J.* **277,** 4775-4788

29. Ugrankar, R., Liu, Y., Provaznik, J., Schmitt, S., and Lehmann, M. (2011) *Mol.Cell Biol.* **31,** 1646-1656

30. Golden, A., Liu, J., and Cohen-Fix, O. (2009) *J.Cell Sci.* **122,** 1970-1978

31. Nakamura, Y., Koizumi, R., Shui, G., Shimojima, M., Wenk, M. R., Ito, T., and Ohta, H. (2009) *Proc.Natl.Acad.Sci.U.S.A* **106,** 20978-20983

32. Eastmond, P. J., Quettier, A. L., Kroon, J. T., Craddock, C., Adams, N., and Slabas, A. R. (2010) *Plant Cell* **22,** 2796-2811

33. Karanasios, E., Han, G.-S., Xu, Z., Carman, G. M., and Siniossoglou, S. (2010) *Proc.Natl.Acad.Sci.U.S.A.* **107,** 17539-17544

34. Choi, H.-S., Su, W.-M., Morgan, J. M., Han, G.-S., Xu, Z., Karanasios, E., Siniossoglou, S., and Carman, G. M. (2011) *J.Biol.Chem.* **286,** 1486-1498

35. O'Hara, L., Han, G. S., Peak-Chew, S., Grimsey, N., Carman, G. M., and Siniossoglou, S. (2006) *J Biol.Chem.* **281,** 34537-34548

36. Ubersax, J. A., Woodbury, E. L., Quang, P. N., Paraz, M., Blethrow, J. D., Shah, K., Shokat, K. M., and Morgan, D. O. (2003) *Nature* **425,** 859-864

37. Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., McCartney, R. R., Schmidt, M. C., Rachidi, N., Lee, S. J., Mah, A. S., Meng, L., Stark, M. J., Stern, D. F., De Virgilio C., Tyers, M., Andrews, B., Gerstein, M., Schweitzer, B., Predki, P. F., and Snyder, M. (2005) *Nature* **438,** 679-684

38. Dephoure, N., Howson, R. W., Blethrow, J. D., Shokat, K. M., and O'Shea, E. K. (2005) *Proc.Natl.Acad.Sci.U.S.A* **102,** 17940-17945

39. Mah, A. S., Elia, A. E., Devgan, G., Ptacek, J., Schutkowski, M., Snyder, M., Yaffe, M. B., and Deshaies, R. J. (2005) *BMC.Biochem.* **6,** 22

40. Siniossoglou, S., Santos-Rosa, H., Rappsilber, J., Mann, M., and Hurt, E. (1998) *EMBO J.* **17,** 6449-6464

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41. Santos-Rosa, H., Leung, J., Grimsey, N., Peak-Chew, S., and Siniossoglou, S. (2005) *EMBO J* **24,** 1931-1941

42. Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K., and Weissman, J. S. (2003) *Nature* **425,** 737-741

43. Wu, W.-I. and Carman, G. M. (1994) *J.Biol.Chem.* **269,** 29495-29501

44. Wu, W.-I., Lin, Y.-P., Wang, E., Merrill, A. H., Jr., and Carman, G. M. (1993) *J.Biol.Chem.* **268,** 13830-13837

45. Finck, B. N., Gropler, M. C., Chen, Z., Leone, T. C., Croce, M. A., Harris, T. E., Lawrence, J. C., Jr., and Kelly, D. P. (2006) *Cell Metab* **4,** 199-210

46. Peterfy, M., Phan, J., and Reue, K. (2005) *J Biol.Chem.* **280,** 32883-32889

47. Reue, K. and Zhang, P. (2008) *FEBS Lett.* **582,** 90-96

48. Rose, M. D., Winston, F., and Heiter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

49. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

50. Garbarino, J., Padamsee, M., Wilcox, L., Oelkers, P. M., D', A. D., Ruggles, K., Ramsey, N., Jabado, O., Turkish, A., and Sturley, S. L. (2009) *J.Biol.Chem.* **284,** 30994-31005

51. Zwietering, M. H., Jongenburger, I., Rombouts, F. M., and van 't, R. K. (1990) *Appl.Environ.Microbiol.* **56,** 1875-1881

52. Madeo, F., Frohlich, E., and Frohlich, K. U. (1997) *J.Cell Biol.* **139,** 729-734

53. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J.Biol.Chem.* **226,** 497-509

54. Schneiter, R. and Daum, G. (2006) *Methods Mol.Biol.* **313,** 41-45

55. Homan, R. and Anderson, M. K. (1998) *J.Chromatogr.B Biomed.Sci.Appl.* **708,** 21-26

56. Carman, G. M. and Lin, Y.-P. (1991) *Methods Enzymol.* **197,** 548-553

57. Bradford, M. M. (1976) *Anal.Biochem.* **72,** 248-254

58. Carman, G. M. and Han, G.-S. (2011) *Ann.Rev.Biochem.* **80,** 859-883

59. Atkinson, K., Fogel, S., and Henry, S. A. (1980) *J.Biol.Chem.* **255,** 6653-6661

60. Bossie, M. A. and Martin, C. E. (1989) *J.Bacteriol.* **171,** 6409-6413

61. Rattray, J. B., Schibeci, A., and Kidby, D. K. (1975) *Bacteriol.Reviews* **39,** 197-231

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62. Rockenfeller, P., Ring, J., Muschett, V., Beranek, A., Buettner, S., Carmona-Gutierrez, D., Eisenberg, T., Khoury, C., Rechberger, G., Kohlwein, S. D., Kroemer, G., and Madeo, F. (2010) *Cell Cycle* **9,** 2836-2842

63. Jonikas, M. C., Collins, S. R., Denic, V., Oh, E., Quan, E. M., Schmid, V., Weibezahn, J., Schwappach, B., Walter, P., Weissman, J. S., and Schuldiner, M. (2009) *Science* **323,** 1693-1697

64. Carmona-Gutierrez, D., Eisenberg, T., Buttner, S., Meisinger, C., Kroemer, G., and Madeo, F. (2010) *Cell Death.Differ.* **17,** 763-773

65. Petschnigg, J., Wolinski, H., Kolb, D., Zellnig, G., Kurat, C. F., Natter, K., and Kohlwein, S. D. (2009) *J.Biol.Chem.* **284,** 30981-30993

66. Connerth, M., Czabany, T., Wagner, A., Zellnig, G., Leitner, E., Steyrer, E., and Daum, G. (2010) *J.Biol.Chem.* **285,** 26832-26841

67. Black, P. N. and Dirusso, C. C. (2007) *Biochim.Biophys.Acta* **1771,** 286-298

68. Lockshon, D., Surface, L. E., Kerr, E. O., Kaeberlein, M., and Kennedy, B. K. (2007) *Genetics* **175,** 77-91

69. Han, G.-S., O'Hara, L., Carman, G. M., and Siniossoglou, S. (2008) *J.Biol.Chem.* **283,** 20433-20442

70. Han, G.-S., O'Hara, L., Siniossoglou, S., and Carman, G. M. (2008) *J.Biol.Chem.* **283,** 20443-20453

71. Rajakumari, S., Grillitsch, K., and Daum, G. (2008) *Prog.Lipid Res.* **47,** 157-171

72. Kohlwein, S. D. (2010) *J.Biol.Chem.* **285,** 15663-15667

73. Goodman, J. M. (2009) *J.Lipid Res.* **50,** 2148-2156

74. Adeyo, O., Horn, P. J., Lee, S., Binns, D. D., Chandrahas, A., Chapman, K. D., and Goodman, J. M. (2011) *J.Cell Biol.* **192,** 1043-1055

75. Toke, D. A., Bennett, W. L., Dillon, D. A., Chen, X., Oshiro, J., Ostrander, D. B., Wu, W.-I., Cremesti, A., Voelker, D. R., Fischl, A. S., and Carman, G. M. (1998) *J.Biol.Chem.* **273,** 3278-3284

76. Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W. I., Voelker, D. R., and Carman, G. M. (1999) *J.Biol.Chem.* **273,** 14331-14338

77. Langner, C. A., Birkenmeier, E. H., Roth, K. A., Bronson, R. T., and Gordon, J. I. (1991) *J.Biol.Chem.* **266,** 11955-11964

78. Langner, C. A., Birkenmeier, E. H., Ben-Zeev, O., Schotz, M. C., Sweet, H. O., Davisson, M. T., and Gordon, J. I. (1989) *J.Biol.Chem.* **264,** 7994-8003

79. Schuller, H. J., Hahn, A., Troster, F., Schutz, A., and Schweizer, E. (1992) *EMBO J.* **11,** 107-114

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80. Chirala, S. S. (1992) *Proc.Natl.Acad.Sci.U.S.A* **89,** 10232-10236

81. Hasslacher, M., Ivessa, A. S., Paltauf, F., and Kohlwein, S. D. (1993) *J.Biol.Chem.* **268,** 10946-10952

82. Chirala, S. S., Zhong, Q., Huang, W., and Al-Feel, W. (1994) *Nucleic Acids Res.* **22,** 412-418

83. Carman, G. M. and Henry, S. A. (2007) *J.Biol.Chem.* **282,** 37293-37297

84. Stukey, J. E., McDonough, V. M., and Martin, C. E. (1990) *J.Biol.Chem.* **265,** 20144-20149

85. Martin, C. E., Oh, C. S., and Jiang, Y. (2007) *Biochim.Biophys.Acta* **1771,** 271-285

86. Hazel, J. R. (1995) *Annu.Rev.Physiol* **57,** 19-42

87. Listenberger, L. L., Han, X., Lewis, S. E., Cases, S., Farese, R. V., Jr., Ory, D. S., and Schaffer, J. E. (2003) *Proc.Natl.Acad.Sci.U.S.A* **100,** 3077-3082

88. Unger, R. H. and Scherer, P. E. (2010) *Trends Endocrinol.Metab* **21,** 345-352

89. Hannun, Y. A. and Obeid, L. M. (2008) *Nat.Rev.Mol.Cell Biol.* **9,** 139-150

90. Fuentes, L., Perez, R., Nieto, M. L., Balsinde, J., and Balboa, M. A. (2003) *J.Biol.Chem.* **278,** 44683-44690

91. Kohlwein, S. D. (2010) *Biochim.Biophys.Acta* **1801,** 222-229

92. Sandager, L., Gustavsson, M. H., Stahl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H., and Stymne, S. (2002) *J.Biol.Chem.* **277,** 6478-6482

93. Oelkers, P., Tinkelenberg, A., Erdeniz, N., Cromley, D., Billheimer, J. T., and Sturley, S. L. (2000) *J Biol.Chem.* **275,** 15609-15612

94. Oelkers, P., Cromley, D., Padamsee, M., Billheimer, J. T., and Sturley, S. L. (2002) *J Biol.Chem.* **277,** 8877-8881

95. Taylor, F. R. and Parks, L. W. (1979) *Biochim.Biophys.Acta* **575,** 204-214

96. Fakas, S., Konstantinou, C., and Carman, G. M. (2011) *J.Biol.Chem.* **286,** 1464-1474

97. Stukey, J. and Carman, G. M. (1997) *Protein Science* **6,** 469-472

98. Brindley, D. N. and Waggoner, D. W. (1998) *J.Biol.Chem.* **273,** 24281-24284

99. Irie, K., Takase, M., Araki, H., and Oshima, Y. (1993) *Mol.Gen.Genet.* **236,** 283-288

100. Loewen, C. J. R., Gaspar, M. L., Jesch, S. A., Delon, C., Ktistakis, N. T., Henry, S. A., and Levine, T. P. (2004) *Science* **304,** 1644-1647

101. Carman, G. M. and Han, G.-S. (2009) *J.Lipid Res.* **50,** S69-S73

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102. Tehlivets, O., Scheuringer, K., and Kohlwein, S. D. (2007) *Biochim.Biophys.Acta* **1771,** 255-270

103. Cowart, L. A. and Obeid, L. M. (2007) *Biochim.Biophys.Acta* **1771,** 421-431

104. Czabany, T., Athenstaedt, K., and Daum, G. (2007) *Biochim.Biophys.Acta* **1771,** 299-309

105. Thomas, B. and Rothstein, R. (1989) *Cell* **56,** 619-630

106. Wimmer, C., Doye, V., Grandi, P., Nehrbass, U., and Hurt, E. C. (1992) *EMBO J.* **11,** 5051-5061

107. Sikorski, R. S. and Hieter, P. (1989) *Genetics* **122,** 19-27

108. Kastaniotis, A. J., Autio, K. J., Sormunen, R. T., and Hiltunen, J. K. (2004) *Mol.Microbiol.* **53,** 1407-1421

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FOOTNOTES

*This work was supported, in whole or in part by, National Institutes of Health Grants GM-28140 (to G.M.C.) and DK-54320 (to S.L.S.). The liquid chromatography-mass spectrometry system was obtained through a National Center for Research Resources Grant RR-021120 from the National Institutes of Health.

[1]Supported by National Institutes of Health-Clinical and Translational Science Award Training Grant RR-02415803.

[2]Supported by National Institutes of Health Grant DK-007647.

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[4]The abbreviations used are: *PA*, phosphatidate; *DAG*, diacylglycerol *TAG*, triacylglycerol; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; and *PS*, phosphatidylserine; *FA*, fatty acids; *14:0*, myristic acid; *16:0*, palmitic acid; *16:1*, palmitoleic acid; *18:0*, stearic acid; *18:1*, oleic acid.

[5]PA phosphatase is distinguished in catalytic activity from the lipid phosphate phosphatase enzymes that dephosphorylate a broad spectrum of substrates (e.g., PA, lysoPA, and DAG pyrophosphate) by a distinct catalytic mechanism that does not require divalent cations (4, 12, 97, 98).

[6]*PAH1* was previously known by the designation *SMP2* (99).

[7]Elevated PA levels induce the expression of UAS_{INO}-containing genes by tethering the Opi1p repressor at the nuclear/ER membrane. This prevents Opi1p translocation into the nucleus where it attenuates transcription of genes by binding to Ino2p within the transcriptional activator Ino2p-Ino4p complex (83, 100).

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FIGURE LEGENDS

FIGURE 1. **Roles of PA phosphatase in the synthesis of TAG and membrane phospholipids in *S. cerevisiae*.** The structures of PA, DAG, and TAG are shown with fatty acyl groups of 16 and 18 carbons with and without a single double bond where indicated. The pathways shown in the figure include the relevant steps discussed in this work. More comprehensive pathways of lipid metabolism in yeast may be found in Refs. 72, 101-104. The activity of PA phosphatase (*PAP*) plays a major role in governing whether cells utilize PA for the synthesis of TAG or whether they utilize PA for the synthesis of membrane phospholipids (*PL*). In addition, PA signals the transcriptional regulation of phospholipid synthesis genes and the growth of the nuclear/ER membrane. The PA phosphatase reaction is counterbalanced by the DAG kinase (*DGK*) reaction. Acyl CoA molecules, which are made *de novo* or derived from exogenous fatty acids (*FA*), are used for the synthesis of PA and for the synthesis of TAG from DAG. Acyl CoA is also used for the synthesis of ergosterol esters (not shown). The *bold* arrows indicate the consequences of the *pah1*Δ mutation.

