REGULATION OF THE *PAH1*-ENCODED PHOSPHATIDATE PHOSPHATASE AND ITS ROLE IN LIPID METABOLISM IN YEAST

By

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and approved by

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ABSTRACT OF THE DISSERTATION

Regulation of the yeast *PAH1*-encoded phosphatidate phosphatase and its role in lipid metabolism in yeast

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The yeast *PAH1*-encoded phosphatidate phosphatase (PAP) catalyzes the penultimate step in the synthesis of triacylglycerol (TAG). PAP plays a crucial role in lipid homeostasis by controlling the relative proportions of its substrate phosphatidate (PA) and its product diacylglycerol (DAG). The cellular amounts of these lipid intermediates influence the synthesis of TAG and the pathways by which membrane phospholipids are synthesized. The importance of this enzyme is exemplified by the severe phenotypes of the *pah1*Δ mutant that include deregulation of phospholipid synthesis, an aberrant expansion of the nuclear/ER membrane, a decrease in lipid droplet formation, a massive reduction in TAG content, defects in vacuole homeostasis and fusion, and increased sensitivity to lipotoxicity. On the other hand, the attenuation of *PAH1*-encoded PAP function is also essential to normal cell physiology. Indeed, the overexpression of an unregulated form of PA phosphatase inhibits cell growth, and this is attributed to the depletion of PA needed for phospholipid synthesis via CDP-DAG and

the accumulation of DAG to a toxic level. TAG accumulates in the stationary phase of growth, and the lipid analysis of the *pah1*Δ mutant established the essential role of Pah1p PAP in this process. While Pah1p PAP activity was elevated in stationary phase cells, the expression of *PAH1* mRNA and Pah1p was maximal in the exponential phase, but declined as cells entered the stationary phase. The levels of Pah1p were stabilized in stationary phase cells treated with the proteasome inhibitor MG132 as well as in several mutants that exhibit a decrease in proteasome function. Catalytic site mutant forms of Pah1p exhibited increased stability in stationary phase, indicating that the balance of PA and DAG acts as a signal for the degradation of the enzyme. Additionally, Pah1p levels were also stabilized in cells overexpressing *DGK1*, pointing to PA as a possible regulator of Pah1p stability. This work reveals a novel mechanism for the control of Pah1p PAP function in yeast.

DEDICATION

To my mom, for showing me it is possible to have a successful career and be a wonderful mother at the same time; you are my role model.

To my dad, for teaching me the value of hard work and perseverance; your efforts and encouragement made this possible.

To my brother, for always being there for me despite the distance.

To my friends and extended family, for keeping me sane throughout this journey.

You are my source of strength and inspiration.

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the pGH339 plasmid directing the expression of *DPP1* driven by the *PAH1* promoter, which was fundamental in our understanding of Pah1p regulation. My deepest appreciation to former lab members for their invaluable help during my early days: Sreenivas Avula, Yu-Fang Chang, and Mal-Gi Choi. Special thanks to Aníbal Soto-Cardalda and Wen-Min Su, with whom I shared laughter and tears, baseball and Taiwanese cooking lessons, and who have become dear and close friends whom I will always treasure. I would also like to acknowledge Aníbal Soto-Cardalda for his contribution to this work: the construction of the *PAH1* promoter truncations and the preliminary experiments that provided the first indication that Pah1p degradation occurred in a regulated manner. To all of the Carman lab members, it has been a privilege and a pleasure working alongside you.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CDP	cytidine diphosphate
CDP-DAG	cytidine diphosphate diacylglycerol
CL	cardiolipin
CMP	cytidine monophosphate
CTP	cytidine triphosphate
DAG	diacylglycerol
DGK	diacylglycerol kinase
DGPP	diacylglycerol pyrophosphate
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
HAD	haloacid dehalogenase
Ino	inositol
KDa	kilo Daltons
LPP	lipid phosphate phosphatase
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	*N*-ethylmaleimide
PA	phosphatidate
PAP	phosphatidate phosphatase
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PG	phosphatidylglycerol
PGP	phosphatidylglycerol phosphate
PI	phosphatidylinositol
P_i	inorganic phosphate
PKA	protein kinase A
PKC	protein kinase C
PLD	phospolipase D
PS	phosphatidylserine
SDS-PAGE	sodium dodecyl sufate polyacrylamide gel electrophoresis
TAG	triacylglycerol
UAS_{INO}	upstream activating sequence, inositol responsive element
UTP	uridine triphosphate

INTRODUCTION

With the increasing prevalence of obesity and its associated diseases, much interest has been placed on the study of mechanisms that control lipid homeostasis. The importance of lipids in normal cell physiology and development is not limited to their role as energy storage molecules. In addition, lipids are key structural components of cell membranes, macromolecule precursors, molecular chaperones, hormones, pigments, reservoirs of lipid signaling molecules, and can also assist in protein-membrane associations (1-5).

The crucial functions of lipids in cells are highly conserved from simple prokaryotes to the most complex of eukaryotes, humans (6). The budding yeast *Saccharomyces cerevisiae* synthesizes TAG and membrane phospholipids by pathways common to those of higher eukaryotic organisms (3, 6, 7), and has thus been extensively used as a model system for the study of lipid synthesis. Moreover, the yeast genome is exceptionally small by eukaryotic standards (8, 9), which has allowed for its complete sequencing and thus, the identification of nearly all of the structural genes responsible for *de novo* lipid synthesis (2, 3, 7, 9-12). Other attractive features of this model organism include its ease of use, since it can be easily and rapidly grown in large quantities (6, 9); its tractable genetics (1, 3, 9), which facilitate genetic manipulations; and the availability of extensive databases (3, 7, 9).

Phospholipids are structural components of cell membranes

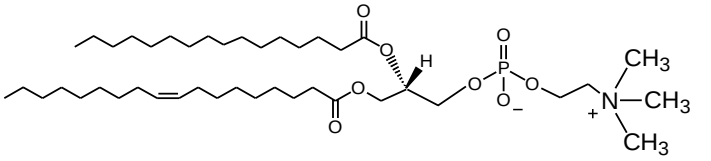
Biological membranes are an essential component of the cell (13). Cell membranes act as selective barriers, defining the periphery of cells and separating their

contents from the surrounding environment, thus maintaining cell integrity as a coordinated biochemical system (1, 8). Other functions of membranes include enabling communication between cells through the transduction of molecular signals by proteins embedded on the cell surface, as well as serving as a platform where crucial biological reactions take place (1, 8, 13).

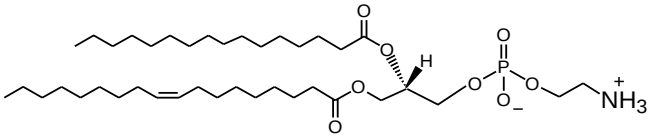
Cell membranes are composed of lipid and protein molecules held together by noncovalent interactions, which form a flexible, hydrophobic layer around the cell (13). Phospholipids are the most abundant class of membrane lipids, and are therefore intimately linked to cellular growth (13). All phospholipids share a common structure, composed of a glycerol molecule, two fatty acyl chains, and a hydrophilic head group (1, 8) (Fig. 1). The two fatty acids are linked to the *sn*-1 and *sn*-2 positions of the glycerol backbone, comprising the hydrophobic portion of the phospholipid molecule, while the third site is occupied by a charged phosphate group, which confers hydrophilic properties to the head portion of the molecule (1, 8, 14) (Fig. 1). The amphipathic property of phospholipids allows for the formation of phospholipid bilayers in cellular membranes (1, 8, 15).

The most abundant membrane phospholipids in *S. cerevisiae* are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) (2, 6, 8, 10, 11, 14) (Fig. 1). These phospholipids are formed by binding of the head groups choline, ethanolamine, serine, or inositol to the phosphate group on the third carbon of the glycerol backbone, respectively (1, 8, 14). In addition, yeast mitochondrial membranes also contain the phospholipids phosphatidylglycerol (PG) and cardiolipin (CL) (2, 6, 10, 11, 14). All subcellular membranes are composed of

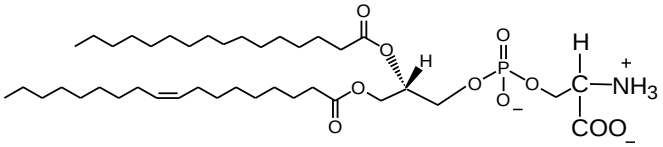
Figure 1. Major phospholipids in *Saccharomyces cerevisiae.*



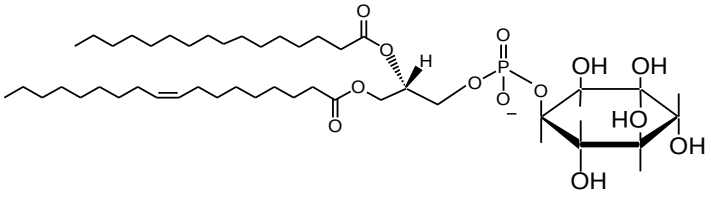
Phosphatidylcholine
(PC)



Phosphatidylethanolamine
(PE)



Phosphatidylserine
(PS)



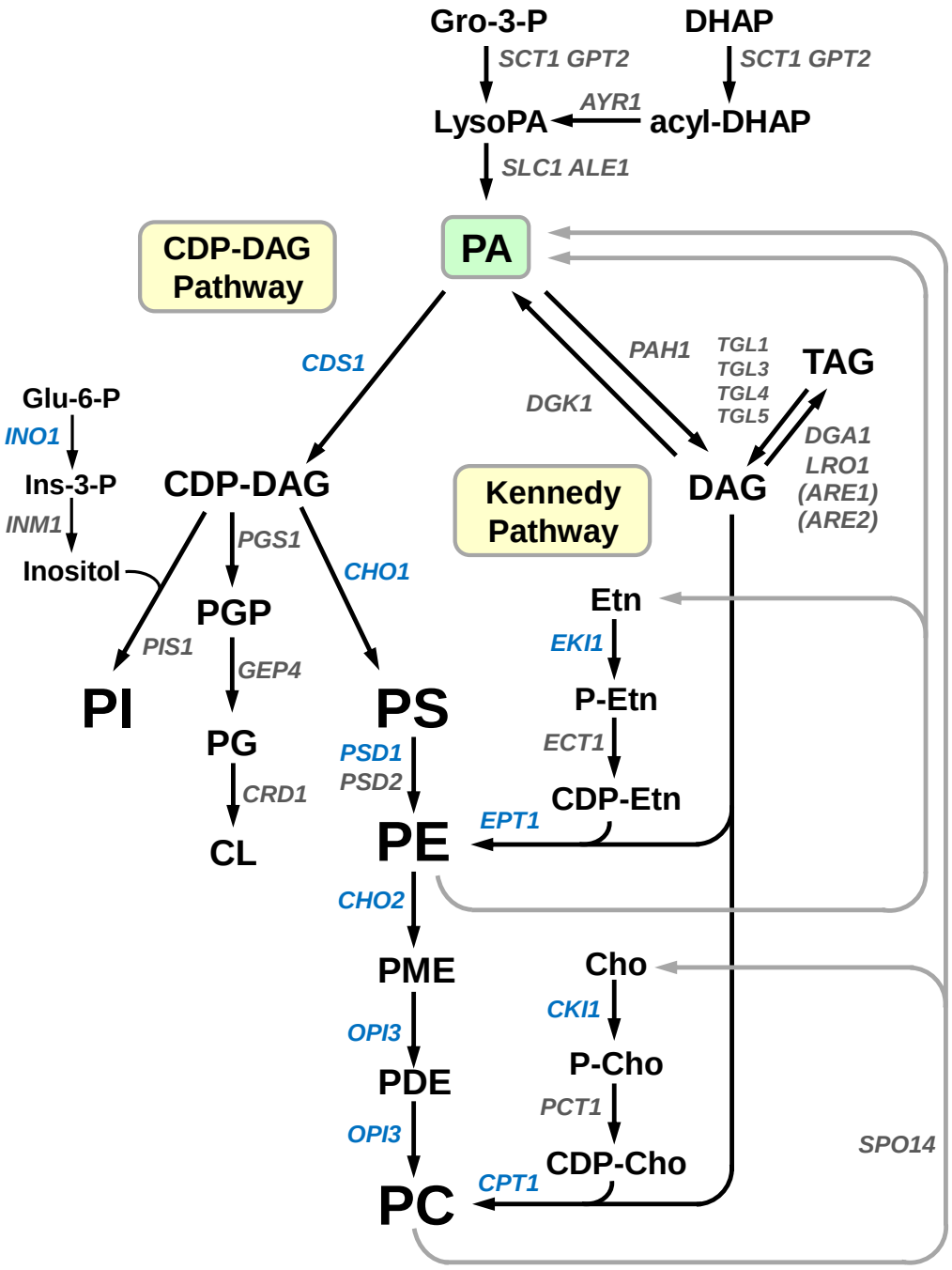
Phosphatidylinositol
(PI)

essentially the same set of phospholipids; however, the relative percentage of individual lipid classes may vary between different organelle membranes or within the same membranes as a result of changes in growth conditions (6, 14, 15). Thus, the average charge of membrane phospholipids must be maintained relatively constant through biochemical and genetic regulation of phospholipid biosynthesis (14).

Phospholipid synthesis is a complex process involving numerous branch points and interconnected pathways by which the precursor PA is partitioned between CDP-DAG and DAG (Fig. 2) (3, 6, 7, 10, 12). In yeast, PC is the end product of phospholipid synthesis and the major membrane phospholipid (4, 7, 10, 15). In addition, it can also serve as reservoir for important secondary lipid messengers (16). PC can be synthesized via two complementary pathways: the primary CDP-DAG pathway, and the auxiliary Kennedy pathway, which becomes essential when enzymes in the CDP-DAG pathway are defective (6, 7, 10, 16).

In the CDP-DAG pathway, the *CDS1*-encoded CDP-DAG synthase catalyzes the formation of the energy-rich intermediate CDP-DAG from PA and CTP (17, 18) (Fig. 2). The CMP in CDP-DAG is then displaced by serine in the reaction catalyzed by the *CHO1/PSS1*-encoded PS synthase, resulting in the synthesis of PS (19-22). The formation of PS by this reaction is the only step that differs from phospholipid synthesis in mammals, where PS is synthesized by an exchange reaction between PE or PC with serine (23). PS is next acted upon by the *PSD1*- and *PSD2*-encoded PS decarboxylases to form PE (19, 24-27). PC is finally synthesized through three sequential methylations of PE, catalyzed by the *CHO2/PEM1*-encoded PE methyltransferase and the *OPI3/PEM2*-encoded phospholipid methyltransferase (28-31).

Figure 2. Phospholipid and TAG biosynthesis in *Saccharomyces cerevisiae*. The CDP-DAG, CDP-choline, and CDP-ethanolamine pathways are shown for the synthesis of phospholipids, and include the relevant steps discussed throughout. The genes that are known to encode enzymes catalyzing individual steps in the lipid synthesis pathway are indicated. *CDP-Cho*, CDP-choline; *CDP-DAG*, CDP-diacylglycerol; *CDP-Etn*, CDP-ethanolamine; *CL*, cardiolipin; *DAG*, diacylglycerol; *DHAP*, dihydroxyacetone phosphate; *Glu-6-P*, glucose-6-phosphate; *Ins*, inositol; *Ins-3-P*, inositol-3-phosphate; *Gro-3-P*, glycerol-3-phosphate; *PA*, phosphatidate; *PC*, phosphatidylcholine; *P-Cho*, phosphocholine; *PE*, phosphatidylethanolamine; *P-Etn*, phosphoethanolamine; *PG*, phosphatidylglycerol; *PGP*, phosphatidylglycerol phosphate; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *TAG*, triacylglycerol.


Gro-3-P
DHAP
SCT1 GPT2
SCT1 GPT2
AYR1
LysoPA
acyl-DHAP
SLC1 ALE1
CDP-DAG Pathway
PA
CDS1
PAH1
TGL1
TGL3
TGL4
TGL5
TAG
DGK1
DGA1
LRO1
(ARE1)
(ARE2)
Glu-6-P
INO1
Ins-3-P
INM1
Inositol
CDP-DAG
Kennedy Pathway
DAG
PGS1
CHO1
PIS1
PGP
GEP4
PI
PG
CRD1
CL
PS
PSD1
PSD2
PE
CHO2
PME
OPI3
PDE
OPI3
PC
Etn
EKI1
P-Etn
ECT1
CDP-Etn
EPT1
Cho
CKI1
P-Cho
PCT1
CDP-Cho
CPT1
SPO14

In addition to the CDP-DAG pathway, PE and PC are synthesized preferentially via the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway when cells are supplemented with exogenous choline and ethanolamine, respectively (6, 10) (Fig. 2). Moreover, choline and/or ethanolamine can be generated through the phospholipase D-mediated turnover of PE or PC, and thus the Kennedy pathway can contribute to the synthesis of these phospholipids even in the absence of choline or ethanolamine supplementation (32-37). This pathway becomes essential for the synthesis of PC in mutants defective in enzymes involved in the CDP-DAG pathway (7, 10, 32, 38, 39). Since the Kennedy pathway is secondary, mutants defective in this pathway have no auxotrophic requirements, unlike those defective in the CDP-DAG pathway (30, 31, 38, 40). In the Kennedy pathway, choline and ethanolamine are phosphorylated by the *CKI1*-encoded choline kinase (41) and the *EKI1*-encoded ethanolamine kinase (42), resulting in the formation of phosphocholine and phosphoethanolamine, respectively (Fig. 2). These intermediates are activated by the *PCT1*/*CCT1*- and *ECT1*-encoded phosphocholine and phosphoethanolamine cytidylyltransferases, respectively, which utilize CTP to form CDP-choline and CDP-ethanolamine (14, 43, 44). CDP-choline and CDP-ethanolamine then react with the DAG derived from the dephosphorylation of PA by the *PAH1*-encoded PAP (14, 34, 45, 46) to form PC and PE by reactions catalyzed by the *CPT1*- and *EPT1*-encoded choline and ethanolamine phosphotransferases (47-50), respectively. The PE generated by this pathway undergoes subsequent methylation to form PC, as described above (28-30).