FIGURE 2. **The *pah1*Δ mutation affects the masses of lipids.** Lipid extracts were prepared from wild type (W303-1A) and *pah1*Δ mutant (GHY57) cells grown to the exponential and stationary phases in YEPD medium. ***A,*** the total amounts of TAG and phospholipids (*PL*) were analyzed by high performance liquid chromatography-mass spectroscopy. The lipid extract was also subjected to thin-layer chromatography; free fatty acids (*FA*) were extracted from the silica gel, subjected to transmethylation, and analyzed by gas-liquid chromatography. ***B***, phospholipids were analyzed by high performance liquid chromatography-mass spectrometry. The data shown in both panels were the averages of three separate experiments ± S.D. (*error bars*).

FIGURE 3. **Stationary phase *pah1*Δ mutant cells exhibit hallmark phenotypes of apoptosis.** ***A,*** spheroplasts of stationary phase wild type (W303-1A) and *pah1*Δ mutant (GHY57) cells were stained with fluorescein isothiocyanate annexin V and propidium iodide to examine by fluorescence microscopy the phenotypes characteristic of apoptosis. The data shown are representative of several fields of view during multiple experiments. *DIC*, differential interference contrast. ***B***, quantitation of the average number of cells stained with annexin V and propidium iodide ± S.E. (*error bars*).

FIGURE. 4. **Fatty acid supplementation is toxic to the growth of *pah1*Δ mutant cells.** Wild type (W303-1A) and *pah1*Δ mutant (GHY57) cells were grown in the absence and presence of the indicated concentrations of palmitic (*16:0*), palmitoleic (*16:1*), stearic (*18:0*), and oleic (*18:1*) acids in YEPD medium. Growth was monitored at $A_{600\ nm}$. Each data point represents the average of three independent experiments, and the average standard deviation for each data point was ± 3%.

FIGURE 5. **The *dgk1*Δ mutation does not suppress the palmitoleic acid-induced toxicity of *pah1*Δ mutant cells.** Wild type (RS453), *pah1*Δ (SS1026), *dgk1*Δ (SS1144), and *dgk1*Δ *pah1*Δ mutant (SS1147) cells were grown in the absence and presence of the indicated concentrations of palmitoleic acid in YEPD medium. Growth was monitored at $A_{600\ nm}$. Each data point represents the average of three independent experiments, and the average standard deviation for each data point was ± 3%.

FIGURE 6. **The *dgk1*Δ mutation suppresses the impairment of lipid droplet formation of *pah1*Δ mutant cells.** *A*, wild type (RS453), *pah1*Δ (SS1026), *dgk1*Δ (SS1144), and *dgk1*Δ

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*pah1*Δ mutant (SS1147) cells grown to stationary phase in YEPD medium were stained with Nile red to image lipid droplets by fluorescence microscopy. The data shown are representative of several fields of view during multiple experiments. The *bar* indicates 5 μm. *DIC*, differential interference contrast. *B*, the number of lipid droplets was counted for 6 frames, with 15-30 cells per frame ± S.E. (*error bars*).

FIGURE 7. **PA phosphatase activity is required to protect cells from palmitoleic acid-induced toxicity.** *pah1*Δ (GHY57) expressing the wild type *PAH1* and the indicated *PAH1* mutant alleles from low copy plasmids were grown in the absence and presence of 0.25 mM palmitoleic acid (*16:1*) in SC-leucine medium. Growth was monitored at $A_{600\ nm}$. Each data point represents the average of three independent experiments, and the average standard deviation for each data point was ± 3%.

FIGURE 8. **Palmitoleic acid supplementation induces the level of PA phosphatase activity.** Wild type (W303-1A) and *pah1*Δ mutant (GHY57) cells were grown to the exponential phase in YEPD medium. Palmitoleic acid (0.25 mM) was then added to the cultures, and at the indicated time intervals, cell extracts were prepared and used for the measurement of PA phosphatase activity under standard assay conditions (indicated by the *closed symbols*). PA phosphatase activity was also measured with a reaction mixture that included 2 mM EDTA instead of 1 mM $MgCl_2$ (indicated by the *dot* inside the *symbol* for the wild type). Wild type cells were also grown without palmitoleic acid supplementation (indicated by the open symbol for the wild type). Each data point represents the average of triplicate determinations from a minimum of two independent experiments ± S.D. (*error bars*).

FIGURE 9. **Palmitoleic acid supplementation induces the synthesis of TAG.** Wild type (W303-1A) (*A*) and *pah1*Δ mutant (GHY57) (*B*) cells were grown to the exponential phase in YEPD medium. Palmitoleic acid (0.25 mM) was added to the cultures, and at the indicated time intervals, lipids were extracted and analyzed by high performance liquid chromatography. The data were the averages of three separate experiments ± S.D. (*error bars*).

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TABLE 1

Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	(105)
GHY57	*pah1*Δ*::URA3* derivative of W303-1A	(2)
RS453	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52*	(106)
SS1026	*pah1*Δ::*TRP1* derivative of RS453	(41)
SS1144	*dgk1*Δ::*HIS3* derivative of RS453	(69)
SS1147	*dgk1*Δ::*HIS3 pah1*Δ::*TRP1* derivative of RS453	(69)
Plasmids		
pRS415	Low copy *E. coli*/yeast shuttle vector with *URA3*	(107)
pGH315	*PAH1* derivative of pRS415	(108)
pGH315-D398E	pGH315 containing the D398E mutation in the *PAH1* coding sequence	This study
pGH315-D400E	pGH315 containing the D400E mutation in the *PAH1* coding sequence	This study
pGH315-G80R	pGH315 containing the G80R mutation in the *PAH1* coding sequence	This study

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TABLE 2

Fatty acid composition in exponential and stationary phase cells

		Total lipid fatty acid, %			
Growth phase	Cell type	16:0	16:1	18:0	18:1
Exp	wild type	16.5 ± 0.7	35.3 ± 1.0	9.4 ± 0.2	38.8 ± 0.4
	*pah1*Δ	16.6 ± 0.8	44.8 ± 0.2	7.8 ± 0.2	30.7 ± 0.5
Stat	wild type	17.4 ± 0.4	39.6 ± 2.5	6.8 ± 2.2	36.1 ± 0.7
	*pah1*Δ	15.9 ± 0.9	48.3 ± 0.8	6.0 ± 0.2	29.9 ± 1.9

Wild type (W303-1A) and *pah1*Δ mutant (GHY57) cells were grown to the exponential and stationary phases in YEPD medium. The total lipid fraction was isolated, subjected to transmethylation, and analyzed by gas-liquid chromatography. The data were the averages of three separate experiments ± S.D. The sum of the four fatty acid species in the table was set to 100%. Abbreviations: Exp, exponential; Stat, stationary.

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TABLE 3

Phospholipid molecular species in exponential and stationary phase cells

Lipid	Growth phase	Cell type	Molecular species, %								
			14:0-14:0	14:0-16:1	16:0-16:1	16:1-16:1	16:0-18:0	16:0-18:1	16:1-18:1	18:0-18:1	18:1-18:1
PC	Exp	wild type	n.d.	n.d.	8.1 ± 0.2	31.4 ± 1.1	n.d.	9.4 ± 0.8	41.8 ± 0.9	2.5 ± 0.3	6.9 ± 0.4
		*pah1*Δ	n.d.	n.d.	9.3 ± 0.2	43.0 ± 0.2	n.d.	7.3 ± 0.2	37.8 ± 0.3	n.d.	2.6 ± 0.3
	Stat	WT	n.d.	n.d.	7.8 ± 0.6	35.1 ± 1.6	n.d.	8.3 ± 0.7	44.2 ± 0.4	n.d.	4.6 ± 0.1
		*pah1*Δ	n.d.	n.d.	8.6 ± 0.5	43.8 ± 0.1	n.d.	6.2 ± 0.1	39.2 ± 0.5	n.d.	2.2 ± 0.1
PE	Exp	wild type	n.d.	n.d.	11.1 ± 0.4	15.7 ± 0.6	n.d.	22.3 ± 0.2	41.1 ± 1.1	n.d.	5.3 ± 0.4
		*pah1*Δ	n.d.	5.1 ± 1.0	16.3 ± 0.6	23.8 ± 0.6	n.d.	16.2 ± 0.2	30.4 ± 0.8	n.d.	2.6 ± 0.2
	Stat	WT	n.d.	n.d.	12.3 ± 0.6	14.1 ± 0.5	n.d.	22.7 ± 0.9	38.6 ± 0.9	n.d.	2.8 ± 0.7
		*pah1*Δ	n.d.	3.9 ± 0.3	15.7 ± 0.3	23.2 ± 0.4	n.d.	16.7 ± 0.5	33.0 ± 1.0	n.d.	n.d.
PI	Exp	wild type	n.d.	n.d.	15.4 ± 0.7	2.5 ± 0.7	n.d.	45.4 ± 0.4	9.2 ± 1.0	17.3 ± 0.2	3.1 ± 0.5
		*pah1*Δ	4.2 ± 0.1	n.d.	23.1 ± 0.2	1.9 ± 0.4	n.d.	40.2 ± 1.5	3.9 ± 0.2	16.5 ± 0.5	n.d.
	Stat	WT	n.d.	n.d.	18.3 ± 1.0	4.4 ± 0.9	n.d.	47.5 ± 0.7	9.9 ± 0.7	14.9 ± 0.8	4.1 ± 0.7
		*pah1*Δ	4.8 ± 0.2	n.d.	23.6 ± 0.9	2.1 ± 0.3	n.d.	39.9 ± 1.4	3.6 ± 0.1	14.9 ± 0.5	n.d.
PS	Exp	wild type	n.d.	n.d.	2.1 ± 0.5	19.8 ± 1.2	n.d.	6.2 ± 0.7	69.4 ± 0.7	n.d.	2.5 ± 0.2
		*pah1*Δ	n.d.	n.d.	3.3 ± 2.6	37.7 ± 2.3	n.d.	5.5 ± 0.3	51.4 ± 1.2	n.d.	2.2 ± 0.2
	Stat	WT	n.d.	n.d.	n.d.	27.9 ± 1.1	n.d.	n.d.	72.1 ± 1.1	n.d.	n.d.
		*pah1*Δ	n.d.	n.d.	n.d.	40.0 ± 1.9	n.d.	5.5 ± 1.3	54.5 ± 1.8	n.d.	n.d.
PA	Exp	wild type	n.d.	n.d.	12.3 ± 0.8	6.9 ± 0.3	2.6 ± 2.2	35.9 ± 2.6	33.9 ± 2.9	3.5 ± 0.5	4.9 ± 0.7
		*pah1*Δ	n.d.	n.d.	18.0 ± 0.4	12.4 ± 0.5	2.8 ± 1.2	27.7 ± 1.3	22.7 ± 1.5	6.2 ± 0.0	2.7 ± 0.2
	Stat	wild type	n.d.	n.d.	16.3 ± 0.4	5.1 ± 0.7	3.3 ± 2.9	49.7 ± 1.1	25.6 ± 2.0	n.d.	n.d.
		*pah1*Δ	n.d.	n.d.	19.7 ± 0.8	9.1 ± 0.5	3.5 ± 0.6	35.4 ± 3.8	20.0 ± 2.7	5.2 ± 0.7	n.d.

Wild type (W303-1A) and *pah1*Δ mutant (GHY57) cells were grown to the exponential and stationary phases in YEPD medium. Lipids were extracted and the molecular species of the indicated phospholipids were analyzed by high performance liquid chromatography-mass spectrometry. The percentages shown for the molecular species of each phospholipid were normalized to the total amount found for that phospholipid. The data were the averages of three separate experiments ± S.D. Abbreviations: Exp, exponential; Stat, stationary; n.d., not detected.

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TABLE 4

TAG molecular species in exponential and stationary phase cells

Growth phase	Cell type	Molecular species, %									
		16:0-16:1-16:1	16:1-16:1-16:1	16:0-16:1-18:1	16:1-16:1-18:1	16:0-18:0-18:0	16:0-18:1-18:1	16:1-18:1-18:1	18:0-18:0-18:0	18:0-18:1-18:1	18:1-18:1-18:1
Exp	wild type	6.9 ± 0.2	17.1 ± 1.8	12.5 ± 0.2	32.3 ± 1.3	n.d.	4.1 ± 0.1	17.8 ± 0.5	n.d.	3.0 ± 0.1	6.4 ± 0.1
	*pah1*Δ	8.0 ± 0.9	18.2 ± 1.4	10.8 ± 0.1	25.0 ± 2.3	n.d.	4.3 ± 0.2	8.8 ± 0.5	n.d.	1.8 ± 0.2	23.1 ± 2.4
Stat	wild type	7.4 ± 1.6	19.7 ± 1.9	10.9 ± 3.8	33.5 ± 7.5	7.8 ± 0.5	n.d.	11.6 ± 3.3	6.2 ± 0.5	n.d.	3.0 ± 0.7
	*pah1*Δ	10.5 ± 0.1	21.6 ± 2.2	14.6 ± 1.7	30.4 ± 0.9	1.4 ± 0.1	n.d.	11.2 ± 0.7	1.1 ± 0.1	n.d.	9.4 ± 1.1

Wild type (W303-1A) and *pah1*Δ mutant (GHY57) cells were grown to the exponential and stationary phases in YEPD medium. Lipids were extracted and the molecular species of TAG were analyzed by high performance liquid chromatography-mass spectrometry. The percentages shown for the molecular species were normalized to the total amount found for TAG. The data were the averages of three separate experiments ± S.D. Abbreviations: Exp, exponential; Stat, stationary; n.d., not detected.

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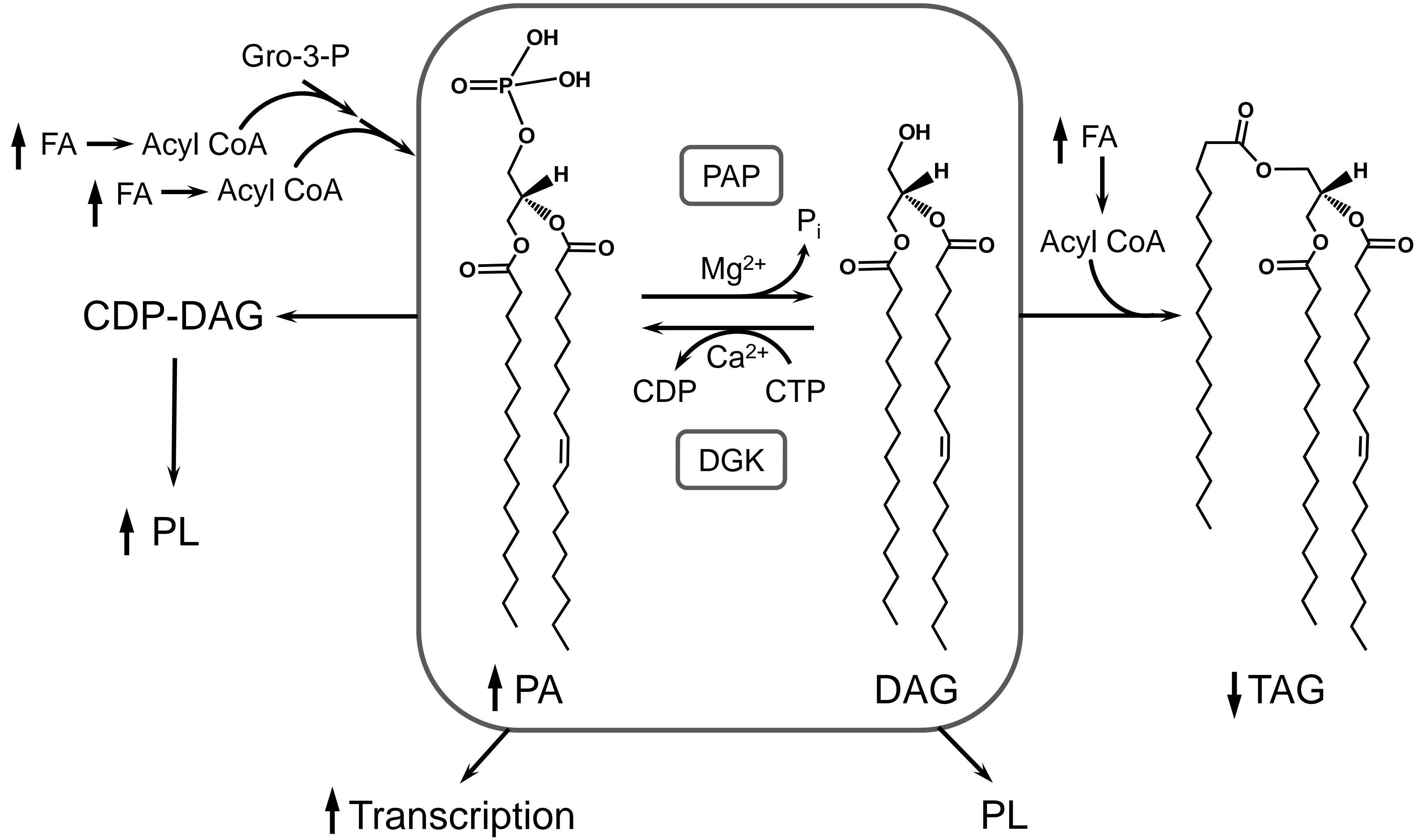