A second branch of the CDP-DAG pathway is involved in the synthesis of PI via the displacement of CMP from CDP-DAG with inositol, in a reaction catalyzed by the

PIS1-encoded PI synthase (51-54) (Fig. 2). The inositol utilized in this reaction is derived from glucose-6-phosphate, which is converted to inositol-3-phosphate by the *INO1*-encoded inositol-3-phosphate synthase in the committed step of inositol synthesis (4, 55-57). Inositol-3-phosphate is then acted upon by the *INM1*-encoded inositol-3-phosphate phosphatase to form inositol (4, 58).

CDP-DAG is also utilized for the synthesis of PG and CL, phospholipids which are confined to inner mitochondrial membranes (7, 59) (Fig. 2). The committed step in the synthesis of CL is catalyzed by the *PGS1*-encoded PG phosphate (PGP) synthase, which synthesizes PGP by displacement of CMP from CDP-DAG with glycerol-3-phosphate (60, 61). PGP is then dephosphorylated by the *GEP4*-encoded PGP phosphatase to synthesize PG (61, 62), which is next used to synthesize CL by the *CRD1*-encoded CL synthase (13).

TAG is the major storage form of cellular energy

The TAG molecule constitutes the most calorie-dense form of metabolic energy, allowing organisms to withstand prolonged periods of nutrient deprivation (63). Moreover, stores of TAG may provide a source of fatty acids and DAG for membrane biosynthesis during cellular growth. This dual function of TAG as a reservoir for energy substrates and membrane lipid precursors makes it a central player in lipid homeostasis (64). The regulation of TAG synthesis and storage is crucial in human health because both an excess and a defect in fat storage results in lipid-associated disorders such as obesity, lipodystrophy, insulin resistance, diabetes, hypertension, cardiovascular disease, and cancer (63).

TAG is comprised of a glycerol backbone and three fatty acyl chains esterified to positions *sn*-1, -2 and -3 (1). Due to its hydrophobicity, TAG is stored in special subcellular organelles called lipid droplets, which consist of a lipophilic core enveloped by a phospholipid monolayer (65). Synthesis of TAG is important for the creation of a highly concentrated form of metabolic energy that can be released through the hydrolysis and subsequent oxidation of its constituent fatty acids (34). As described in the previous section, the breakdown products of TAG are also required during cell growth as precursors for the synthesis of phospholipids, a process which is especially important in yeast cells upon growth resumption from stationary phase (34, 66, 67). In addition, the synthesis of TAG also serves to channel potentially toxic excess free fatty acids into neutral lipids, thereby protecting cells from fatty acid-induced lipotoxicity (68-70).

As in phospholipid synthesis, PA serves as the major precursor in *de novo* TAG synthesis (Fig. 2). This pathway consists of several steps, beginning with the acylation of glycerol-3-phosphate or dihydroxyacetone phosphate (DHAP), intermediates in the glycolytic pathway, catalyzed by the *SCT1*- and *GPT2*-encoded acyltransferases to form lysoPA and acyl-DHAP, respectively (71-73). In an additional step, acyl-DHAP is converted to lysoPA by the *AYR1*-encoded NADPH-dependent acyl-DHAP reductase (74). LysoPA is then in turn acylated at position 2 by the *SLC1*- and *ALE1*-encoded lysophospholipid acyltransferases, generating PA (71-73, 75-77). The dephosphorylation of PA by the *PAH1*-encoded PAP gives rise to DAG (78, 79), which is then acylated to TAG by the *DGA1*- and *LRO1*-encoded acyltransferases (64, 80-82). The *ARE1*- and *ARE2*-encoded acyltransferases, which are primarily involved in the synthesis of ergosterol esters, can also catalyze the acylation of DAG (64). Under nutrient-limiting

conditions or when growth resumption is required, TAG can be hydrolyzed to DAG and free fatty acids by the *TGL1*-, *TGL3*-, *TGL4*-, and *TGL5*-encoded TAG lipases (64, 83-85), and DAG converted to PA by the *DGK1*-encoded DAG kinase (86, 87).

PAH1-encoded PAP is the link between phospholipid and TAG synthesis

The *PAH1*-encoded PAP is uniquely positioned at a branch point in lipid synthesis, as it catalyzes the dephosphorylation of PA to yield DAG and P_i in the penultimate step in TAG synthesis (88) (Fig. 2). As described in previous sections, in *de novo* lipid synthesis in *S. cerevisiae*, the DAG generated in the reaction is used for the synthesis of TAG as well as the phospholipids PC and PE via the Kennedy pathway (89-92), while the reaction substrate PA serves as a precursor for all major phospholipids via the CDP-DAG pathway (89-91). In mammalian cells, however, the phospholipids PS, PE and PC are derived from DAG (23). In addition, both the substrate and the product of this reaction have lipid signaling functions. PA is implicated in transcription, activation of cell growth, membrane proliferation, secretion, and vesicular trafficking, while DAG is primarily involved in the activation of protein kinase C (PKC) in higher eukaryotes (93-102). By the nature of the reaction, PAP activity controls the cellular concentrations of these two important lipid mediators, playing a role in lipid signaling. Thus, the regulation of PAP activity may govern whether cells make storage lipids or membrane phospholipids, determine the pathways by which these lipids are synthesized, and control the cellular levels of important signaling lipids. Genetic and biochemical studies in yeast and mammalian cells have revealed PAP as a major regulator of lipid metabolism and cell physiology (63, 70, 78, 79, 92, 103-112).

PAP activities and their involvement in lipid metabolism and signaling

The PAP reaction was first described in animal tissues by Smith et al. in 1957 (88), providing a link between the neutral and phospholipid synthesis pathways in mammalian cells (91, 113). Subsequent studies demonstrated that this enzymatic reaction requires Mg^{2+} ions, and that the majority of activity resides in the soluble fraction of cell lysates (114), in contrast to other enzymes in the TAG and phospholipid synthesis pathways, which are integral membrane proteins (115-119). Furthermore, PAP activity was found to translocate from the cytosol to the membrane fraction of cells treated with fatty acids (89, 120, 121). Due to the instability of the mammalian enzyme, the isolation of PAP remained elusive for more than three decades.

In 1984, Hosaka and Yamashita identified PAP activity in the cytosolic and membrane fractions of the yeast *S. cerevisiae* (114, 122). The use of this model organism allowed for the purification of a 91-kDa PAP enzyme from the total membrane fraction in 1989 (123). Additionally, 104-kDa, 75-kDa, and 45-kDa forms of PAP were isolated from microsomes, cytosol, and mitochondria, respectively (114, 124). The 91-kDa enzyme was later shown to be a degradation product of the 104-kDa form (124), while precursor-product relationships do not exist between the 75-kDa, 45-kDa, and 104-kDa proteins (124). Characterization studies indicated that all forms of the PAP enzyme require Mg^{2+} ions for catalysis, and they are highly specific for PA as a substrate (114, 123, 124). Unfortunately, none of the schemes used to purify these enzymes resulted in sufficient amounts of protein for sequencing by classical Edman degradation analysis, and so the gene(s) encoding them could not be identified.

In subsequent studies, Wu and coworkers isolated a 34-kDa protein from yeast

microsomes that exhibited PAP activity (125). Characterization of the enzyme revealed that it catalyzes the removal of the β-phosphate from DAG pyrophosphate (DGPP) to form PA, and subsequently dephosphorylates PA to produce DAG; thus, the enzyme was termed DGPP phosphatase (125). In contrast to the enzymes discussed above, this phosphatase enzyme does not require Mg^{2+} ions for catalysis (125). Sufficient enzyme was purified to obtain amino acid sequence information, which was matched to a gene in the *S. cerevisiae* database and designated *DPP1* (126). Dpp1p[1] is 289 amino acids in length and has a molecular mass of 33.5 kDa (126), which is in close agreement with the size of the purified DGPP phosphatase (125). The identification of a conserved phosphatase sequence motif contained within several lipid phosphatases (127) revealed DGPP phosphatase shares sequence homology with a mammalian Mg^{2+}-independent PAP (designated PAP2) believed to be involved in lipid signaling (128-131). Shortly after this discovery, the *LPP1* gene was identified based on protein sequence homology with Dpp1p (132). Lpp1p is 274 amino acids in length and has a predicted molecular mass of 31.6 kDa (132). Studies with the *dpp1*Δ *lpp1* Δ double mutant show that the *DPP1* and *LPP1* genes encode essentially all Mg^{2+}-independent PAP activity in yeast, with *DPP1* being the major contributor (126, 132, 133). Detailed characterization studies showed that the PAP activity of these enzymes is distinct from the conventional Mg^{2+}-dependent PAP enzymes, whose gene(s) had yet to be identified. In particular, the broad substrate specificity (discussed in later sections) of the Mg^{2+}-independent Dpp1p and Lpp1p enzymes has resulted in their designation as lipid phosphate phosphatase (LPP) enzymes (134).

In a fortunate twist of scientific fate, a preparation of the 91-kDa enzyme was

recovered from frozen storage, analyzed for enzymatic activity, and sequenced by mass spectrometry (78), a much more sensitive method than Edman degradation. The deduced protein sequence matched that of the deduced product of the *SMP2* gene, which had been implicated in plasmid maintenance and respiration (135). The molecular function of Smp2p, however, had yet to be established. Overexpression of *SMP2* was reported to complement the aberrant nuclear membrane expansion phenotype of *nem1*Δ and *spo7*Δ mutants lacking the ER-associated Nem1p-Spo7p phosphatase complex (104, 136). Upon identification of the molecular function as a PAP enzyme, the *SMP2* gene was renamed *PAH1* (for *p*hosphatidic *a*cid phospho*h*ydrolase) (78). Pah1p is 862 amino acids in length and has a predicted molecular mass of 95-kDa; however, when expressed in *S. cerevisiae* it migrates as a 124-kDa protein upon SDS-PAGE (78). While phosphorylation of Pah1p results in a shift in electrophoretic mobility to a position of a slightly higher molecular mass (104, 105, 107, 108), Pah1p expressed in *E. coli* migrates as a 114-kDa protein product upon SDS-PAGE analysis (78). Therefore, the discrepancy in the predicted vs. observed size of Pah1p cannot be attributed solely to modification by phosphorylation.

The identification and characterization of yeast Pah1p revealed its homology to the mammalian proteins known as lipins, encoded by the murine *Lpin1*, *2*, and *3* genes (78, 137). Pah1p and mouse lipin-1 share structural similarity in two conserved regions: the NLIP and CLIP domains, found at the N-terminus and C-terminus, respectively (137). *Lpin1* was identified as the gene whose mutation is responsible for the transient fatty liver dystrophy (*fld*) phenotype of mice (137, 138). A loss of lipin-1 was shown to prevent normal adipose tissue development, resulting in lipodystrophy and insulin resistance,

while an excess of lipin-1 promotes obesity and insulin sensitivity (137, 139). However, the molecular function of this protein was not known at the time. In view of the observation that Pah1p PAP activity is dependent on a haloacid dehalogenase (HAD)-like domain possessing the D*X*D*X*(T/V) catalytic motif (78, 79), which is contained in the CLIP domain, lipin-1 was characterized as a PAP enzyme (78, 140). Further enzymological studies confirmed that all isoforms of lipin-1 (α, β, and γ splice variants), as well as lipin-2 and -3, also exhibit PAP activity (140, 141). *LPIN1* mutations in humans are associated with metabolic syndrome, type 2 diabetes, and recurrent acute myoglobinuria in children, while mutations in *LPIN2* result in anemia and inflammatory disorders associated with Majeed syndrome (63, 111, 142). Little is known about the consequences of *LPIN3* mutations. Mammalian lipin-1 and -2 were shown to complement phenotypes exhibited by yeast *pah1*Δ mutant cells (143), indicating the functions of PAP enzymes are evolutionarily conserved. Indeed, the discovery of yeast Pah1p led to the identification of genes encoding PAP enzymes in humans (78, 141), mice (137, 140), flies (144, 145), worms (146), and plants (147, 148), suggesting PA phosphatases fulfill a fundamental cellular function. All PAP enzymes have the HAD-like domain that contains a D*X*D*X*(T/V) catalytic motif and the NLIP domain of unknown function (78, 79, 137, 149). The reader is directed to recent reviews that summarize our current understanding of mammalian lipins (103, 112, 113, 150, 151).

Despite these exciting discoveries, analysis of the *lpp1*Δ *dpp1*Δ *pah1*Δ triple mutant revealed it still contains Mg^{2+}-dependent PAP activity (78), indicating the presence of additional unidentified enzymes. The residual PAP enzyme was shown to associate with membranes as a peripheral membrane protein, and to be susceptible to

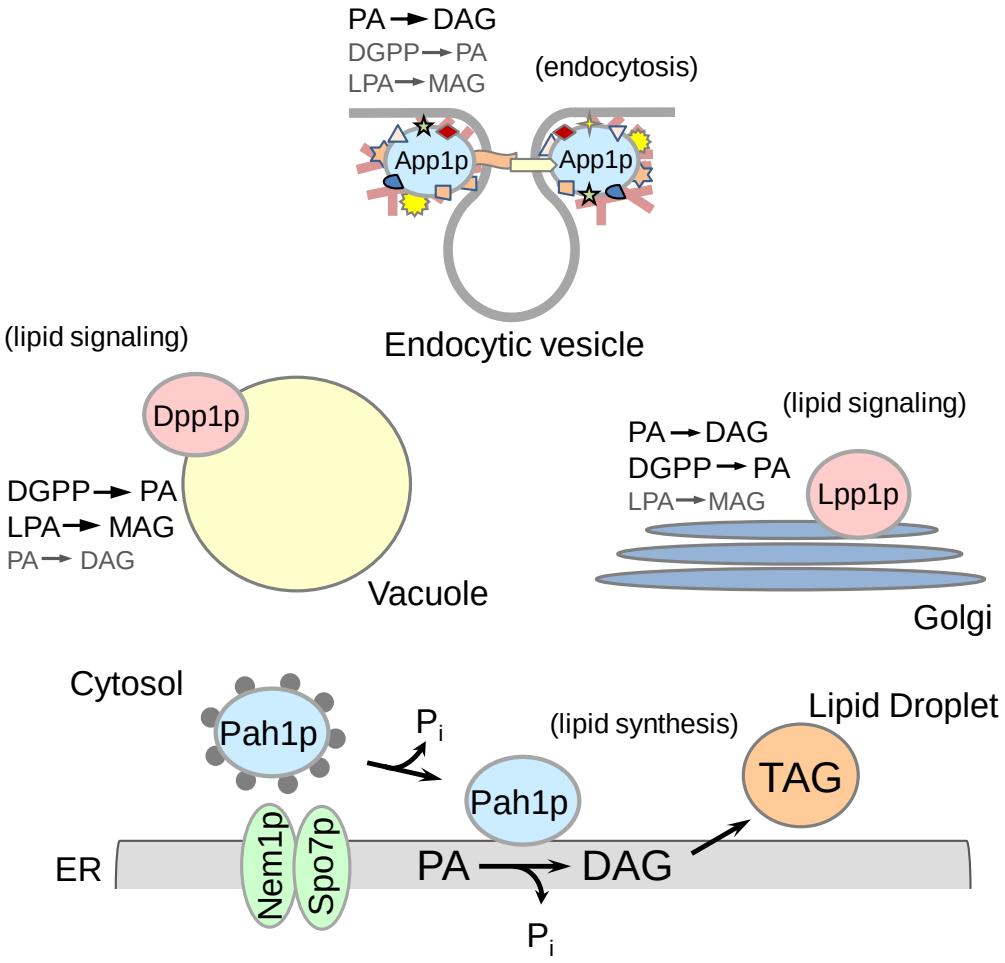
inhibition by the thioreactive agent *N*-ethylmaleimide (NEM) (78). Efforts to isolate this activity have recently led to the discovery of a second Mg^{2+}-dependent PA phosphatase in yeast encoded by the *APP1* gene (152). Its predicted protein product is 587 amino acids in length, and has a minimum subunit molecular mass of 66.1 kDa (152). The *dpp1*Δ *lpp1*Δ *pah1*Δ *app1*Δ quadruple mutant has no remaining PAP activity, confirming that all genes encoding PAP activity in yeast are now known.

Biochemical, enzymological and structural properties of PAP enzymes

Since the initial characterization of the PAP reaction in 1957 (88), both Mg^{2+}-dependent and -independent enzymes have been identified in yeast (78, 126, 132, 152). Besides the difference in their cofactor requirement, these enzymes are distinguished by several other properties (Figs. 3 and 4).

Dpp1p and Lpp1p are relatively small integral membrane proteins confined to the vacuole and Golgi membranes, respectively (Fig. 3). Both proteins possess six transmembrane domains distributed over their polypeptide sequences (126, 132). In contrast, Pah1p is a much larger protein that contains no transmembrane domains in its sequence; it is primarily found in the cytosol, but must translocate to the ER membrane to access its substrate PA for catalysis (78) (Fig. 3). In addition, Pah1p has also been found to localize to the nucleus where studies have indicated it interacts with the promoter of phospholipid synthesis genes (103, 104). App1p is also a cytosolic protein which associates with endocytic proteins at cortical actin patches (153) (Fig. 3). Thus, the spatial differences in the subcellular localization of the PAP and LPP enzymes point to distinct cellular functions.

Figure 3. Cellular locations and functions of PAP and LPP activities in yeast. The cartoon shows the integral membrane enzymes Dpp1p and Lpp1p in the vacuole and Golgi, respectively. App1p is a cytosolic protein, which can localize to actin patches in endocytic vesicles. Pah1p in the cytosol is phosphorylated on multiple sites (symbols decorating enzyme). At the ER membrane, Pah1p is dephosphorylated by the Nem1p–Spo7p phosphatase complex, which allows for its interaction with the membrane where its substrate PA resides. The dephosphorylated form of Pah1p catalyzes the dephosphorylation of PA to generate DAG for the synthesis of TAG that is stored in lipid droplets.