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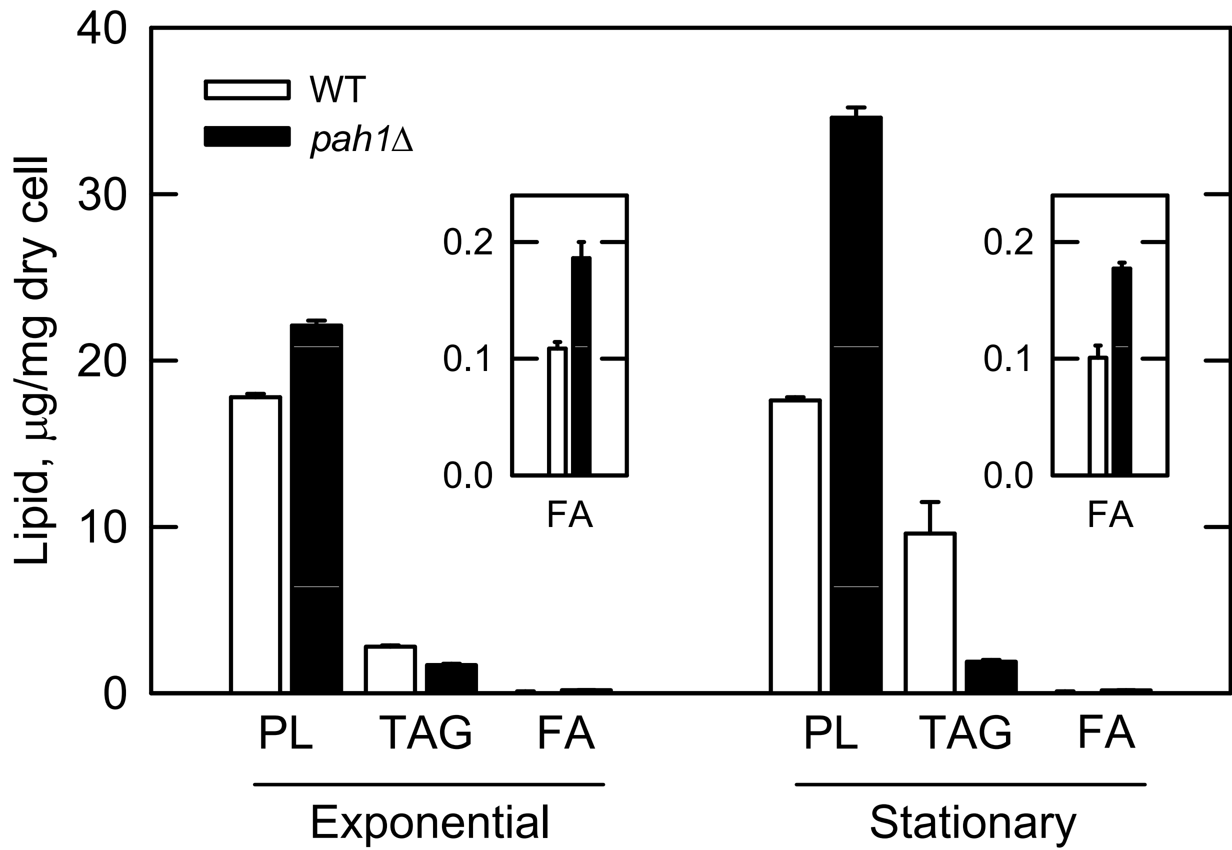

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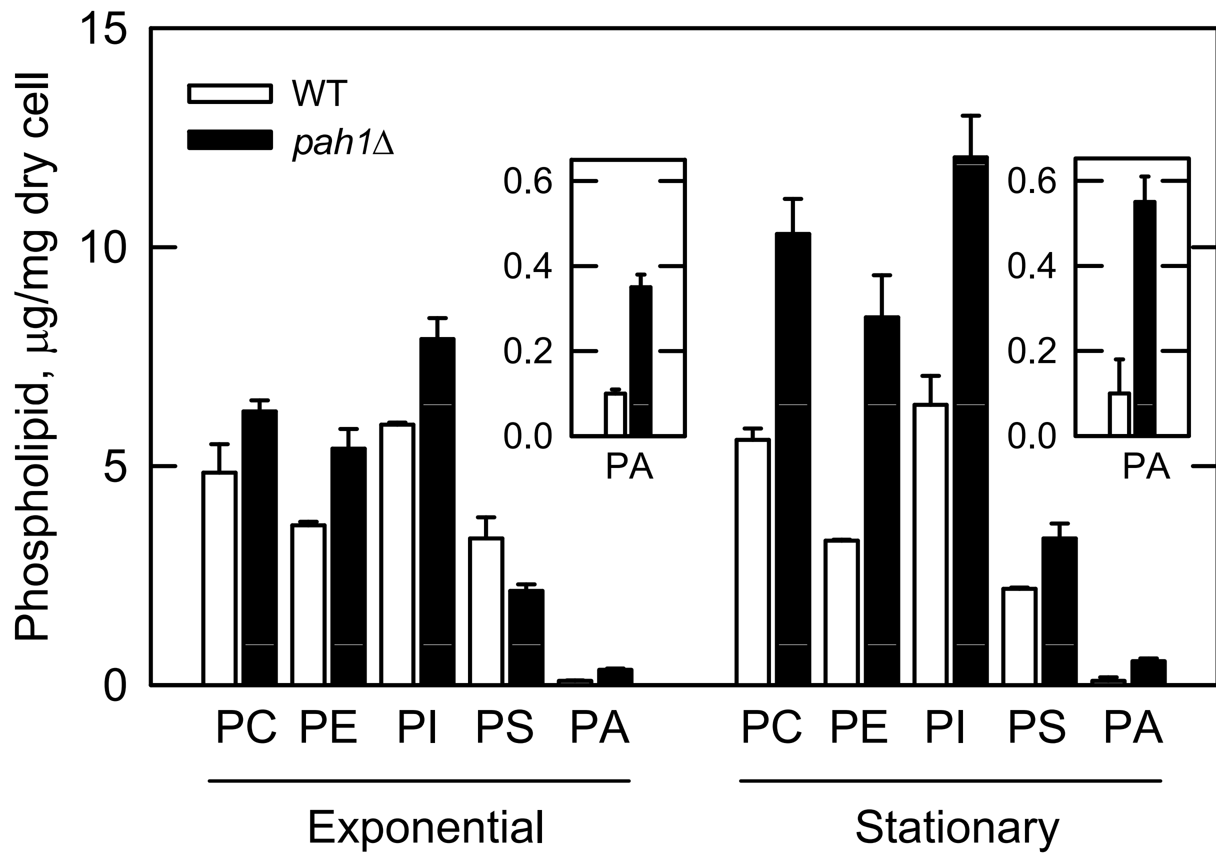