PA → DAG
DGPP → PA
LPA → MAG
(endocytosis)
App1p
App1p
Endocytic vesicle
(lipid signaling)
Dpp1p
DGPP → PA
LPA → MAG
PA → DAG
Vacuole
(lipid signaling)
PA → DAG
DGPP → PA
LPA → MAG
Lpp1p
Golgi
Cytosol
Pah1p
Pi
(lipid synthesis)
Lipid Droplet
TAG
Pah1p
ER
Nem1p
Spo7p
PA → DAG
Pi

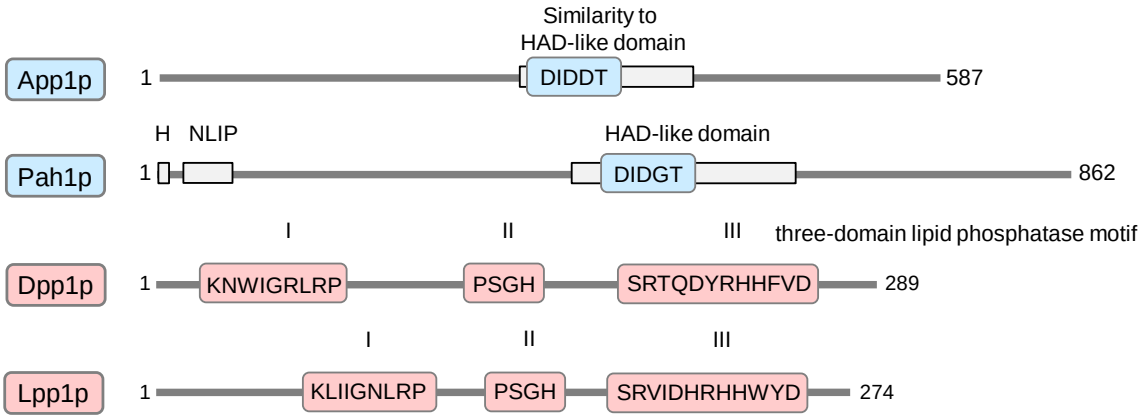
While mammalian Mg^{2+}-dependent PAP and Mg^{2+}-independent LPP enzymes are also differentiated based on their sensitivity to NEM (89, 128), this distinction is not applicable to their yeast counterparts. In fact, Dpp1p activity is insensitive to NEM (125), whereas the activity of Lpp1p is potently inhibited by this compound (154). Moreover, NEM has no effect on the PAP activity of Pah1p (78), while it is inhibitory to that of App1p (153). Similarly, the synthetic compound propranolol inhibits Pah1p (124), App1p (153), and Lpp1p (154), even though it is thought to hinder activity by interacting with the Mg^{2+} binding site of enzymes (155). Therefore, inhibition by NEM or propranolol should not be used to distinguish yeast PAP and LPP enzymes.

Dpp1p and Lpp1p do not require divalent cations for activity (125, 132, 154), while maximum activities of Pah1p and App1p are dependent on Mg^{2+} ions (78, 153). This distinction in cofactor requirement can be explained by the differences in the catalytic motifs that govern the activity of each class of enzymes (Fig. 4). The PAP activity of Pah1p is governed by a D*X*D*X*(T/V) motif within an HAD-like domain found in members of a superfamily of Mg^{2+}-dependent phosphatase enzymes (156, 157) that include mammalian lipin PAP enzymes (63, 78). Similarly, App1p contains a D*X*D*X*(T/V) catalytic motif within a region of weak sequence similarity to an HAD-like domain (158). Consistent with other Mg^{2+}-independent LPP enzymes (127, 159, 160), Dpp1p and Lpp1p contain a three-domain lipid phosphatase motif comprised of the consensus sequences KX_6RP (I), PSGH (II) and SRX_5HX_3D (III) (127), which confers these proteins their enzymatic activity (161).

Finally, the PAP and LPP enzymes in yeast differ with respect to their substrate specificities (Fig. 3). Dpp1p and Lpp1p utilize a variety of lipid phosphate substrates,

Figure 4. Distinguishing characteristics of PAP and LPP enzymes. The basic characteristics of the yeast PAP (App1p and Pah1p) and LPP (Dpp1p and Lpp1p) enzymes, including their catalytic motifs are summarized in the figure. The diagrams of each protein are not drawn to scale. *H*, amphipathic helix; *HAD*, haloacid dehalogenase.

Mg^{2+}-dependent	Mg^{2+}-independent
App1p (66 kDa) – Cytosolic (associates with actin patches)	Dpp1p (34 kDa) - Vacuole (integral membrane protein)
Pah1p (95 kDa) - Cytosolic (translocates to ER membrane)	Lpp1p (32 kDa) – Golgi (integral membrane protein)



including PA, DGPP, lysoPA, sphingoid base phosphates, and isoprenoid phosphates (125, 126, 132, 133, 154, 162); however, only DGPP and PA have been shown to be substrates *in vivo* (132). In addition, the enzymological properties of Lpp1p differ significantly from those of Dpp1p. While Dpp1p can utilize PA in the absence of DGPP, it has a 10-fold higher specificity for DGPP (125). Conversely, PA is the preferred substrate for Lpp1p, followed by DGPP and lysoPA (132, 154). Moreover, the affinity of Lpp1p for PA, DGPP, and lysoPA as substrates is greater than the affinity of Dpp1p for these substrates (154). App1p utilizes DGPP and lysoPA as substrates, albeit with specificity constants 4- and 7-fold lower, respectively, when compared with PA (153). In contrast, Pah1p is specific for PA (114, 123-125).

Both the substrates and products of the reactions catalyzed by the LPP enzymes are important signaling molecules, suggesting that these enzymes are involved in lipid signaling, and are not responsible for the *de novo* synthesis of phospholipids and TAG that occurs in the ER (91, 126, 132). Moreover, App1p is thought to regulate local PA and DAG levels at cortical actin patches, facilitating membrane fission/fusion events and regulating enzymes that govern vesicular trafficking, thus playing a role in endocytosis (152). Additionally, the *app1p*Δ, *dpp1*Δ, and *lpp1*Δ mutations, singly or in combination, do not affect lipid synthesis, whereas the *pah1*Δ mutation affects both the synthesis of TAG as well as that of phospholipids (70, 78, 152). Thus, the synthesis of the DAG required for phospholipid and TAG synthesis in *S. cerevisiae* is attributed solely to the Pah1p PAP enzyme (70, 78, 152, 163).

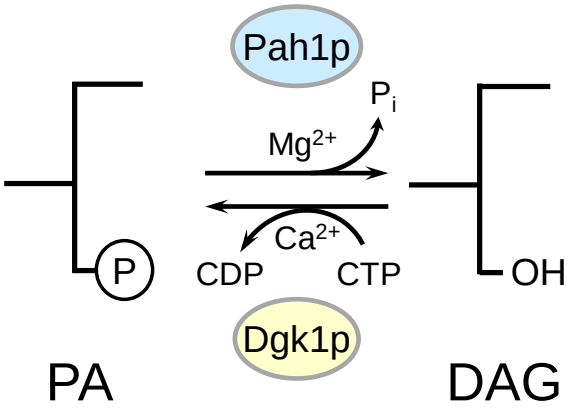
Pah1p PAP is a key player in *de novo* lipid synthesis

The essential role of PAP in *de novo* lipid metabolism has been established through studies using the yeast *pah1*Δ mutant (79, 104, 105, 133). Consistent with a lack of enzyme activity, this mutant shows elevated levels of PA and decreased levels of DAG and TAG (70, 78, 79). Additionally, the amounts of phospholipids, fatty acids, and sterol esters are also elevated in response to the *pah1*Δ mutation (70, 78, 79), indicating that PAP regulates overall lipid synthesis. The effects on TAG (> 90 % decrease) are most pronounced in the stationary phase of growth (78, 79), where the synthesis of TAG is predominant over the synthesis of membrane phospholipids (164).

In addition to the alteration in lipid metabolism, phenotypes of the *pah1*Δ mutant include slow growth (104), aberrant expansion of the nuclear/ER membrane (104), respiratory deficiency (78), defects in lipid droplet formation (109) and morphology (165), vacuole homeostasis and fusion (110), fatty-acid induced lipotoxicity (70), and a growth sensitivity to elevated temperature (78, 104) (Fig. 5). Catalytically inactive mutant forms of PAP, with mutations in either a conserved glycine (e.g., G80R) at the N-terminus or DIDGT catalytic motif residues (D398E or D400E) exhibit the same phenotypes associated with the *pah1*Δ mutation, indicating these effects are specifically linked to the loss of PAP activity (70, 79, 109, 110).

A contributing factor for the increased amounts of phospholipids and fatty acids in the *pah1*Δ mutant is the derepression of lipid synthesis genes in response to elevated PA levels (78, 79, 104, 166-170) (Fig. 6). The promoter region of the genes thus affected contains an inositol-responsive upstream activating sequence, or UAS_{INO} element, which serves as binding site for the Ino2p-Ino4p complex that stimulates expression of lipid synthesis genes (166). The PA-mediated regulation of these genes is controlled by the

Figure 5. Summary of *pah1*Δ mutant phenotypes. The reactions catalyzed by Pah1p PAP and Dgk1p DAG kinase control the balance of PA and DAG. The *pah1*Δ phenotypes suppressed and not suppressed by the *dgk1*Δ mutation are listed in the figure.



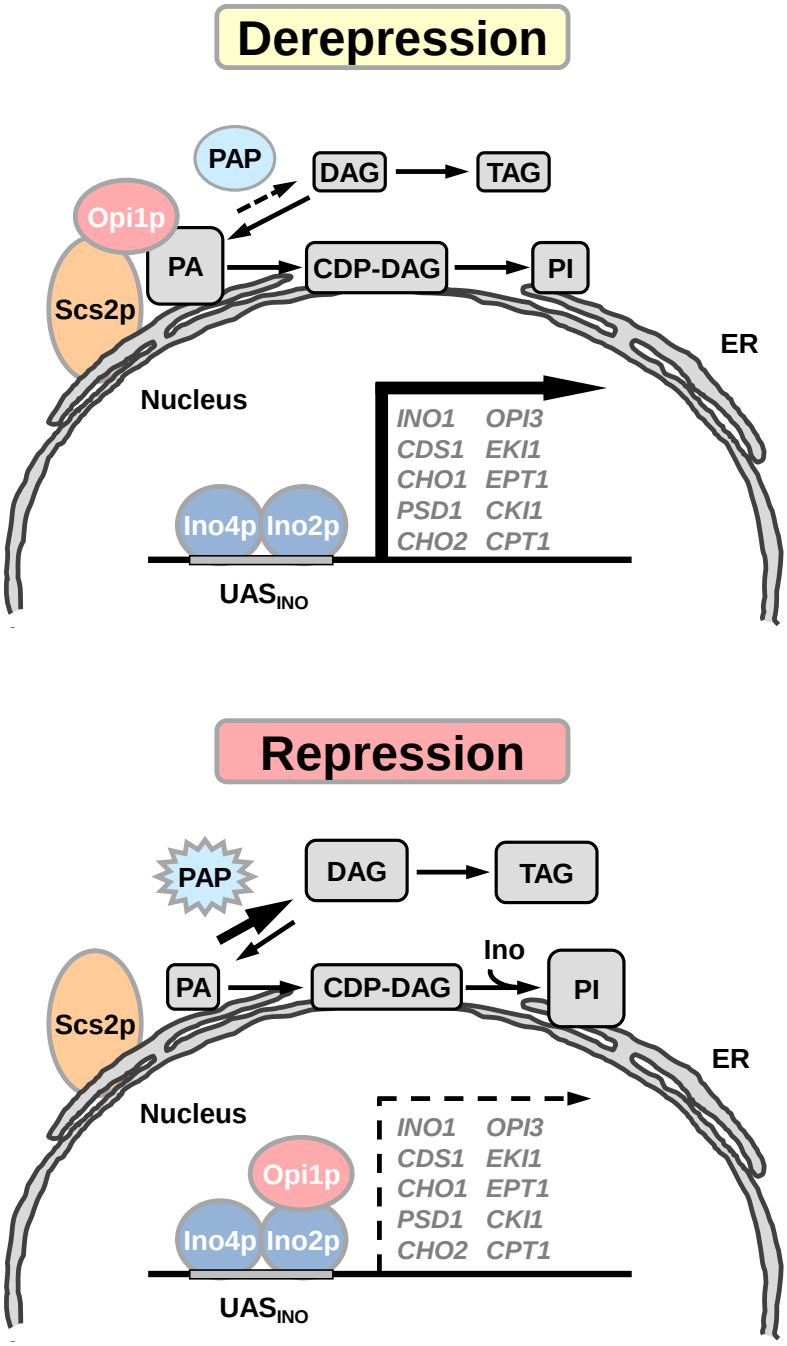
Phenotypes of the *pah1*Δ mutant

- ↑ Nuclear/ER membrane expansion
- ↑ Phospholipid synthesis gene expression
- ↑ Phospholipid content
- ↓ Lipid droplet formation
- ↑ Fatty acid content
- ↑ Fatty acid toxicity
- ↑ Temperature sensitivity
- ↓ TAG synthesis

Suppressed by *dgk1*Δ

Not suppressed by *dgk1*Δ

Figure 6. PA-mediated regulation of phospholipid biosynthetic genes. The model describes the mechanism by which PAP activity affects Opi1p-mediated regulation of UAS_{INO}-containing phospholipid biosynthetic genes. Under conditions inhibitory to PAP activity, Opi1p remains tethered to the ER membrane through interactions with PA and Scs2p, effectively derepressing expression of the UAS_{INO}-containing genes. In contrast, induction of PAP activity results in a reduction in PA concentrations and the subsequent release of Opi1p from the ER membrane, rendering it free to enter the nucleus and bind Ino2p, repressing expression of phospholipid biosynthetic genes. *CDP-DAG*, CDP-diacylglycerol; *DAG*, diacylglycerol; *Ino*; inositol; *PA*, phosphatidate; *PI*, phosphatidylinositol; *TAG*, triacylglycerol.


Derepression
PAP
DAG
TAG
Opi1p
PA
CDP-DAG
PI
Scs2p
ER
Nucleus
INO1 OPI3
CDS1 EKI1
CHO1 EPT1
PSD1 CKI1
CHO2 CPT1
Ino4p Ino2p
UAS INO
Repression
PAP
DAG
TAG
Ino
PA
CDP-DAG
PI
Scs2p
ER
Nucleus
INO1 OPI3
CDS1 EKI1
CHO1 EPT1
PSD1 CKI1
CHO2 CPT1
Opi1p
Ino4p Ino2p
UAS INO

transcriptional repressor Opi1p (171), whose function is determined by its localization. In its inactive state, Opi1p is tethered to the nuclear/ER membrane through interactions with Scs2p and PA (172, 173). Upon induction of PAP activity, the resulting reduction in PA levels destabilizes this interaction, allowing for the translocation of Opi1p into the nucleus where it suppresses transcription of UAS_{INO}-containing genes by binding to the Ino2p subunit of the Ino2p-Ino4p activator complex (173, 174). Additionally, the decreased capacity of *pah1*Δ mutant cells to incorporate fatty acids into TAG may contribute to the observed alterations in phospholipids, fatty acids, and sterol esters. This misregulation of lipid metabolism may also affect the susceptibility of the *pah1*Δ mutant to fatty acid-induced toxicity (70).

The yeast *DGK1*-encoded DAG kinase (Dgk1p) has recently been identified as an enzyme whose function counterbalances that of the Pah1p PAP (87) (Fig. 5). This nuclear/ER integral membrane enzyme is unique in that, in contrast to other DAG kinases in bacteria, plants, and animals (97, 175-178), it utilizes CTP rather than ATP as the phosphate donor in the phosphorylation reaction (87). Cells bearing a *dgk1*Δ mutation do not exhibit any remarkable phenotypes under standard growth conditions (87). However, like the *pah1*Δ mutation, overexpression of Dgk1p causes the accumulation of PA at the nuclear/ER membrane and the derepression of UAS_{INO}-containing genes (87), providing evidence that Dgk1p activity antagonizes that of the Pah1p enzyme by regulating the cellular levels of PA (Fig. 5).

The aberrant membrane expansion phenotype of the *pah1*Δ mutant has been attributed to the abnormal increase in phospholipid synthesis associated with this mutation (104); however, data indicate that increased expression of phospholipid

biosynthetic genes alone is not sufficient for nuclear/ER membrane expansion (87, 105). In addition, *DGK1* overexpression results in anomalous nuclear/ER membrane morphology (87), while the introduction of the *dgk1*Δ mutation to the *pah1*Δ background restores PA levels, the suppression of UAS_{INO}-containing genes, and a normal nuclear/ER membrane structure (87). Thus, increased phospholipid synthesis coupled to increased PA levels result in the aberrant nuclear/ER morphology displayed by the *pah1*Δ mutant. Among the phenotypes associated with the *pah1*Δ mutant, the defect in lipid droplet formation that results from loss of Pah1p function can also be complemented by the *dgk1*Δ mutation (70, 109), indicating that elevated PA levels might be the basis for this phenotype. In support of this hypothesis, a recent study has implicated PA as an important regulator of lipid droplet morphology (165). The defect in lipid droplet formation and structure associated with the *pah1*Δ mutation has been attributed to the decreased DAG levels caused by loss of PAP activity (109). Thus, this phenotype might result from a combination of both an elevated PA content and reduced DAG levels.

In contrast with the *pah1*Δ mutant phenotypes described above, the defect in TAG synthesis (87), increase in fatty acid content (87), fatty acid-induced toxicity (70), and temperature sensitivity[2] cannot be suppressed by loss of Dgk1p function. These observations seem to indicate that these phenotypes are related to depletion of DAG rather than increase in PA content. Another phenotype exhibited by the *pah1*Δ mutant is the defect in vacuole homeostasis and fusion (110). While this phenotype is attributed to the loss of Pah1p PAP activity (110), it is unknown whether it is based on alterations in PA and/or DAG. The analysis of vacuole morphology in the *pah1*Δ *dgk1*Δ double mutant might shed light on the mechanism underlying this phenotype.

[2] Unpublished observations from the laboratories of S. Siniossoglou and G. M. Carman

Pah1p function is regulated at several levels

The involvement of PAP enzymes in lipid homeostasis and lipid-associated disorders in animals has prompted interest in understanding how their expression and activity are regulated. In addition to the *pah1*Δ mutant phenotypes discussed above, overexpression of a phosphorylation-deficient form of Pah1p (7A, discussed in later sections) in *S. cerevisiae* is also detrimental to cell growth (87, 105, 107). The cause of this phenotype appears to be consistent with a reduction in PA and accumulation of DAG to a toxic level. Thus, a balance in PA and DAG levels mediated by Pah1p PAP activity must be achieved to maintain lipid homeostasis and normal cell physiology. In view of the variety of cellular processes in which PA and DAG play a role, the regulatory mechanisms governing PAP activity are complex, and occur at many levels, affecting Pah1p transcription, posttranslational modification, subcellular localization, and biochemical properties.

Regulation of gene expression

The regulation of phospholipid biosynthetic gene expression has been extensively studied, and is known to be triggered by different conditions, including nutrient availability and growth phase (7, 179, 180). For instance, numerous studies have shown that the major metabolic signal that coordinates regulation of phospholipid biosynthetic gene expression through their UAS_{INO} elements is the presence of the soluble phospholipid precursor molecules inositol and choline (12). This response is reasonable, since the presence of inositol eliminates the need to synthesize it *in vivo*, while the availability of choline allows for the synthesis of PC through the more energetically

favorable salvage pathway (12). Inositol supplementation results in elevated PI synthesis through direct allosteric inhibition of the *CHO1*-encoded PS synthase (181), which leads to the activation of the *PIS1*-encoded PI synthase (182). This in turn draws upon PA content through the utilization of CDP-DAG for PI synthesis, and results in the translocation of Opi1 into the nucleus and consequent repression of UAS_{INO}-containing genes (166, 173). It is important to note that the extent of transcriptional regulation through the UAS_{INO} element varies; furthermore, some genes containing UAS_{INO} elements (e.g., *PIS1* and *INO4*) are not regulated in response to inositol (12).

Growth phase is also known to affect the expression of many genes in *S. cerevisiae*, including phospholipid biosynthetic genes. Thus, maximal expression of UAS_{INO}-containing genes occurs during exponential phase and in the absence of inositol and choline, and they are repressed as cells enter stationary phase, even in the absence of inositol (183). This repression leads to a decrease in the activities of the phospholipid biosynthetic enzymes in the primary route of biosynthesis from PA to PC, which results in accumulation of TAG at the expense of total phospholipid synthesis (122, 164, 183). Conversely, expression of the genes *INM1* (184), *AUR1* (inositol phosphorylceramide synthase) (185), and *DPP1* (186) is increased by inositol supplementation and in the stationary phase (7).

The *Lpin1/2*-encoded mammalian PAP enzymes are regulated at the transcriptional level in response to various conditions. For instance, several transcription factors have been shown to interact with the *Lpin1* promoter, including the glucocorticoid receptor (GR) (187-189), sterol response element binding protein-1 (SREBP-1) (190), and cAMP response element binding protein (CREBP) (191), while *Lpin2* is known to be

induced by fasting (188, 192) in a cAMP- or glucocorticoid-independent manner (188).

In contrast with phospholipid synthesis genes or mammalian orthologs of PAP, relatively little is known about the transcriptional regulation of the yeast Pah1p enzyme. Recent work by Soto-Cardalda and colleagues has shown that the expression of *PAH1*-encoded PAP activity is affected by intracellular levels of zinc (46). This essential nutrient in *S. cerevisiae* and higher eukaryotes (193, 194) serves as a cofactor for numerous enzymes, and is a structural component of many proteins (193, 195, 196). Tight control of intracellular zinc levels must therefore be exerted through the action of zinc transporters located in the plasma, vacuole, ER, and mitochondrial membranes (196-205). A deficiency in zinc causes an induction in the expression of many of these transporters, which is accompanied by changes in membrane phospholipid composition that result from the transcriptional regulation of various phospholipid synthesis genes (46, 180, 182, 194, 206-209). *PAH1* expression is induced in response to zinc depletion in a Zap1p-dependent manner through its interaction with zinc-responsive upstream activating sequences (UAS_{ZRE}) in the *PAH1* promoter (46). This induction correlates with an increase in Pah1p PAP activity and elevated TAG levels (46). In addition, microarray data has indicated that *PAH1* expression is induced upon transition from glucose-based fermentative growth to glycerol- and ethanol-based respiratory growth (210). Furthermore, despite the observed increase in Mg^{2+}-dependent PAP activity in stationary phase cells (122), reports suggest that *PAH1* is repressed during the diauxic shift (211). Additional studies are needed to determine the effect of growth phase and inositol supplementation on *PAH1* expression.

Regulation of transcription and mRNA stability

Once transcription has taken place in the nucleus, eukaryotic mRNAs must be transported to the cytoplasm, where they undergo many processes (212). Thus, eukaryotic gene expression may also be controlled by posttranscriptional modifications, which affect pre-mRNA processing, mRNA nucleo-cytoplasmic transport, mRNA translation, and mRNA degradation (212, 213).

The process of transcription can be affected by numerous factors. Of particular relevance to this work is the effect of growth phase on transcription of phospholipid synthetic genes, since it is known that overall transcription decreases dramatically as cells enter stationary phase (214). During this process, the nuclear chromatin undergoes a characteristic rearrangement of its conformation, and thus, the higher-order structure of the DNA may prevent the efficient transcription of particular genes (214).

Additionally, the level of expression of a gene is dependent upon the steady-state level of mRNA transcript that is available for translation, which in turn is determined by the combined effects of its rate of synthesis and degradation (212, 215). Thus, an important control point of gene expression is the regulation of mRNA stability (213, 215), which is affected by several factors including metabolic, developmental, and environmental signals (212, 213, 216-219). There are some examples of regulation of lipid synthesis by mRNA stability. For instance, a reduction in mRNA steady-state levels has been observed in response to inositol supplementation for transcripts of the *INO1*, *CHO1*, *CHO2*, *OPI3* and *CKI* genes and, to a much lesser extent, for transcripts of the *FAS1*, *FAS2*, and *FAS3/ACC1* genes encoding fatty acid synthases (168, 170, 220-222). Additionally, the *OLE1*-encoded Δ-9 fatty acid desaturase mRNA is destabilized by fatty

acid supplementation (19, 223, 224), and *SPO11* and *SPO13* transcripts are degraded upon shift from acetate- to glycerol-based medium (225). Moreover, *CHO1* mRNA stability is known to increase in response to a defect in mitochondrial respiration (226).

Whether Pah1p function is regulated at the transcriptional level or through mRNA stability is yet to be determined.

Biochemical regulation

1. Regulation by lipids and nucleotides

Pah1p PAP activity is stimulated by the phospholipids CDP-DAG, PI and CL, which act as mixed competitive activators of PAP activity by decreasing the K_m for PA (227). On the other hand, PAP activity is inhibited by the sphingoid bases sphingosine, phytosphingosine, and sphinganine (227) in a parabolic competitive mechanism, by which more than one inhibitor molecule contributes to the exclusion of PA from the enzyme.

The nucleotides ATP and CTP, precursors of phospholipid synthesis (7), inhibit Pah1p PAP activity by a complex mechanism which affects both the V_{max} and K_m for PA and might also involve chelation of the Mg^{2+} cofactor (228). Moreover, cellular ATP and CTP levels correlate with synthesis of phospholipids and TAG (228): high ATP levels favor increased PA content and phospholipid synthesis, while low levels result in reduced PA content and increased TAG synthesis; high CTP levels increase PA content and thus result in derepression of UAS_{INO}-containing genes (229).

2. Regulation by phosphorylation/dephosphorylation

Covalent modification by the addition of phosphate groups can result in

alterations in the overall charge and/or conformation of an enzyme, thereby dramatically affecting its substrate binding ability, catalysis, and cellular localization, and thus controlling its function (1).

The phosphorylation of phospholipid synthetic enzymes plays a major role in their regulation (7, 10-12). Past studies have shown that several enzymes involved in phospholipid synthesis (e.g., PAP, PS synthase, choline kinase, and CTP synthetases) are regulated by phosphorylation (105, 230). The major kinases responsible for the phosphorylation of enzymes in the phospholipid synthetic pathways include PKC, protein kinase A (PKA), and cyclin-dependent kinases (Cdc28p and Pho85p) (104, 105, 108, 230-233). In *S. cerevisiae*, Cdc28p is essential and sufficient for cell cycle progression, while the non-essential Pho85p supports many additional functions (231). Cross-talk between these two kinases allows for regulation of cell morphology, gene expression, macromolecular metabolism, and signaling in response to environmental stimuli (231, 234-236). PKA is required for normal growth and progression through the cell cycle (237, 238), while PKC is crucial for cell cycle progression and cell wall formation (232, 233, 239).

At 3,910 molecules per cell (240), Pah1p is a relatively abundant enzyme in *S. cerevisiae* (119). While Pah1p is found mostly in the cytosol, its substrate PA resides in the nuclear/ER membrane, and therefore translocation of the enzyme is vital for *in vivo* function (78, 106-108). Recent studies have demonstrated that phosphorylation/dephosphorylation of Pah1p governs it subcellular localization, thus serving as the major regulator of PAP activity (105-108). Phosphorylated Pah1p resides in the cytosol, while its dephosphorylated form is associated with the membrane (107)

(Fig. 3). Recruitment of the phosphorylated enzyme to the nuclear/ER membrane is dependent on the Nem1p-Spo7p protein phosphatase complex, which dephosphorylates PAP and thus allows for its association with the membrane through a process mediated by a short N-terminal amphipathic helix (104, 106, 107, 136) (Figs. 4 and 7).

The nuclear/ER membrane-associated Nem1p-Spo7p complex was identified in studies that showed it is essential for the formation of a spherical nucleus (104). Further work revealed the protein phosphatase activity of the complex is dependent on the catalytic motif D*X*D*X*(T/V) in Nem1p, as well as binding of the regulatory subunit Spo7p to Nem1p (104). A defect in either Nem1p or Spo7p results in the same aberrant nuclear/ER membrane expansion phenotype exhibited by the *pah1*Δ mutant, indicating both subunits of the phosphatase complex are required for PAP function *in vivo*, and confirming that Pah1p is dephosphorylated exclusively by Nem1p-Spo7p (104, 136). Overexpression of the Nem1p-Spo7p complex is lethal only in the presence of its substrate Pah1p (104), suggesting that dephosphorylation of Pah1p by Nem1p-Spo7p is a key modulator of PAP function. Moreover, the expression level of Nem1p is 10-fold lower than that of Pah1p (240), supporting this theory. Thus, under normal conditions, the level of membrane-associated Pah1p may be controlled by the amount of the Nem1p-Spo7p complex on the membrane, which would result in low PAP activity.

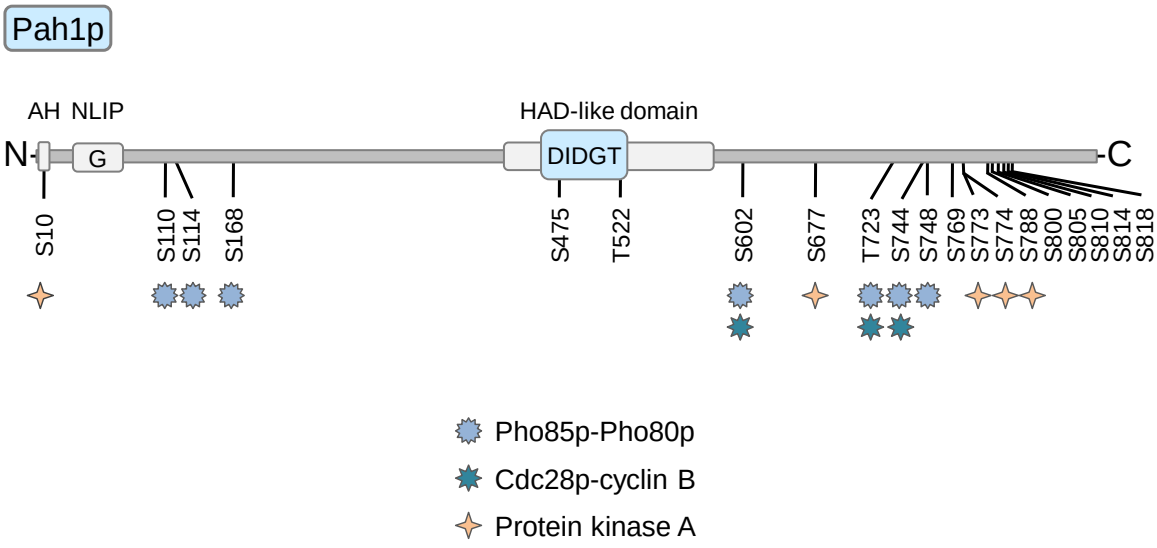
Large-scale analysis of the yeast proteome has identified Pah1p as a phosphoprotein with target sites for several protein kinases, including those encoded by *PHO85* (241, 242) and *CDC28* (243). Mass spectrometry and immunoblot analyses of yeast purified Pah1p have identified 14 sites of phosphorylation, seven of which (Ser^{110}, Ser^{114}, Ser^{168}, Ser^{602}, Thr^{723}, Ser^{744}, and Ser^{748}) are contained within the minimal

(Ser/Thr)-Pro motif that is a target for cell cycle-regulated protein kinases (105). In fact, all seven sites are phosphorylated by Pho85p-Pho80p (108), and three of the sites (Ser^{602}, Thr^{723}, and Ser^{744}) are also targets of Cdc28p-cyclin B (107) (Fig. 7). The phosphorylation efficiency of the three common sites is much greater for Pho85p-Pho80p when compared with Cdc28p-cyclin B (108). Phosphorylation by Pho85p-Pho80p results in a 6-fold reduction in the catalytic efficiency (V_{max}/K_m) of PAP (108), whereas activity is not affected by phosphorylation via Cdc28p-cyclin B (107). That these kinases phosphorylate Pah1p *in vivo* is supported by the analysis of Pah1p phosphorylation in mutants lacking Pho85p or functional Cdc28p (104, 108).

Pah1p is also a substrate for PKA (232, 244), the principal mediator of signals transmitted through the *RAS*/cAMP pathway in *S. cerevisiae* (237, 238). PKA phosphorylates Pah1p on Ser^{10}, Ser^{677}, Ser^{773}, Ser^{774}, and Ser^{788} with specificity similar to that shown for Pho85p-Pho80p and Cdc28p-cyclin B (244) (Fig. 7). The PKA-mediated phosphorylation of Pah1p inhibits its PAP activity by decreasing catalytic efficiency (1.8-fold), but to a lesser extent as that observed for Pho85p-Pho80p (108, 244). The inhibitory effect of PKA on PAP activity is primarily conferred by phosphorylation at Ser^{10} (244).

Analysis of phosphorylation-deficient forms of Pah1p has provided insight into the biochemical and physiological roles of phosphorylation by Pho85p-Pho80p, Cdc28p-cyclin B, and PKA (105, 107, 108, 244). The purified 7A mutant enzyme, where all seven (Ser/Thr)-Pro sites are mutated to nonphosphorylatable alanine, exhibits elevated PAP activity and increased interaction with phospholipid vesicles (105, 107). *In vivo*, expression of the 7A mutant enzyme complements the *pah1*Δ *nem1*Δ double mutant

Figure 7. Pah1p is a highly phosphorylated protein. The yeast Pah1p basic characteristics, including its catalytic motif and phosphorylation sites, are illustrated in the diagram. *AH*, amphipathic helix; *HAD*, haloacid dehalogenase; ✹, approximate positions of the sites (Ser^{110}, Ser^{114}, Ser^{168}, Ser^{602}, Thr^{723}, Ser^{744}, and Ser^{748}) phosphorylated by Pho85p-Pho80p; ✸, approximate positions of the sites (Ser^{602}, Thr^{723}, and Ser^{744}) phosphorylated by Cdc28p-cyclin B; ✦, approximate positions of the sites (Ser^{10}, Ser^{677}, Ser^{773}, Ser^{774}, and Ser^{788}) phosphorylated by PKA.


Pah1p
AH
NLIP
HAD-like domain
N-
G
DIDGT
-C
S10
S110
S114
S168
S475
T522
S602
S677
T723
S744
S748
S769
S773
S774
S788
S800
S805
S810
S814
S818
Pho85p-Pho80p
Cdc28p-cyclin B
Protein kinase A

phenotypes that include temperature sensitivity, nuclear/ER membrane expansion, and derepression of phospholipid synthesis genes (105, 107). Moreover, the 7A mutations facilitate the translocation of Pah1p from the cytosol to the membrane, and in a *nem1*Δ mutant background, cause an increase in the synthesis of TAG (106, 107). Cells lacking the Nem1p-Spo7p complex exhibit reduced TAG due to loss of PAP function, thus indicating that lack of phosphorylation of the seven sites renders Pah1p capable of bypassing the Nem1p-Spo7p requirement for *in vivo* function (107). Simultaneous mutation of the three Cdc28p-cyclin B phosphorylation sites (3A) only partially mimics the physiological consequences of the 7A mutations (107).

Analysis of the S10A and S10D mutations (mimicking dephosphorylation and phosphorylation, respectively, by PKA), alone or in combination with the 7A mutations, indicate that phosphorylation at Ser^{10} inhibits its association with membranes, PAP activity, and TAG synthesis (244). In fact, the S10A mutation enhances the physiological effects caused by the 7A mutations, whereas the S10D mutation attenuates the effects of the 7A mutations (244). Thus, the PKA-mediated phosphorylation of Ser^{10} functions in conjunction with the phosphorylations mediated by Pho85p-Pho80p and Cdc28p-cyclin B, and that Ser^{10} should be dephosphorylated for proper PAP function (244).

In addition to Pho85p-Pho80p, Cdc28p-cyclin B, and PKA, Pah1p is phosphorylated by PKC and casein kinase II (232). These protein kinases are also known to regulate phospholipid synthesis in yeast (233). Phosphorylation of Pah1p by PKC and casein kinase II decreases its interaction with model membranes (232), suggesting an inhibitory effect of phosphorylation on PAP activity *in vivo*. Further studies identifying the PKC and casein kinase II phosphorylation sites and characterizing their effects on

PAP activity are yet to be performed.

Like the yeast Pah1p PAP, the phosphorylation of mammalian lipin-1 and -2 affects their subcellular localization, thereby indirectly inhibiting PAP activity (245). For instance, the phosphorylation of lipin-1 in response to insulin and amino acids in rat and mouse adipocytes is mTOR-dependent and promotes cytosolic versus membrane localization (246), thus hindering PAP *in vivo* function by limiting access of the enzyme to its substrate. In addition, reduced PAP activity during mitosis has been linked to lipin-1 and -2 phosphorylations (143), confirming an evolutionarily conserved role of phosphorylation as a modulator of PAP activity. Like yeast Pah1p, lipin-1 is specifically dephosphorylated by the Nem1p human ortholog C-terminal domain nuclear envelope phosphatase 1 (CTDNEP1, formerly called dullard) (246, 247). In addition, recent work indicates CTDNEP1 can dephosphorylate lipin-1α, -1β, and -2 only in the presence of envelope phosphatase 1-regulatory subunit 1 (NEP1-R1), the metazoan ortholog of Spo7p (247).