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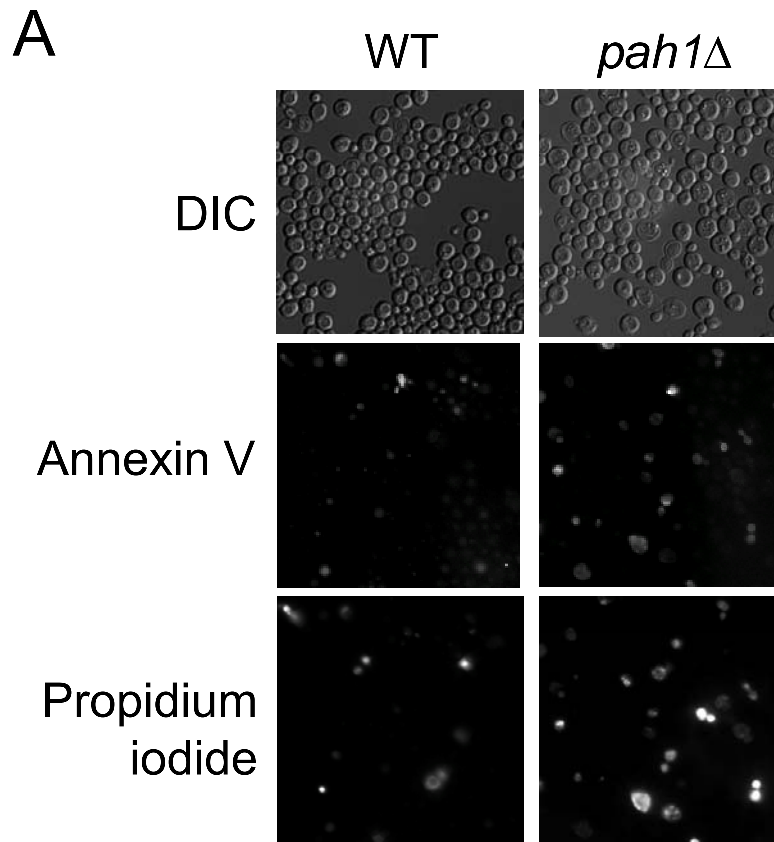

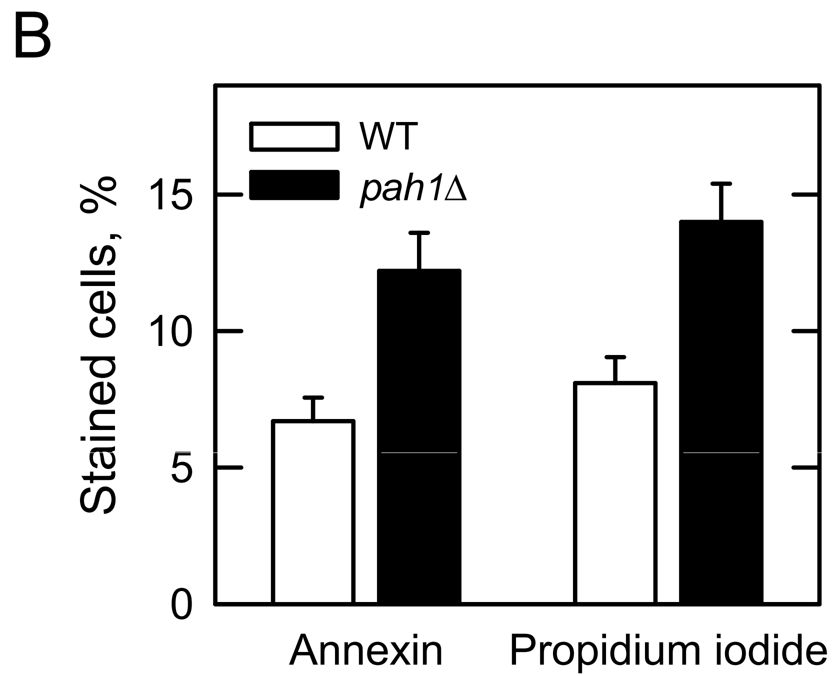

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4

A

WT

16:0 16:1 18:0 18:1

$A_{600\ nm}$

1.2
0.8
0.4
0.0

0 12 24 0 12 24 0 12 24 0 12 24

Time, h

○ 0.00 mM
◇ 0.10 mM
□ 0.25 mM
△ 0.50 mM

B

pah1Δ

16:0 16:1 18:0 18:1

$A_{600\ nm}$

1.2
0.8
0.4
0.0

0 12 24 0 12 24 0 12 24 0 12 24

Time, h

○ 0.00 mM
◇ 0.10 mM
□ 0.25 mM
△ 0.50 mM

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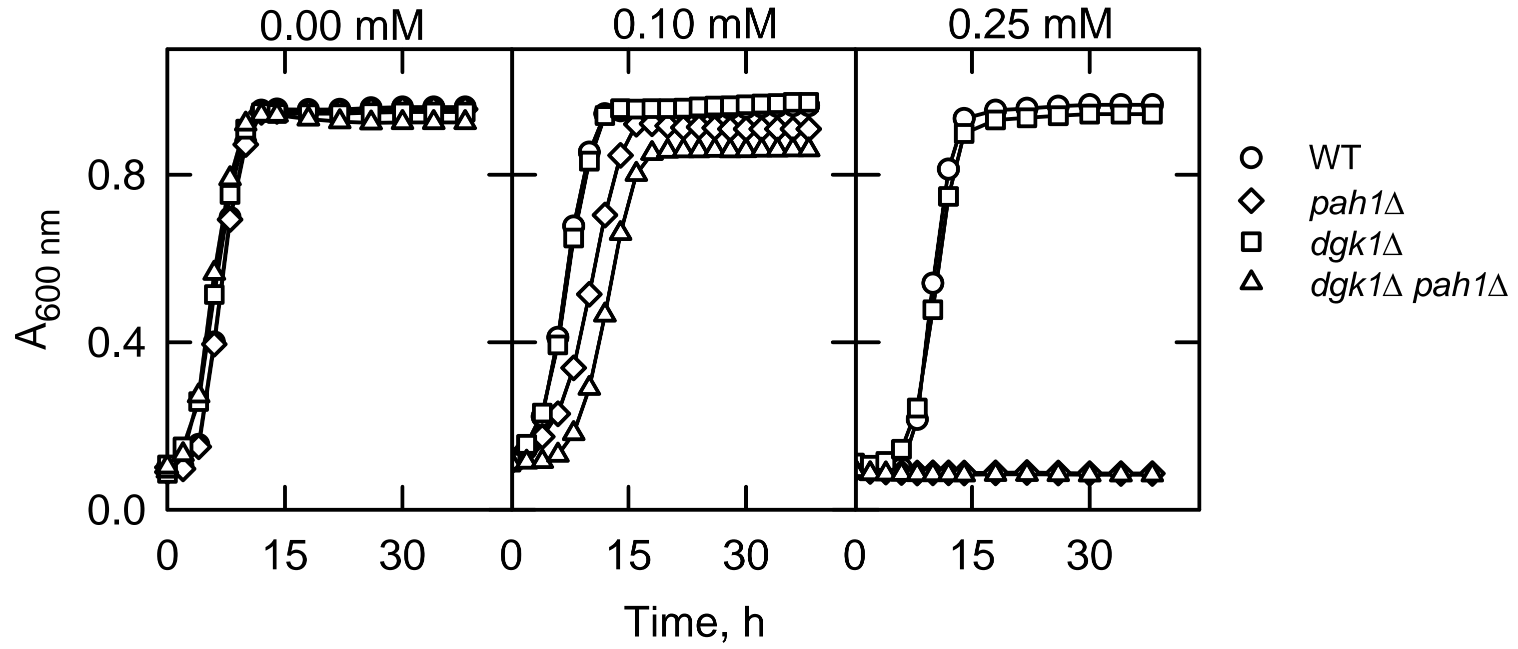