Regulation by protein stability

Stationary phase is the stage of growth where the synthesis of TAG predominates over the synthesis of phospholipids (164), and this change in lipid metabolism correlates with an increase in PAP activity (122, 164). That the *pah1*Δ mutation results in elevated phospholipids and a dramatic decrease in TAG in stationary phase (70, 78) supports the notion that Pah1p PAP is a major contributor to the regulation of lipid synthesis in response to growth phase. Thus in exponential phase, where membrane phospholipid synthesis is essential, PAP function should be attenuated to allow partitioning of PA into

phospholipids. On the other hand, the increased synthesis of TAG that occurs when cells progress into stationary phase should require stimulation of PAP function for channeling PA into the storage lipid TAG. As discussed above, phosphorylation/dephosphorylation is a major modulator of PAP function. We speculate that attenuation of Pah1p function in exponential phase is mediated by phosphorylation of the enzyme, whereas in cells entering stationary phase PAP activity is stimulated by dephosphorylation.

Ironically, phosphorylation deficiency caused by the 7A mutations or by the loss of Pho85p-Pho80p phosphorylation of Pah1p in *pho85*Δ mutant cells cause dramatic reductions (50-60 %) in Pah1p abundance (107, 108). In addition, the abundance of the enzyme decreases in cells progressing from the early- to late-exponential phase, but this effect is attenuated in *nem1*Δ mutant cells lacking the Nem1p-Spo7p phosphatase complex (108). These observations indicate that phosphorylation stabilizes Pah1p, whereas dephosphorylation causes loss of abundance. We know that controlling excess PAP activity is important because the overexpression of the 7A mutant form of Pah1p (87, 105, 107), as well as the overexpression of Nem1p-Spo7p (104), is deleterious to growth (87, 104, 105, 107). Thus, the paradoxical effects of phosphorylation/dephosphorylation on Pah1p function and enzyme abundance appear to be a mechanism by which cells control the levels of PA and DAG to maintain homeostasis of lipids. It remains to be determined whether Pah1p abundance is regulated by means of programmed proteolysis.

Protein degradation within eukaryotic cells is compartmentalized, either in macromolecular structures known as proteasomes or in degradative organelles such as lysosomes (248, 249). Proteolysis within lysosomes is largely nonselective, and is

primarily reserved for the degradation of long-lived proteins and for the increased protein degradation observed under starvation conditions (249). On the other hand, proteasomes are responsible for the turnover of most short-lived proteins (1, 249, 250). Thus, in eukaryotes much of the regulatory proteolysis is mediated by the ubiquitin-proteasome pathway (249), a complex and highly regulated mechanism by which proteins are targeted for degradation by proteasomes through their ligation to ubiquitin (1, 249). In addition, a third degradation pathway occurs in the ER, where both misfolded and normal proteins are directed in an ubiquitin-mediated mechanism (251).

In particular, this ER-associated degradation (ERAD) pathway is involved in the proteolysis of the essential enzyme 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, HMGR), the most representative example of regulation of lipid synthesis by protein degradation (252-254). The ER-localized HMGR is the rate-limiting enzyme in the synthesis of cholesterol and related sterols. Its regulation is achieved at several levels, including feedback-regulated ER degradation, which is conserved from yeast to mammals (251, 253, 254). Yeast expresses two isoforms of HMGR, Hmg1p and Hmg2p, which differ primarily in stability: Hmg1p is quite stable, while Hmg2p undergoes ubiquitin-mediated ER degradation (251). Thus, when flux through the sterol pathway is high, Hmg2p degradation is fast, and steady state levels are low, whereas decreased sterol pathway flux results in decreased Hmg2p degradation and high protein levels (251, 252, 254). Given the number of separate avenues by which ERAD seems to occur, it is highly likely that other proteins are similarly regulated. In fact, a recent study has reported that the yeast *OLE1*-encoded Δ-9 desaturase undergoes regulated ER degradation, and is therefore a plausible candidate for regulated ERAD (252, 255).

Yet another example related to the control of lipid metabolism through protein degradation is the *ITR1*-encoded inositol permease, which undergoes endocytic degradation in the vacuole in response to inositol supplementation (256).

HYPOTHESIS

With the identification and characterization of the yeast *PAH1* gene and its protein product, monumental advances have been made in establishing the role of PAP in lipid homeostasis. The importance of understanding the mechanisms that regulate PAP activity is underscored by the involvement of this enzyme in lipid-based disorders in human physiology. Genetic and biochemical studies with yeast Pah1p PAP have provided insights into the basic biochemical properties of the enzyme and how its activity is controlled by effector molecules, subcellular localization, and posttranslational modifications.

Despite the extensive characterization of the yeast Pah1p PAP and its mammalian homolog lipin 1, much remains to be known about the regulation of the yeast enzyme, particularly with respect to growth phase. While it has been shown that TAG is synthesized throughout growth and its accumulation in stationary phase cells has been attributed to an observed increase in PAP activity, the exact gene/enzyme responsible is yet to be identified. Given the severe reduction in TAG levels in the *pah1*Δ, a phenotype that is accentuated in the stationary phase of growth, we set out to investigate the involvement of the Pah1p PAP in TAG synthesis, and its regulation in response to growth phase. In this work, we addressed the hypothesis that Pah1p PAP activity is induced in stationary phase, and that this increased activity has a direct effect on the observed accumulation of TAG. Moreover, we investigated whether this regulation occurred at the transcriptional or posttranslational levels.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. Growth medium supplies were obtained from Difco. Ampicillin, ammonium hydroxide, aprotinin, benzamidine, bovine serum albumin, cycloheximide, diethyl ether, diethyl pyrocarbonate (DEPC), EDTA, EGTA, glycerol, glycine, imidazole-HCl, hexane, leupeptin, magnesium chloride, carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132), NEM, octyl-β-D-glucopyranoside, O-nitrophenyl β-D-galactopyranoside (ONPG), pepstatin, phenol buffer, phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), Triton X-100 and β-mercaptoethanol were supplied by Sigma-Aldrich. Acetic acid, chloroform, chloroform/isoamyl alcohol (24:1), dimethyl sulfoxide (DMSO), hydrochloric acid, and methanol were from Fisher. Polymerase chain reaction (PCR) primers were prepared by Genosys Biotechnologies. Invitrogen was the source of the yeast deletion consortium strain collection, mouse anti-phosphoglycerate kinase (Pgk1p) antibodies, and UltraPure™ agarose. New England Biolabs was the source of modifying enzymes, recombinant $Vent_R$® DNA polymerase, restriction endonucleases, nucleotides, ssRNA size standards, and the NEBlot® kit. The QuikChange® site-directed mutagenesis kit was purchased from Stratagene. DNA gel extraction and plasmid DNA purification kits were obtained from Qiagen. The Yeastmaker™ transformation kit was purchased from Clotech. Bio-Rad was the supplier of acrylamide solutions, DNA size ladders, protein assay, electrophoretic and immunochemical reagents, protein molecular mass standards, and Zeta-Probe® blotting membranes. Polyvinylidene difluoride (PVDF) membrane, ProbeQuant G-50 columns, and the enhanced chemiluminescence Western blotting

detection kit were purchased from GE Healthcare. Custom rabbit anti-Pah1p (107), anti-Cho1p (257), anti-Dpp1p (186), and anti-Dgk1p (86) antibodies were prepared at BioSynthesis Inc. Rabbit anti-Pah1p antibodies were prepared against a mixture of peptides found at the N- (residues 130-147) and C- (residues 778-794) terminal portions of the protein (107), and the IgG fraction isolated from antisera by protein A-sepharose chromatography (258); rabbit anti-Cho1p antibodies were prepared against residues 1–15 at the N-terminal end (257); rabbit anti-Dpp1p antibodies were raised against residues 263–279 at the C-terminal end, and the IgG fraction was isolated from antisera by protein A-Sepharose chromatography (186); and anti-Dgk1p antibodies were prepared against residues 133–145 and residues 188–201, and the IgG fraction was isolated from antisera by protein A-Sepharose (86). Alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies and alkaline phosphatase-conjugated goat anti-mouse IgG antibodies were obtained from Thermo Scientific and Pierce, respectively. Radiochemicals were obtained from PerkinElmer Life Sciences, and scintillation counting supplies and acrylamide solutions from National Diagnostics. Lipids and silica gel 60 thin-layer chromatography plates were from Avanti Polar Lipids and EM Science, respectively. Thiolutin was a gift from Pfizer.

Strains and growth conditions

The strains used in this work are listed in Table 1. Plasmid amplification and maintenance was performed in *Escherichia coli* strain DH5α. *E. coli* cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7) at 37 °C, and ampicillin (100 μg/ml) was added to select for cells carrying plasmids. Yeast cultures were

generally grown at 30 °C in YEPD medium (1% yeast extract, 2% peptone, 2% glucose), or in standard synthetic complete medium (2% glucose) with the appropriate amino acids omitted for selection purposes (259). To evaluate the effect of inositol, cells were cultured in synthetic medium prepared using an inositol-free yeast nitrogen base (260), which was supplemented with inositol where indicated. A modified synthetic medium was utilized for optimum growth of wild-type and mutant cells of the BY family (261). *GAL1/10*-dependent overexpression was achieved by supplementing log phase cells grown in 2% raffinose with 2% galactose. Cell numbers in liquid cultures were determined spectrophotometrically at an absorbance of 600 nm. The growth medium was supplemented with agar (2% for yeast, 1.5% for *E. coli*) for growth in plates.

DNA manipulations and plasmid constructions

Standard protocols were used for the isolation and manipulation of genomic and plasmid DNA, and the digestion, ligation and PCR amplification of DNA (262, 263). All mutations were confirmed by DNA sequencing. Plasmid transformations of *E. coli* (262) and yeast (264) were performed as described previously.

The plasmids used in this study are listed in Table 2. The pFP1 plasmid contains 1000 bp of the putative *PAH1* gene promoter fused to the coding sequence of the *lacZ* gene of *E. coli*. This plasmid was constructed by replacing the *DPP1* gene promoter fragment in pJO2 with the *PAH1* promoter sequence at the *Eco*RI/*Kpn*I sites. The putative *PAH1* promoter was obtained by PCR (primers listed in Table 3) using the genomic DNA of strain W303-1A as template. The PCR primer used in the forward direction corresponds to -1000 bp to the start codon, and the primer used in the reverse

direction corresponds to +3 bp to the start codon. The correct orientation of the *PAH1* promoter in pFP1 was confirmed by restriction enzyme digestion and DNA sequencing. The pFP1 plasmid was introduced into W303-1A and β-galactosidase activity measured as an indication of *PAH1* expression. Using appropriate primers, A. Soto-Cardalda constructed a series of P_{PAH1}-*lacZ* deletion plasmids by PCR amplification using plasmid pFP1 as the template. Each PCR product was digested with *Eco*RI and *Kpn*I and substituted for the 1.0-kb *Eco*RI/*Kpn*I fragment in pFP1. Plasmid constructions were confirmed by *Eco*RI/*Kpn*I digestion, and the promoter truncation plasmids introduced into W303-1A cells for analysis of β-galactosidase activity. Plasmids pFP2 and pFP3 are derivatives of pFP1 in which the sequences of a putative Rph1p binding site and an Rph1p/Gis1p binding site (5'-AGGG(A/G)-3') (265) in the *PAH1* promoter were mutated to the nonconsensus sequence 5'-AAAAA-3'. Site-specific mutations were generated with the Stratagene QuikChange® site-directed mutagenesis kit according to manufacturer's instructions, using the appropriate mutagenic primers listed in Table 4 and plasmid pFP1 as template. Samples were digested with DpnI after PCR amplification to eliminate template DNA, and the plasmids amplified, purified, and sequenced to confirm the desired mutations were obtained.

The pGH311 (78), pRIP1PGK (266), and pAS103 (267) plasmids were used to synthesize radiolabeled probes for Northern blot analysis of *PAH1*, *PGK1* and *CHO1* mRNA, respectively. The pGH312 construct and its derivatives pGH312-G80R, pGH312-D398E, and pGH312-D400E, express the wild-type and catalytic site mutant versions of HA-tagged *PAH1* (79). The plasmid pGH339 directs the expression of the *DPP1* gene driven by the *PAH1* promoter, and was constructed by G.-S. Han by insertion

of the *DPP1* ORF and 3'-UTR from plasmid pGH201 into the *Aat*II/*Spe*I sites in plasmid pGH316 containing the *PAH1* promoter. The high-copy plasmid YCplac111-*GAL1/10-DGK1* construct directs the overexpression of *DGK1* (87).

Labeling and analysis of lipids

Cells were grown to the indicated growth phases in 5 ml of culture medium with 1 μCi/ml of [2-^{14}C]acetate for steady-state labeling of lipids. Lipid synthesis was followed by labeling cells at the indicated phases of growth with 5 μCi/mL of [2-^{14}C]acetate for 20 minutes. Total lipids were extracted from labeled cells by the method of Bligh and Dyer (268). Lipids were analyzed by one-dimensional thin layer chromatography on silica gel plates using the solvent system hexane/ diethyl ether/ acetic acid (40:10:1, v/v) (269). The identity of the labeled lipids on the thin-layer chromatography plates was confirmed by comparison with standards after exposure to iodine vapor. Radiolabeled lipids were visualized by phosphorimaging analysis, and their relative quantities analyzed using ImageQuant software. Signals were in the linear range of detectability.

Preparation of cell extracts and subcellular fractionation

All steps were performed at 4 °C. Cell extracts were prepared by disruption of yeast cells with glass beads (0.5-mm diameter) in a Biospec Products Mini BeadBeater-16 (270). The lysis buffer contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3 M sucrose, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 mM NaF, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. Glass beads and cell debris were removed by removed by centrifugation at 1,500 x *g* for 5 min, and the supernatant

used as the cell extract. The cytosol (supernatant) and total membrane (pellet) fractions were separated by centrifugation at 100,000 x *g* for 70 min (270), and the membrane pellets resuspended in the lysis buffer to the same volume as the cytosol fraction. Protein concentration was determined by the Bradford method (271) using bovine serum albumin as the standard.

PAP activity assay

The ^{32}P-labeled PA used to measure PAP activity was enzymatically synthesized from DAG and [γ-^{32}P]ATP using *E. coli* purified DAG kinase, and the radioactive product was purified by thin-layer chromatography (270). PAP activity was measured by following the release of water-soluble $^{32}P_i$ from chloroform-soluble [^{32}P]PA (10,000-15,000 cpm/nmol) for 20 min at 30 °C (270). 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. All enzyme assays were conducted in triplicate, and the average standard deviation was ± 5%. The reactions were linear with time and protein concentration. A unit of PAP activity was defined as the amount of enzyme that catalyzed the dephosphorylation of 1 nmol of PA/min. Specific activity was defined as units/mg of protein.

β-galactosidase activity assay

β-galactosidase activity was measured for 5 min at room temperature by following the release of *O*-nitrophenol from ONPG at 410 nm. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 100 mM

β-mercaptoethanol, 3 mM ONPG, and enzyme in a total volume of 0.1 ml. A unit of β-galactosidase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol *O*-nitrophenol/min. Specific activity was defined as units/mg of total protein. All enzyme assays were conducted in triplicate. The average standard deviation of the assays was ± 5%. Enzyme reactions were linear with time and protein concentration.

RNA isolation, preparation of radiolabeled probes and Northern blotting

Total RNA was isolated using the methods of Schmitt et al. (272) and Herrick et al. (273). Additional experiments were performed using a modified hot acidic phenol RNA isolation method to maximize extraction efficiency (274, 275). Cell pellets were resuspended in lysis buffer (50 mM sodium acetate, 10 mM EDTA, pH 8), treated with 10% SDS, 0.3-0.4 g of glass beads (0.5-mm diameter) and hot acidic citrate-buffered phenol (pH 4.3, preheated at 65 °C), and vortexed vigorously at room temperature for 1 min, followed by incubation at 65 °C for 5 min. This was repeated for a total of six vortex cycles over a period of 30 min. The samples were cooled briefly on ice, and the organic and aqueous phases separated by addition of chloroform/isoamyl alcohol (24:1) and centrifugation at 17,000 x *g* for 5 min at 4 °C. The aqueous phase was recovered and RNA precipitated overnight with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol at – 20 °C overnight, followed by centrifugation at 17,000 x *g* for 10 min. The RNA pellet was then washed with 70% ethanol, dried at room temperature for 15 min, and resuspended in nuclease-free water.

RNA was resolved on a 1.1% formaldehyde gel at 22 V for 15 h and transferred to Zeta Probe membranes by vacuum blotting (276). For the detection of *PAH1* mRNA,

a radiolabeled probe was synthesized using 3.8-kb fragment from the *PAH1* coding sequence isolated from the plasmid pGH311. For internal controls, a 1.0-kb fragment of the *PGK1* gene (266), and a 1.2-kb fragment of the *CHO1* gene (267) were used. The purified DNA fragments were labeled to high-specific activity with [α-^{32}P]dTTP using the NEBlot random primer labeling kit, and unincorporated nucleotides were removed using ProbeQuant G-50 columns. The specific activity of labeled probes was determined by liquid scintillation counting. Pre-hybridization, hybridization with the probes, and washes to remove nonspecific binding were carried out according to the manufacturer's instructions. Images of radiolabeled species were acquired by phosphorimaging analysis, and relative densities of the images were analyzed using ImageQuant software. Signals were in the linear range of detectability. The level of total rRNA was used as a loading control.

SDS-PAGE and immunoblot analysis

SDS-PAGE (277) and immunoblotting (278) with polyvinylidene difluoride membrane were performed by standard protocols. Rabbit anti-Pah1p (107), rabbit anti-Cho1p (279), and mouse anti-Pgk1p antibodies were used at a concentration of 2 μg/ml. Rabbit anti-Dpp1p (186) and anti-Erg25p antibodies were used at dilutions of 1:1,000, while rabbit anti-Dgk1p (86) was used at a 1:1,500 dilution. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies and alkaline phosphatase-conjugated goat anti-mouse IgG antibodies were used at a dilution of 1:5,000. For Erg25p, horseradish peroxidase conjugated anti-IgG secondary antibodies were used at a 1:2000 dilution. Immune complexes were detected using the enhanced chemifluorescence Western

blotting detection kit. Fluorimaging was used to acquire images from immunoblots, and the relative densities of the images were analyzed using ImageQuant software. Signals were in the linear range of detectability.

Protein and mRNA stability

Analysis of *PAH*1 mRNA decay was determined after the arrest of transcription with thiolutin (15 μg/ml) as described by Gonzalez and Martin (223). After treatment, Northern blot analyses were performed as described above. Pah1p protein stability was evaluated after translation arrest with cycloheximide (100 μg/mL), followed by immunoblot analysis.

Proteasome inhibition with MG132

MG132 was prepared as a 50 mM stock solution in DMSO, and added to cultures to obtain a 50 μM final concentration of MG132. An equal volume of DMSO was added to the treatment control cultures. Cells were grown in the presence of MG132 upon initial inoculation, and harvested at the indicated times of growth. In complementary experiments, cells were grown to 16 h ($A_{600\ nm}$/mL ~ 2.0-2.5), treated with MG132 (50 μM) or DMSO (vehicle), and collected 0, 4 and 8 h after treatment.

Data analyses

The Student's *t*-test (SigmaPlot software) was used to determine statistical significance, and p values < 0.05 were taken as a significant difference.

TABLE I

Strain	Genotype or relevant characteristics	Source or Ref.
E. coli		
DH5α	F$^-$ φ80*dlac*ZΔM15 Δ(*lacZYA-arg*F) U169 *deo*R *rec*A1 *end*A1 *hd*R17(r_k^- m_k^+) *pho*A *sup*E44 l$^-$*thi*-1 *gyr*A96 *rel*A1	(262)
S. cerevisiae		
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	(280)
GHY57	*pah1*Δ::*URA3* derivative of W303–1A	(78)
DTY1	*dpp1*Δ::*TRP1/Kan^r* derivative of W303-1A	(126)
TBY1	*dpp1*Δ::*TRP1/Kan^r lpp1*Δ::*HIS3/Kan^r* derivative of W303–1A	(132)
GHY58	*pah1*Δ::*URA3 dpp1*Δ::*TRP1/Kan^r lpp1*Δ::*HIS3/Kan^r* derivative of W303–1A	(78)
GHY65	*app1*Δ::*natMX4 dpp1*Δ::*TRP1/Kan^r lpp1*Δ::*HIS3/Kan^r* derivative of W303–1A	(152)
SH304	*MAT***a** *his3*Δ*200 leu2*Δ*1 trp1*Δ*63 ura3-52 opi1*Δ*::LEU2*	S. A. Henry
SH303	*MAT***a** *his3*Δ*200 leu2*Δ*1 trp1*Δ*63 ura3-52 ino2*Δ*::TRP1*	S. A. Henry
SH307	*MATα his3*Δ*200 leu2*Δ*1 trp1*Δ*63 ura3-52 ino4*Δ*::LEU2*	S. A. Henry
JOY37	*gis1*Δ*::LEU2 derivative of W303-1A*	(281)
JOY38	*rph1*Δ*::HIS3 derivative of W303-1A*	(281)
RS453	*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52*	(87)
SS1026	*pah1*Δ::*TRP1* derivative of RS453	(104, 282)
SS1002	*nem1*Δ::*HIS3* derivative of RS453	(136)
SS1144	*dgk1*Δ::*HIS3* derivative of RS453	(87)
BY4741	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	(283)
*pdr5*Δ	*pdr5*Δ*::KanMX* derivative of BY4741	Deletion consortium
*rpn4*Δ	*rpn4*Δ*::KanMX* derivative of BY4741	Deletion consortium
*blm10*Δ	*blm10*Δ*::KanMX* derivative of BY4741	Deletion consortium
*ump1*Δ	*ump1*Δ*::KanMX* derivative of BY4741	Deletion consortium
KMY576, "WT"	*MAT***a** *his3*Δ*300 trp1*Δ*63 lys2-801 ura3-52 leu2-2, 112 Gal+*	K. Madura
pre1-1 pre2-1	*MATα pre1-1 pre2-2 ura3*Δ*5 his3-11, 15 leu2-3, 112*	K. Madura
*hrd1*Δ	*hrd1*Δ::*KanMX* derivative of BY4741	Deletion consortium
*ubc4*Δ	*ubc4*Δ::*KanMX* derivative of BY4741	Deletion consortium
*ubc8*Δ	*ubc8*Δ::*KanMX* derivative of BY4741	Deletion consortium
*doa4*Δ	*doa4*Δ::*KanMX* derivative of BY4741	Deletion consortium

TABLE II

Plasmid	Relevant characteristics	Source or Ref.
pJO2	P_{DPP1}-*lacZ* reporter construct on a multicopy plasmid containing *URA3*	(186)
pFP1	P_{PAH1}-*lacZ (1000 bp)* reporter construct on a multicopy plasmid containing *URA3, derivative of pJO2*	This study
pJH359	P_{INO1}-*lacZ* reporter construct on a multicopy plasmid containing *URA3*	(284)
pFP2	Derivatives of pFP1, mutations in UAS_{PDS} element 1	This study
pFP3	Derivative of pFP1, mutations in UAS_{PDS} element 2	This study
P_{PAH1}-*lacZ, -800*	P_{PAH1}-*lacZ deletion construct, 800 bp promoter*	A. Soto-Cardalda
P_{PAH1}-*lacZ, -500*	P_{PAH1}-*lacZ deletion construct, 500 bp promoter*	A. Soto-Cardalda
P_{PAH1}-*lacZ, -300*	P_{PAH1}-*lacZ deletion construct, 300 bp promoter*	A. Soto-Cardalda
P_{PAH1}-*lacZ, -200*	P_{PAH1}-*lacZ deletion construct, 200 bp promoter*	A. Soto-Cardalda
pGH311	*PAH1* gene inserted into the *Xba*I/*Sph*I sites in Yep351	(78)
pRIP1PGK	Plasmid containing a 1.0-kb fragment of the *PGK1* gene	(266)
pAS103	Plasmid containing a 1.2-kb fragment of the *CHO1* gene	(267)
YEp351	Multicopy *E. coli*/yeast shuttle vector containing *LEU2*	(285)
pGH312	HA-tagged *PAH1* gene inserted into the XbaI/SphI sites of YEp351	(78)
pGH312-G80R	pGH312 containing the G80R mutation in the *PAH1* coding sequence	(79)
pGH312-D395E	pGH312 containing the D398E mutation in the *PAH1* coding sequence	(79)
pGH312-D400E	pGH312 containing the D400E mutation in the *PAH1* coding sequence	(79)
pRS415	Single copy *E. coli*/yeast shuttle vector with *LEU2*	(286)
pGH316	HA-tagged *PAH1* gene inserted into the XbaI/HindIII sites of pRS415	(79)
pGH201	HA-tagged *DPP1* gene inserted into the *Sal*I site of pRS415	(206)
pGH339	pGH316 derivative containing the *Aat*II-*Spe*I fragment of pGH201	G.-S. Han
YCplac111-*GAL1/10-DGK1*	*DGK1* under control of *GAL1/10* promoter into *CEN/LEU2* vector	(87)

TABLE III

Primers used to construct the pFP1plasmid

Oligonucleotide	Sequence
PAH1-lacZ F	5’- GCGGTACCTAGAGTCCAAACTCAACAGCC -3’
PAH1-lacZ R	5’- GCCGGAATTCATAATCGACCGATGTGTC -3’

TABLE IV

Primers used for site-directed mutagenesis

Plasmid	Primer	Sequence
pFP2	UAS_{PDS}[3] 1 F	5’-CGGACGGTCTACCAATCTAAAAAAAAAGTTAGGTTTCTTTAGTGGC-3’
	UAS_{PDS} 1 R	5’-GCCACTAAAGAAACCTAACTTTTTTTTAGATTGGTAGACCGTCCG-3’
pFP3	UAS_{PDS} 2 F	5’-CCAATCTGAAGGTGTGAAAAAAAACTTTAGTGGCGTTGGCC-3’
	UAS_{PDS} 2 R	5’-GGCCAACGCCACTAAAGTTTTTTTTCACACCTTCAGATTGG-3’

[3]UAS_{PDS} for *u*pstream *a*ctivating *s*equences during *p*ost-*d*iauxic *s*hift

*Underlined sequences indicate the positions of putative Rph1p binding sites that are to be mutated to the nonconsensus sequence 5’-AAAAA-3’.

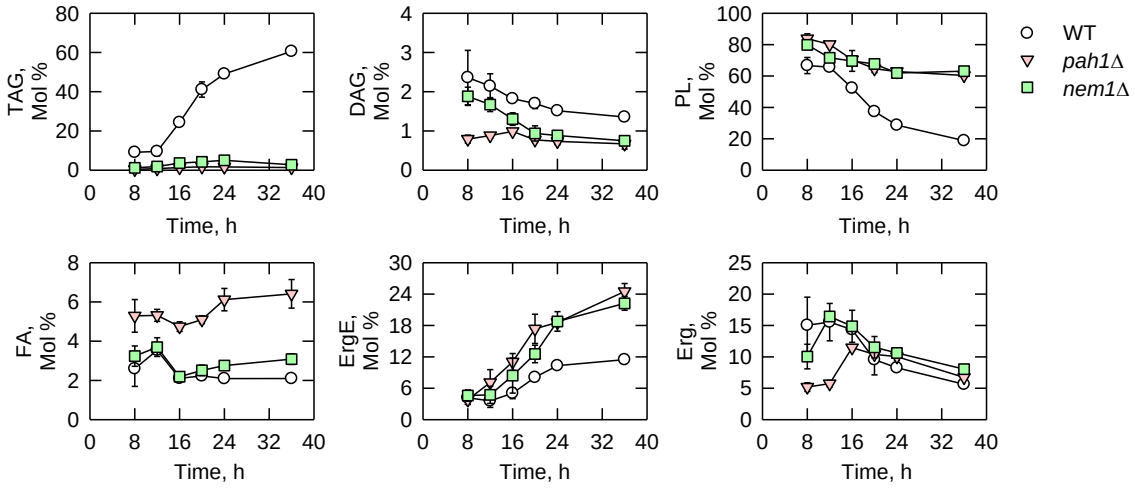
RESULTS

Effect of Pah1p PAP activity on lipid synthesis during growth

Studies by Taylor and Parks in the late 1970s indicated that TAG is synthesized from its precursor PA throughout the exponential phase of growth, and that its levels increase as wild type cells progress to stationary phase (164). In contrast, the *pah1*Δ mutant shows a marked decrease in DAG and TAG, particularly in stationary phase, where TAG content is drastically reduced to 10% of wild type levels (78), pointing to a central role of the *PAH1*-encoded PAP in lipid synthesis. Therefore, we examined the contribution of Pah1p to TAG content and synthesis during growth in wild type cells, *pah1*Δ mutant cells lacking Pah1p PAP activity, and *nem1*Δ mutant cells defective in Pah1p dephosphorylation and hence Pah1p *in vivo* function (Figs. 8 and 9).

Phospholipids and neutral lipids were extracted from cells grown to the indicated time points that were labeled to steady state with [2-^{14}C]acetate (Fig. 8). In wild type cells, TAG content increased throughout growth, and was highest in stationary phase, where TAG levels were 6.8-fold higher than in exponential phase. Moreover, this increase in TAG appeared to occur at the expense of phospholipids, which decreased to 75% of exponential phase levels in stationary wild type cells. TAG content was reduced to < 10% of wild type levels in stationary *pah1*Δ and *nem1*Δ mutant cells, while fatty acids, phospholipids and ergosterol esters were higher in the mutants defective in Pah1p function. In particular, phospholipid levels were reduced by only 31% and 20% in stationary *pah1*Δ and *nem1*Δ mutant cells, respectively, which resulted in 3.4- and 3.8-fold higher phospholipid content in the mutant strains. In addition, DAG levels were reduced in *pah1*Δ and *nem1*Δ mutant cells in both the exponential and stationary phases

Figure 8. Effect of the Pah1p PAP on neutral and phospholipid content. Wild type (*WT*), *pah1*Δ and *nem1*Δ mutant cells were grown in synthetic complete media in the presence of [2-^{14}C]acetate (1 μCi/mL). Lipids were extracted, separated by thin-layer chromatography, and the phosphorimages were subjected to ImageQuant analysis. The percentage of each individual lipid was normalized to the total ^{14}C-labeled chloroform fraction. Each *data point* represents the average of three experiments ± S.D. (*error bars*).

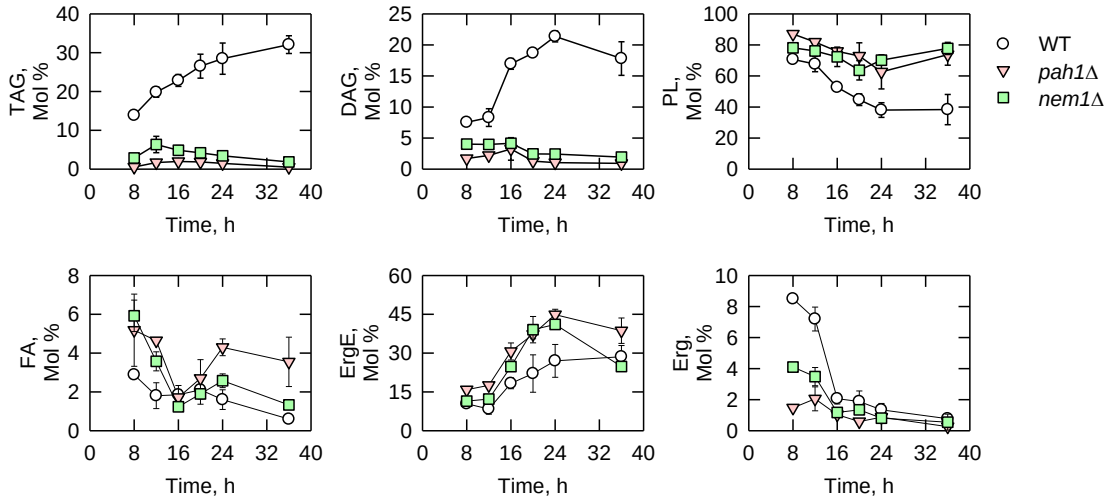

TAG, Mol %
DAG, Mol %
PL, Mol %
FA, Mol %
ErgE, Mol %
Erg, Mol %
Time, h
WT
pah1Δ
nem1Δ

of growth as compared to wild type cells. These results confirmed that Pah1p PAP activity is essential for lipid synthesis throughout growth, and in particular, for the observed increase in TAG levels in stationary phase cells.

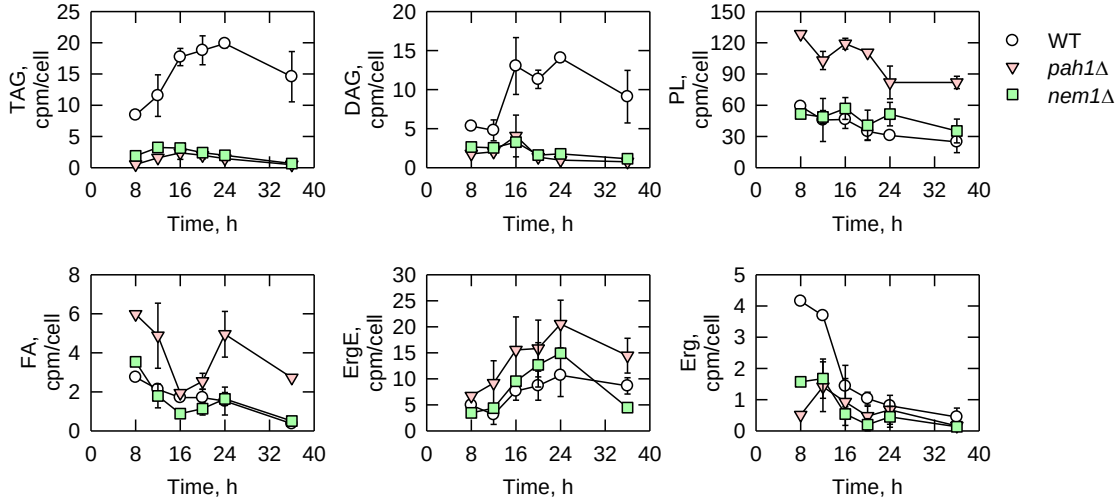
In order to determine whether the accumulation of TAG in stationary phase is due to increased synthesis or decreased TAG hydrolysis, lipid synthesis rates were analyzed in wild type, *pah1*Δ and *nem1*Δ mutant cells (Fig. 9). Cells grown to different times in the growth phase were labeled with [2-^{14}C]acetate for 20 min, lipids extracted and analyzed as described above. Fig. 9*A* shows lipid synthesis rates calculated as the percentage of each individual lipid normalized to the total ^{14}C-labeled lipid fraction. TAG synthesis rates increased by 131% in stationary phase in wild type cells, while it remained relatively constant in the *pah1*Δ and *nem1*Δ mutants. In addition, DAG synthesis was induced by 136% in stationary wild type cells compared to exponential phase cells, whereas it decreased by 54% and 48% in *pah1*Δ and *nem1*Δ mutant cells, respectively. Wild type cells showed a 54% reduction in the phospholipid synthesis rate in stationary phase, which correlates with the observed decrease in phospholipid steady state levels in these cells. In contrast, phospholipid synthesis was decreased by < 15% in the *pah1*Δ and *nem1*Δ mutants, accounting for their higher phospholipid content. Fatty acid synthesis rates were 2.0-fold higher in both mutant strains compared to wild type cells. While both wild type and *nem1*Δ mutant cells showed a > 75% decrease in fatty acid synthesis in stationary phase, fatty acid synthesis in the *pah1*Δ mutant was reduced by only 31%, which explains the observed accumulation of fatty acids in these cells. Lipid synthesis rates reported as total mass amounts of each lipid species (Fig. 9*B*) show the same trends as those calculated as percentages of total lipids, with the exception of

Figure 9. Effect of the Pah1p PAP on neutral and phospholipid synthesis. Wild type (*WT*), *pah1*Δ and *nem1*Δ mutant cells were grown in synthetic complete media to the indicated time points in the growth phase. Cells were then harvested, normalized to equal optical densities, and incubated with [2-^{14}C]acetate (5.0 μCi/mL) for 20 min. Lipids were extracted, separated and visualized by phosphorimaging analysis, and their relative quantities analyzed using ImageQuant software. The values reported are the average of two separate experiments ± S.D. (*error bars*). *A*, the percentage of each individual lipid was normalized to the total ^{14}C-labeled chloroform fraction. *B*, lipid synthesis rates were calculated using total mass amounts of each lipid species. *PL*, phospholipids; *FA*, fatty acids; *Erg*, ergosterol; *ErgE*, ergosterol esters.