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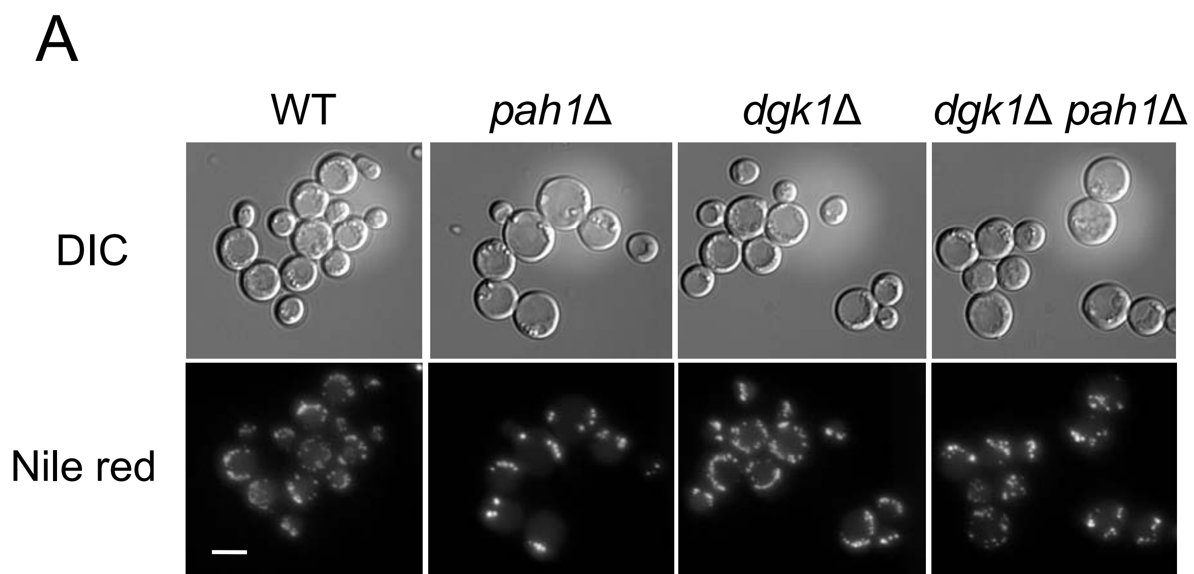

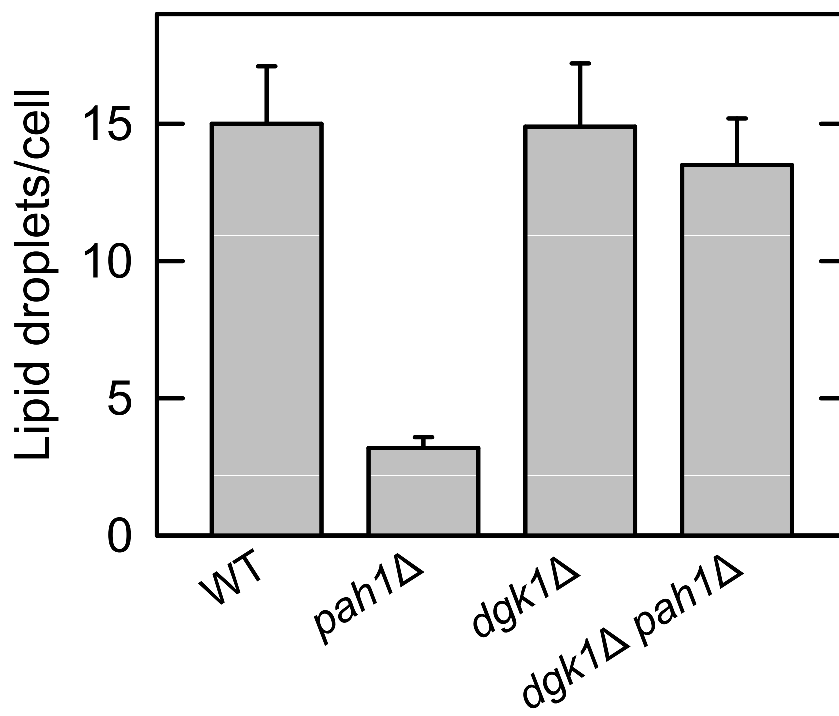

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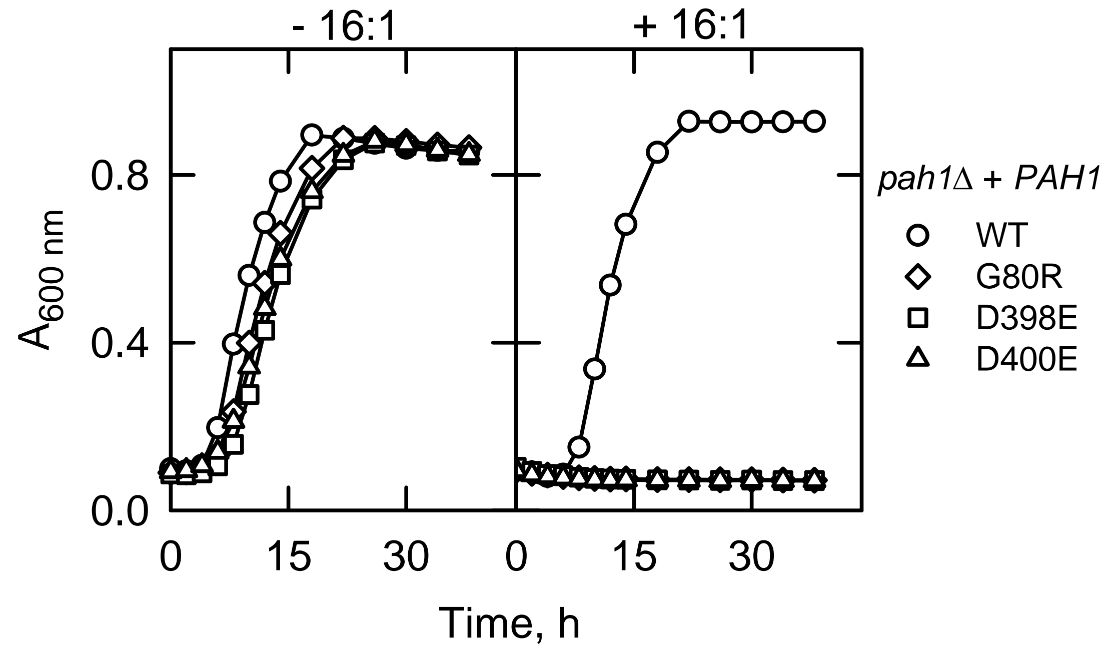