A


TAG, Mol %
DAG, Mol %
PL, Mol %
FA, Mol %
ErgE, Mol %
Erg, Mol %
Time, h
WT
pah1Δ
nem1Δ

B


TAG, cpm/cell
DAG, cpm/cell
PL, cpm/cell
FA, cpm/cell
ErgE, cpm/cell
Erg, cpm/cell
Time, h
WT
pah1Δ
nem1Δ

phospholipid and ergosterol ester synthesis. While the initial phospholipid synthesis rates (*8h*) of *nem1*Δ cells were comparable to those of wild type cells, *pah1*Δ mutant cells exhibited 118% and 149% higher phospholipid synthesis rates than wild type and *nem1*Δ mutant cells, respectively. However, the decrease in phospholipid synthesis in wild type cells (58%) was 1.8- and 1.6-fold higher than in *pah1*Δ (32%) and *nem1*Δ (36%) mutant cells, respectively, which correlates to the increased phospholipid synthesis rates values when expressed as percentage of total lipids in these mutants compared to that of wild type cells. In addition, ergosterol ester synthesis rates in *nem1*Δ mutant cells were much lower than those *pah1*Δ mutant cells, which may contribute to the differences in synthesis rates values calculated as percentage of total lipids vs mass amounts. Taken together, these results indicate that TAG accumulation in stationary phase is due, at least in part, to a Pah1p-mediated increase in its rate of synthesis.

Regulation of Pah1p PAP activity by growth phase

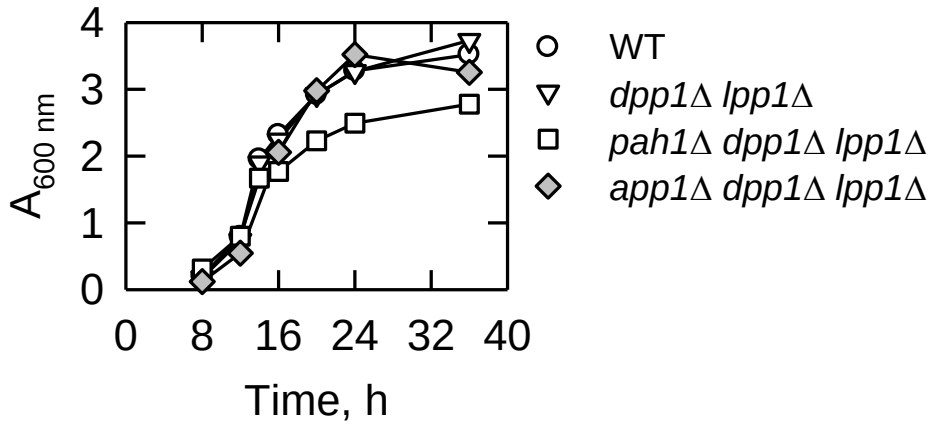
In early PAP characterization studies, Hosaka and Yamashita showed PAP activity increases as cells progress throughout growth (122). Moreover, the induction in PAP activity was correlated to the accumulation of TAG observed in stationary phase cells (122, 164). At this point in time, however, the genes encoding the PAP and LPP enzymes were unknown, and thus the particular enzyme(s) responsible for the increase in PAP activity and TAG synthesis remains to be identified. Having shown the direct involvement of Pah1p PAP function on TAG synthesis and content, we set out to investigate whether this role is directly related to a growth phase-mediated regulation of Pah1p enzymatic activity.

The growth rate and PAP activity levels of wild type, *dpp1*Δ *lpp1*Δ, *pah1*Δ *dpp1*Δ *lpp1*Δ, and *app1*Δ *dpp1*Δ *lpp1*Δ mutant cells were analyzed throughout growth (Fig. 10*A*). The *dpp1*Δ *lpp1*Δ and *app1*Δ *dpp1*Δ *lpp1*Δ mutations did not result in significant growth defects, while the *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant showed significantly slower growth compared to all other strains, demonstrating the importance of Pah1p function in normal cell development and physiology. Cells were collected at the indicated time points and extracts prepared and assayed for PAP activity (Fig. 10*B*). There was a marked (6.1-fold) increase in PAP activity as wild type cells progress from exponential to stationary phase, peaking at 24 h and decreasing by 25% at 36 h. In addition, PAP activity in the *dpp1*Δ *lpp1*Δ double mutant, which lacks the non-specific, Mg^{2+}-independent PAP activities, was also shown to increase but to a lesser extent than in wild type cells (3.3-fold), suggesting that the increase in PAP activity in wild type cells can be attributed in part to the *DPP1*- and *LPP1*-encoded enzymes. In fact, Dpp1p levels and PAP activity have been shown to increase in stationary phase cells (186). While the *app1*Δ *dpp1*Δ *lpp1*Δ triple mutant shows slightly higher PAP activity levels as those observed in the *dpp1*Δ *lpp1*Δ double mutant, the increase in PAP activity is abrogated in the *pah1*Δ *dpp1*Δ *lpp1*Δ triple mutant. Thus, these results indicate that the Pah1p PAP activity is induced as cells progress to stationary phase, and is primarily responsible for TAG synthesis and accumulation throughout growth.

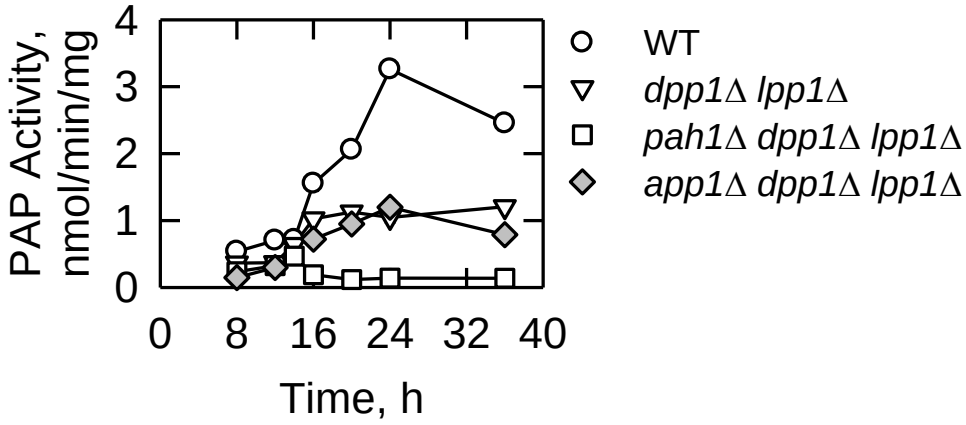
Given that PAP activity was shown to translocate from the cytosol to the membrane fraction of cells in previous studies (89, 120, 121), and considering the interrelationship between Pah1p function and its translocation to the membrane via Nem1p-Spo7p-dependent dephosphorylation, we examined Pah1p PAP activity in

Figure 10. Effect of growth phase on Pah1p PAP activity. *A*, the growth of wild type (*WT*), *dpp1*Δ *lpp1*Δ, *pah1*Δ *dpp1*Δ *lpp1*Δ, and *app1*Δ *dpp1*Δ *lpp1*Δ mutant cells grown in synthetic media was monitored by recording their optical density at 600 nm ($A_{600\ nm}$) at the indicated times. Each *data point* represents the average of triplicate absorbance determinations ± S.D. (*error bars*). *B*, PAP activity from cells harvested at the indicated time of growth. Each *data point* represents the average of triplicate enzyme determinations from two independent experiments ± S.D. (*error bars*).

A


4
3
2
1
0
$A_{600\ nm}$
0
8
16
24
32
40
Time, h
WT
dpp1Δ lpp1Δ
pah1Δ dpp1Δ lpp1Δ
app1Δ dpp1Δ lpp1Δ

B


4
3
2
1
0
PAP Activity,
nmol/min/mg
0
8
16
24
32
40
Time, h
WT
dpp1Δ lpp1Δ
pah1Δ dpp1Δ lpp1Δ
app1Δ dpp1Δ lpp1Δ

cytosolic and membrane fractions at different times in the growth phase. Wild type and *dpp1*Δ *lpp1*Δ mutant cells were grown to the indicated time points and the PAP activity of cell extracts, cytosol, and membrane fractions was measured (Fig. 11). As discussed above, the Pah1p-dependent PAP activity of extracts derived from stationary phase cells was 4.4-fold higher than that of exponential cells. Moreover, the Pah1p PAP activity of membrane fractions derived from exponential and stationary phase cells was 10- and 5.4-fold higher than that of cytosolic fractions, respectively, pointing to a translocation of Pah1p PAP activity as cells progress throughout growth.

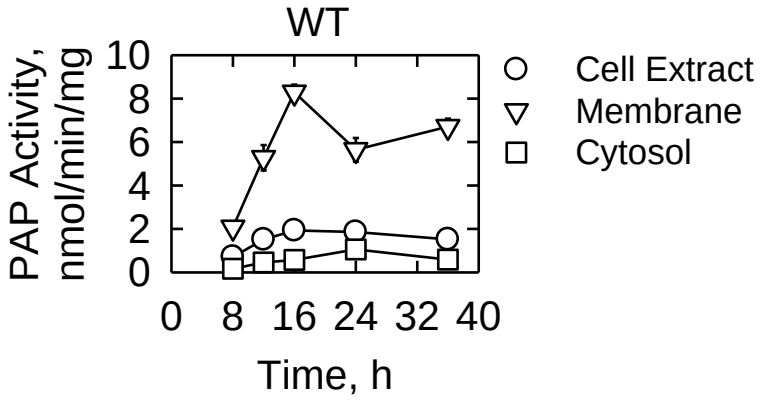
Growth phase-mediated regulation of *PAH1* expression

Given the importance of TAG synthesis throughout growth and the role that Pah1p PAP activity plays in this process, we examined whether this enzyme was regulated by a transcriptional mechanism.

The effect of growth phase on *PAH1* expression was examined by the use of a P_{PAH1}-*lacZ* reporter gene in plasmid pFP1, which was constructed by fusing the *PAH1* promoter in frame with the coding sequence of the *E. coli lacZ* gene (Fig. 12). Thus, the expression of β-galactosidase activity is driven by the *PAH1* promoter, serving as an indicator of *PAH1* expression. The β-galactosidase activity in cell extracts derived from wild type cells bearing the P_{PAH1}-*lacZ* reporter gene was linear with time and protein concentration (data not shown), and increased steadily as cells progressed throughout growth (Fig. 12*A*). Expression of *PAH1* as determined by this reporter assay was 12-fold higher in stationary phase cells compared to exponentially growing cells.

Control experiments were performed to exclude the possibility that the results

Figure 11. Effect of growth phase on the translocation of Pah1p PAP activity. Wild type (*WT*) and *dpp1*Δ *lpp1*Δ mutant cells were harvested at the indicated time of growth. Cell extracts were prepared and separated into cytosol and membrane fractions by centrifugation, and the PAP activity of each subcellular fraction was assayed. Each *data point* represents the average of triplicate enzyme determinations from two independent experiments ± S.D. (*error bars*).


WT
PAP Activity, nmol/min/mg
10
8
6
4
2
0
0 8 16 24 32 40
Time, h
Cell Extract
Membrane
Cytosol

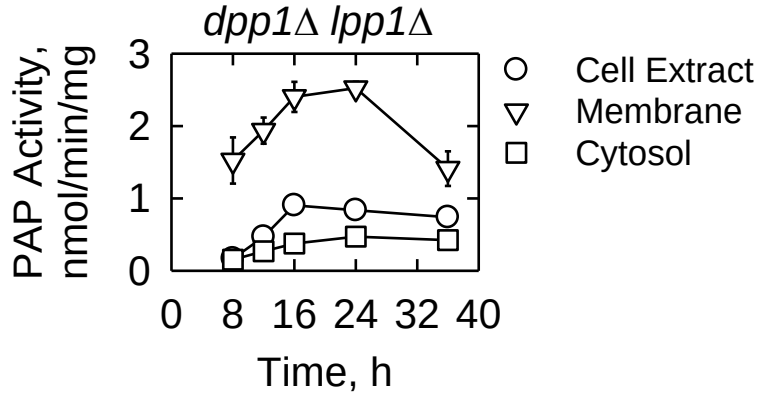
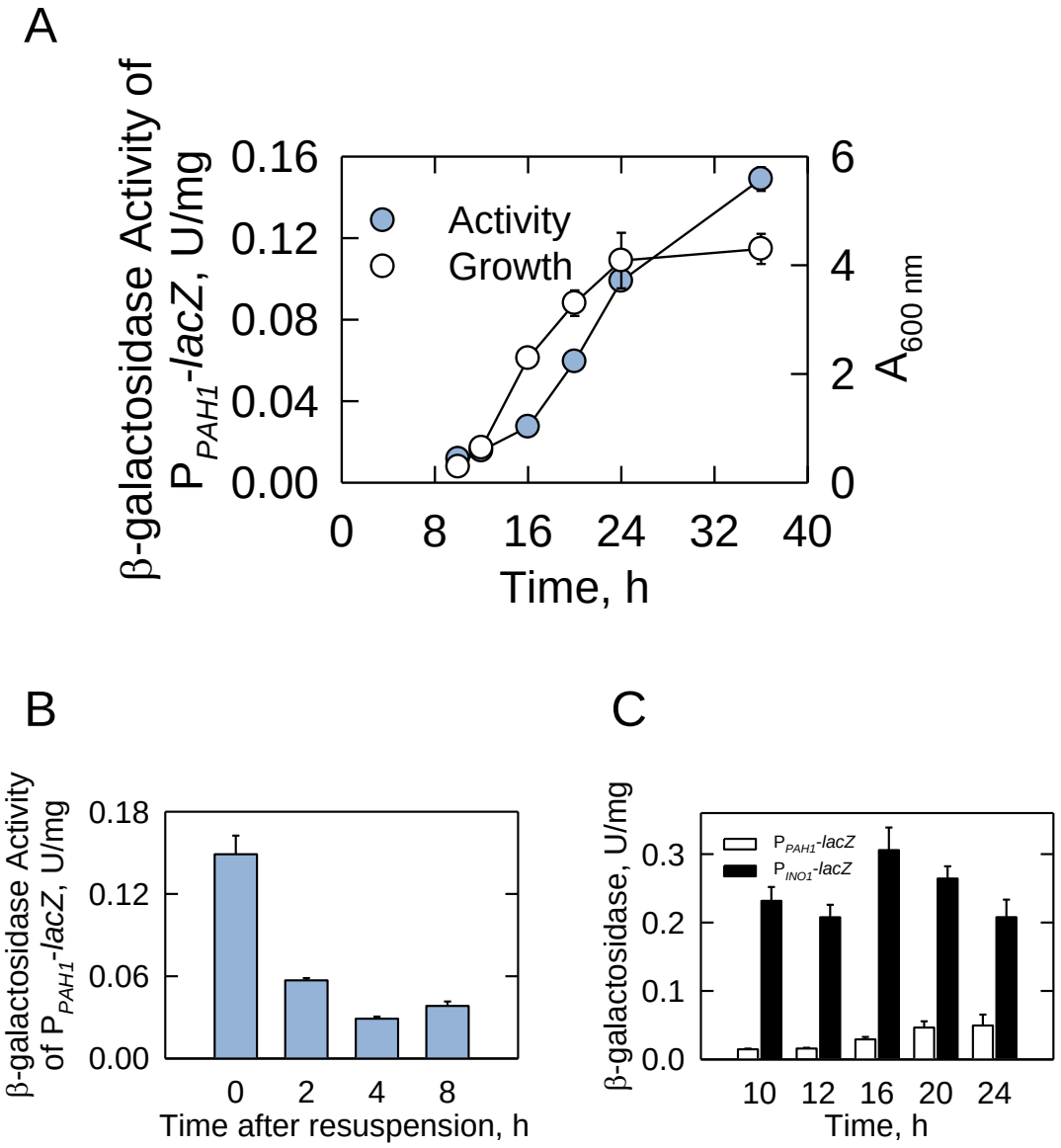

dpp1Δ lpp1Δ
PAP Activity, nmol/min/mg
3
2
1
0
0 8 16 24 32 40
Time, h
Cell Extract
Membrane
Cytosol

Figure 12. Effect of growth phase on *PAH1* expression. *A,* wild type cells bearing the P_{PAH1}-*lacZ* reporter were harvested at the indicated time points, and cell extracts prepared and assayed for β-galactosidase activity. *B,* wild type cells bearing the P_{PAH1}-*lacZ* reporter plasmid were grown to stationary phase, harvested and resuspended in fresh media for the indicated time intervals, and cell extracts were assayed for β-galactosidase activity. *C,* wild type cells bearing the P_{PAH1}-*lacZ* and P_{INO1}-*lacZ* reporters were grown in parallel in the absence of inositol, harvested at the indicated time points, and cell extracts prepared and assayed for β-galactosidase activity. Each *data point* represents the average of triplicate enzyme determinations from two independent experiments ± S.D. (*error bars*).


A
β-galactosidase Activity of P PAH1 -lacZ, U/mg
0.16
0.12
0.08
0.04
0.00
Activity
Growth
6
4
2
0
A600 nm
0
8
16
24
32
40
Time, h
B
β-galactosidase Activity of P PAH1 -lacZ, U/mg
0.18
0.12
0.06
0.00
0
2
4
8
Time after resuspension, h
C
β-galactosidase, U/mg
P PAH1 -lacZ
P INO1 -lacZ
0.3
0.2
0.1
0.0
10
12
16
20
24
Time, h

observed for *PAH1* expression were an artifact of the reporter assay due to the relative stability of the β-galactosidase protein (Fig. 12*B*). For these studies, wild type cells expressing the P_{PAH1}-*lacZ* reporter gene were harvested at the stationary phase of growth, washed and resuspended in fresh media, and incubated for an additional 2, 4, and 8 h. The β-galactosidase activity at time zero was high, and decreased by 62% and 81% after 2 and 4 h of incubation in fresh media, respectively, while cells incubated for 8 h showed a trend towards increasing β-galactosidase activity. These results confirm that the growth phase-mediated induction of *PAH1* expression observed in the reporter assay is not due to an accumulation of a stable *lacZ*-fusion protein.