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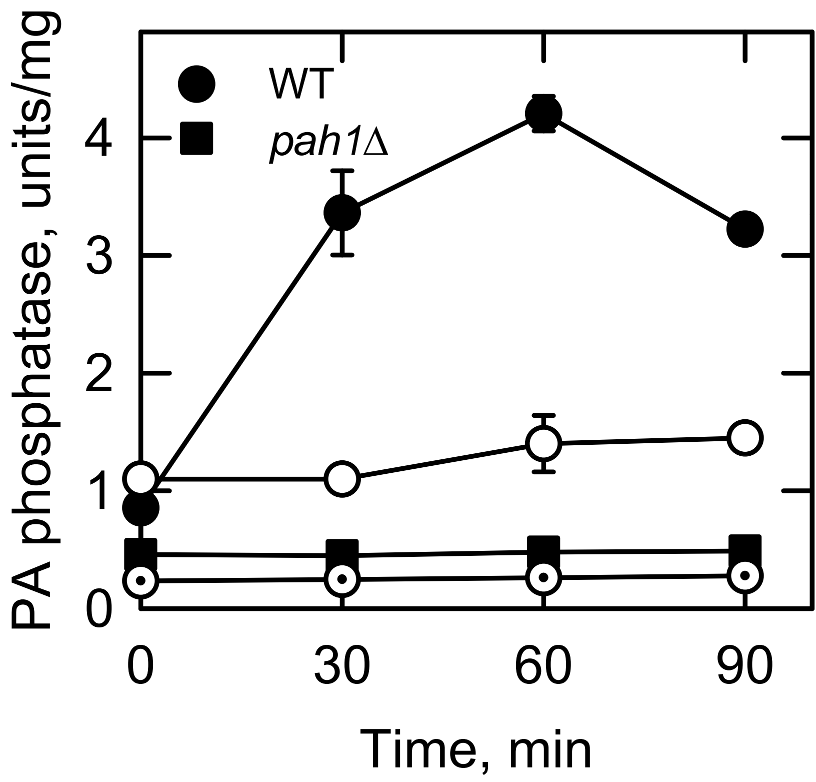

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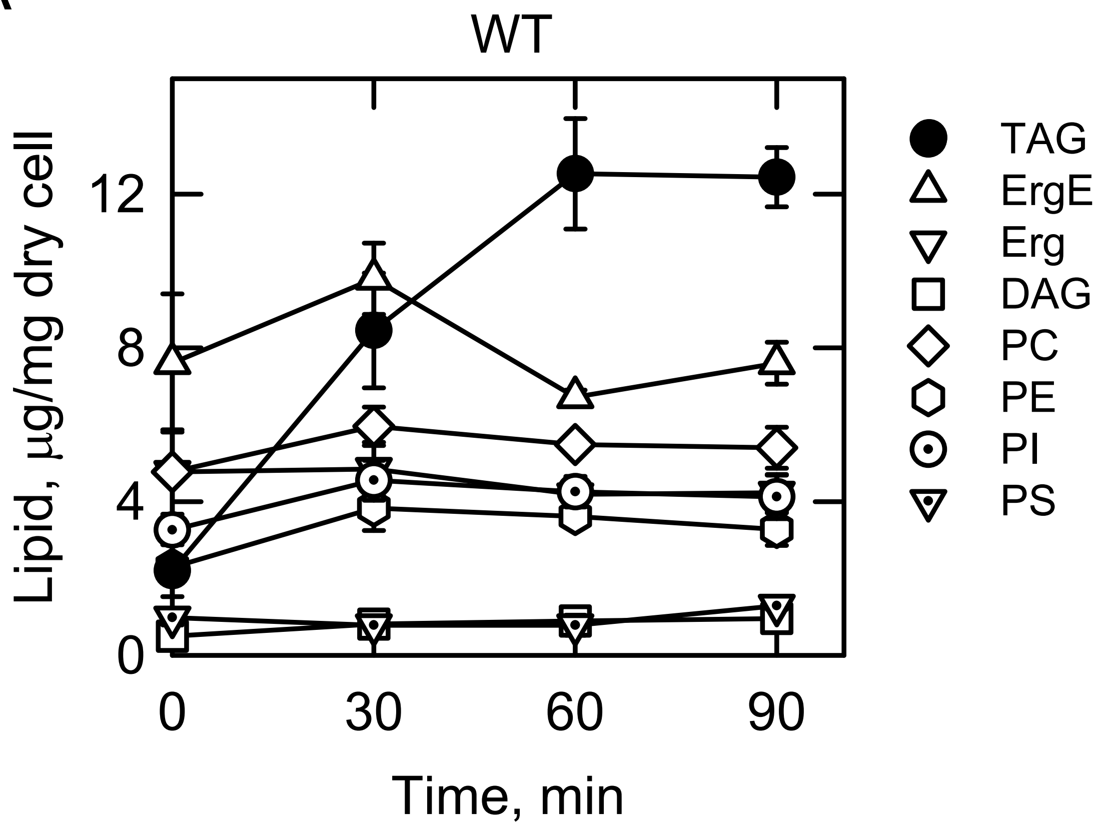

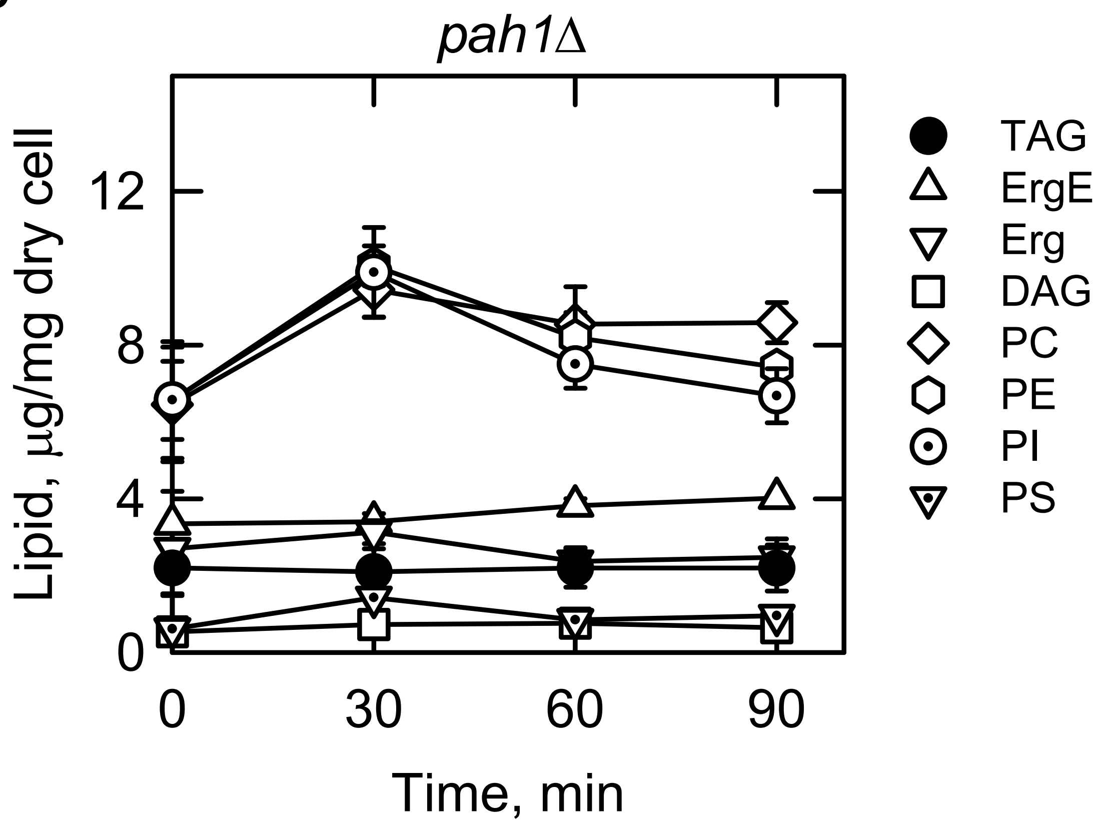

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