To further corroborate these results, P_{INO1}-*lacZ* expression was used as a control for our experimental conditions (Fig. 12*C*). The expression of *INO1* has been shown to be relatively constant until 24 h of growth, and to be repressed by inositol supplementation (12, 179). Thus, wild type cells expressing the P_{INO1}-*lacZ* and P_{PAH1}-*lacZ* reporter plasmids were grown in parallel in the absence of inositol and harvested at various time intervals during growth. *INO1* expression was relatively stable throughout growth while that of *PAH1* doubled, confirming that the growth phase-mediated induction in expression is specific to *PAH1*. Moreover, the absence of inositol resulted in 2.0-fold lower β-galactosidase activity levels in stationary phase cells (Fig. 12, *A* vs *C*), suggesting inositol supplementation may have an effect on *PAH1* expression.

Effect of inositol supplementation on the growth phase-mediated regulation of *PAH1* expression

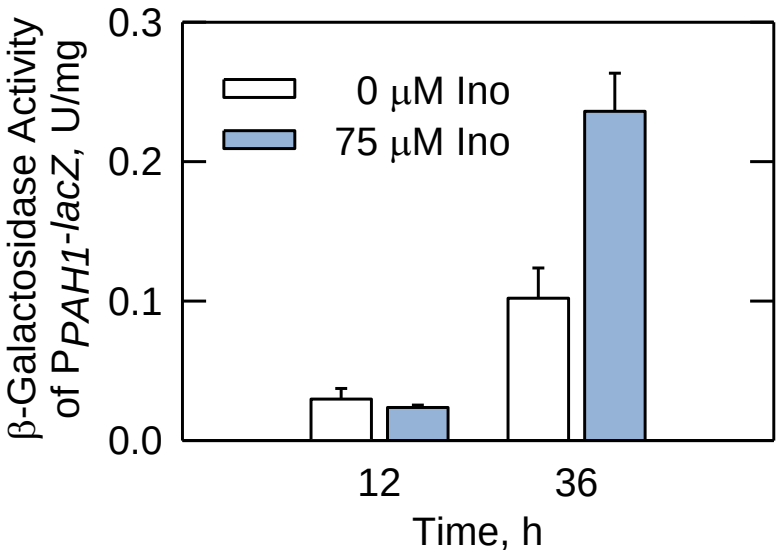
Early studies on the regulation of Pah1p PAP by Morlock et al. showed that

addition of inositol to the growth medium results in a 2-fold increase in Pah1p PAP activity, and that this increase is not due to a direct effect of inositol on enzymatic activity but rather to an inositol-mediated regulation of Pah1p at the genetic level (124, 287). In this work we have shown that the increase in Mg^{2+}-dependent PAP activity in stationary phase cells described in early studies can be attributed exclusively to the Pah1p enzyme. Moreover, the results from the reporter assays described in the above sections indicated that *PAH1* expression is also induced in response to growth phase, and suggested that supplementation with inositol may further increase this induction. Thus, we set out to examine whether the growth phase-mediated regulation of *PAH1* expression is affected by inositol supplementation.

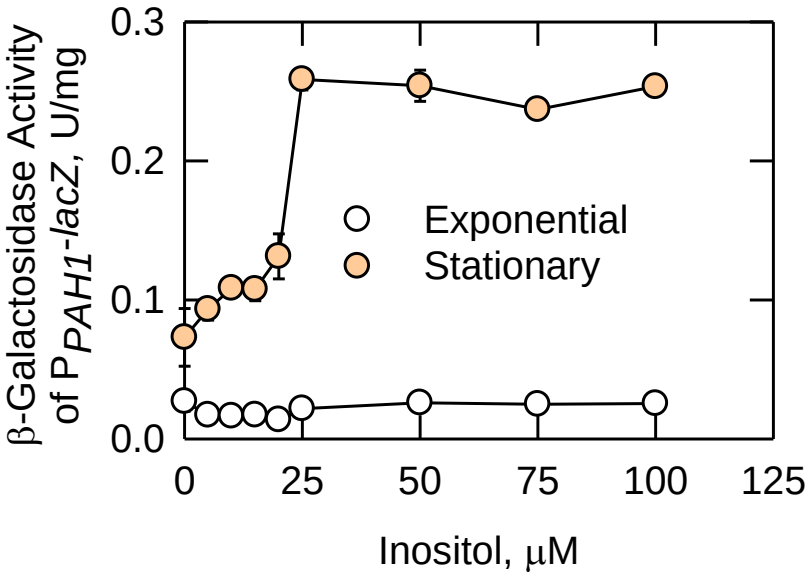
The effect of inositol supplementation on *PAH1* expression was examined using the P_{PAH1}-*lacZ* reporter construct described above (Fig. 13*A*). Wild type cells expressing the reporter plasmid were grown in the absence or presence (75 μM) of inositol and harvested in exponential and stationary phases of growth. As observed previously, *PAH1* expression was induced in response to growth phase. While inositol supplementation did not affect the expression of *PAH1* in the exponential phase of growth, it resulted in a 2.3-fold increase in β-galactosidase activity in stationary phase cells. In the absence of inositol, the β-galactosidase activity driven by the reporter gene was 3.4-fold higher in stationary phase cells when compared with exponential phase cells, whereas inositol supplementation resulted in a 9.7-fold induction in β-galactosidase activity in stationary phase. To study this effect in greater detail, wild type cells bearing the reporter gene were grown in the presence of increasing concentrations of inositol and harvested in exponential and stationary phases of growth (Fig. 13*B*). As expected, inositol

Figure 13. Effect of inositol on the regulation of *PAH1* expression. *A*, wild type cells bearing the P_{PAH1}-*lacZ* reporter were grown in the absence (*0 μM Ino*) and presence (*75 μM Ino*) of inositol, harvested in exponential (*12 h*) and stationary (*36 h*) phases, and cell extracts prepared and used for the β-galactosidase activity assay. *B*, wild type cells bearing the P_{PAH1}-*lacZ* reporter were grown in the indicated concentrations of inositol, harvested in exponential and stationary phases, and cell extracts were used for the β-galactosidase activity assay. Each *data point* represents the average of triplicate enzyme determinations from two independent experiments ± S.D. (*error bars*).

A


β-Galactosidase Activity
of P*PAH1-lacZ*, U/mg
0.3
0.2
0.1
0.0
0 μM Ino
75 μM Ino
12
36
Time, h

B


β-Galactosidase Activity
of P*PAH1-lacZ*, U/mg
0.3
0.2
0.1
0.0
Exponential
Stationary
0
25
50
75
100
125
Inositol, μM

supplementation did not have an effect on *PAH1* expression in exponential phase. In stationary phase cells, however, there was a 2.7-fold increase in *PAH1* expression in the absence of inositol compared to exponential phase cells, and supplementation with increasing concentrations of inositol resulted in a dose-dependent increase in *PAH1* induction, which peaked at 25 μM inositol. Taken together, these results support the conclusion that inositol supplementation has an additive effect on the growth-mediated induction of *PAH1* expression.

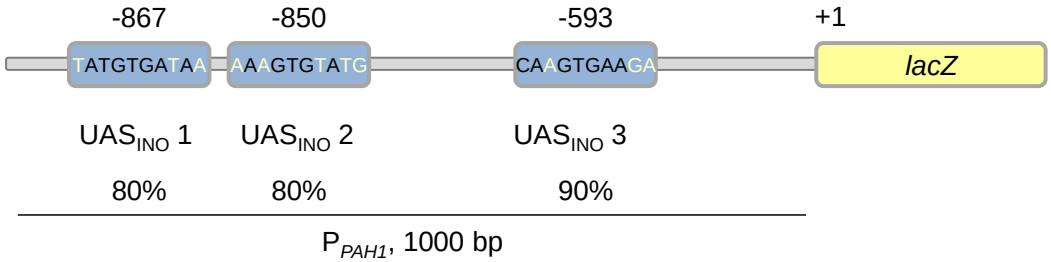
Regulation of *PAH1* by inositol and growth phase in mutants defective in the *INO2, INO4,* and *OPI1* regulatory genes

The *PAH1* promoter contains three putative UAS_{INO} elements of varying similarity to the consensus sequence (Fig. 14*A*), suggesting the *PAH1* gene may undergo transcriptional regulation in response to inositol via a PA-mediated mechanism involving the transcriptional regulators Ino2p, Ino4p, and Opi1p (166, 171, 173, 174) (Fig. 6). Therefore, we analyzed the effects of the *ino2*Δ, *ino4*Δ and *opi1*Δ mutations on *PAH1* expression.

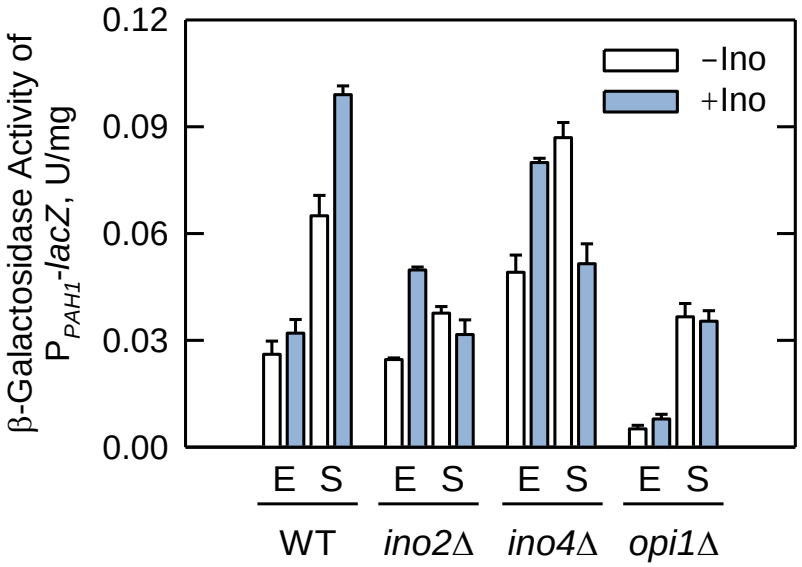
Wild type and mutant cells carrying the P_{PAH1}-*lacZ* reporter plasmid were grown in the absence or presence of inositol, harvested at exponential and stationary phases of growth, and assayed for β-galactosidase activity (Fig. 14*B*). For cells grown to exponential phase in the absence of inositol, the expression of P_{PAH1}-*lacZ* driven β-galactosidase activity was 5.1-fold lower in *opi1*Δ mutant cells when compared with their wild type counterparts. Moreover, *PAH1* expression was not affected by inositol supplementation in either the exponential or stationary phases of growth in this mutant.

Figure 14. Effect of *ino2*Δ, *ino4*Δ and *opi1*Δ mutations on the inositol- and growth phase-mediated regulation of *PAH1* expression. *A*, the *PAH1* promoter has three putative UAS_{INO} elements which share 80-90% homology with the consensus sequence 5'-CATGTGAAAT-3'. Mutations in the consensus sequence are highlighted in *white*. *B*, wild type, *ino2*Δ, *ino4*Δ and *opi1*Δ mutant cells bearing the P_{PAH1}-*lacZ* reporter were grown in the absence (-*Ino*) and presence (+*Ino*, 75 μM) of inositol and harvested in exponential (*E*) and stationary (*S*) phases of growth. Cell extracts were prepared and assayed for β-galactosidase activity. Each *data point* represents the average of triplicate enzyme determinations from two independent experiments ± S.D. (*error bars*).

A


-867
-850
-593
+1
TATGTGATAA
AAAGTGTATG
CAAGTGAAGA
lacZ
UAS$_{INO}$ 1
UAS$_{INO}$ 2
UAS$_{INO}$ 3
80%
80%
90%
P$_{PAH1}$, 1000 bp

B


β-Galactosidase Activity of P$_{PAH1}$-*lacZ*, U/mg
0.12
0.09
0.06
0.03
0.00
−Ino
+Ino
E S
E S
E S
E S
WT
*ino2*Δ
*ino4*Δ
*opi1*Δ

Cells lacking Ino2p and Ino4p are inositol auxotrophs (260), and thus were grown in the presence of 10 μM and 75 μM inositol (186). The 10 μM concentration of inositol is analogous to the growth condition of wild type cells grown in the absence of inositol (288). Under inositol deprivation conditions, the expression of β-galactosidase activity in exponential *ino2*Δ mutant cells was comparable with that of the wild type, whereas it was 1.9-fold higher in exponential *ino4*Δ mutant cells compared with wild type cells. Moreover, in contrast with wild type cells, the *ino2*Δ and *ino4*Δ mutations resulted in significant increases in β-galactosidase activity upon inositol supplementation in exponential phase. While there was no effect of inositol on *PAH1* expression in *ino2*Δ stationary phase cells, addition of inositol resulted in a 2.8-fold decrease in β-galactosidase activity levels in stationary phase *ino4*Δ mutant cells. Finally, *PAH1* expression was induced in stationary phase in the absence of inositol in all three mutant strains examined, consistent with a growth phase-mediated regulation that is independent of inositol. These results are not consistent with the inositol-mediated regulation of UAS_{INO}-containing genes, which are repressed upon inositol supplementation through a PA-mediated mechanism (166, 171, 173, 174) (Fig. 6). Moreover, the expression of the phospholipid biosynthetic enzymes regulated in this manner is not affected by inositol supplementation in *opi1*Δ, *ino2*Δ, and *ino4*Δ mutant cells, and while mutants defective in Opi1p exhibit increased expression of the inositol-regulated phospholipid biosynthetic enzymes, they are repressed in *ino2*Δ and *ino4*Δ mutant strains (2, 10, 12, 289).

Closer inspection of the putative UAS_{INO} elements in the *PAH1* promoter revealed the presence of mutations in the consensus sequence 5'-CATGTGAAAT-3' (Fig. 14*A*) which have been shown to result in dramatic reductions or even obliteration of UAS_{INO}

function (290), and thus might explain the unexpected effects of the *opi1*Δ, *ino2*Δ, and *ino4*Δ mutations on *PAH1* expression. For instance, mutations containing a T or an A as the first base in the 5′ position of the 10-bp element (*i.e.* 5′-(T/A)ATGTGAAAT-3′, such as those found on UAS_{INO} 1 and 2 in the *PAH1* promoter) cause ~80%-90% decrease in UAS_{INO} function, and base substitutions containing an A at position three (*i.e.* 5′-CAAGTGAAAT-3′, UAS_{INO} 3) result in > 98% decrease in UAS_{INO} activity (290).

Regulation of *PAH1* expression by Gis1p and Rph1p transcription factors

There are other examples of lipid synthesis genes whose expression is regulated by inositol or growth phase in a manner similar to that observed for *PAH1*. In particular, the expression of *DPP1* has been shown to be induced by inositol and in the stationary phase of growth (186), and to be controlled by the transcription factor Gis1p and to a lesser extent Rph1p, which act as repressors of its transcription (281). Gis1p can function as both a transcriptional repressor and activator, and is maximally expressed as cells progress into stationary phase (281, 291). Rph1p shares 35% similarity with Gis1p, but the zinc finger regions of Gis1p and Rph1p that interact with DNA are 93% identical, suggesting the two transcription factors recognize identical DNA sequences (265, 281). Moreover, the consensus binding site for Rph1p (5'-AGGGG-3') is nearly identical to the core (5'-AGGGA-3') of the Gis1p-binding site (281).

Inspection of the *PAH1* promoter revealed nine putative binding sites for the transcription factor Gis1p and six for Rph1p, all of which share 75% similarity with their respective consensus sequences. To analyze the possible involvement of Gis1p and Rph1p in the regulation of *PAH1* expression, *gis1*Δ and *rph1*Δ mutant cells bearing the

P_{PAH1}-*lacZ* reporter gene were grown to the exponential and stationary phases of growth and cell extracts assayed for β-galactosidase activity (Fig. 15). The lack of Gis1p and Rph1p did not affect *PAH1* expression in exponential phase cells, but the inositol and growth-phase mediated induction in reporter gene activity observed in wild type stationary phase cells was abolished, suggesting that Gis1p and Rph1p are involved in the regulation of *PAH1* expression as transcriptional activators.

Expression of P_{PAH1}-*lacZ* reporter genes with a series of promoter deletions

To identify the region in the *PAH1* promoter involved in its transcriptional regulation by inositol and growth phase, we examined the β-galactosidase activity resulting from a series of P_{PAH1}-*lacZ* reporter genes with deletions from the 5′ end of the *PAH1* promoter (Fig. 16*A*). Wild type cells bearing the reporter plasmids were grown to the exponential and stationary phases of growth in the absence and presence of 75 μM inositol, and cell extracts were assayed for β-galactosidase activity (Fig. 16*B*). As described above, the expression of *PAH1* was greater in stationary phase cells when compared with exponential phase cells, and further induced upon inositol supplementation in stationary phase. Deletion of the promoter to -800 or -500 resulted in the loss of the inositol-mediated induction in *PAH1* expression in stationary phase cells, suggesting the existence of an inositol-sensitive regulatory element between positions -1000 and -800. Cells expressing reporter genes with promoter deletions to -300 and -200 showed a ~1.5-fold increase in *PAH1* expression in exponential phase with respect to the wild type promoter, and this induction was exacerbated in stationary phase when the same regions of the promoter were deleted. In the absence of inositol, stationary phase

Figure 15. Effect of *gis1*Δ and *rph1*Δ mutations on the inositol- and growth phase-mediated regulation of *PAH1* expression. Wild type, *gis1*Δ and *rph1*Δ mutant cells bearing the P_{PAH1}-*lacZ* reporter were grown in the absence (-*Ino*) and presence (+*Ino*, 75 μM) of inositol and harvested in exponential (*E*) and stationary (*S*) phases of growth. Cell extracts were prepared and assayed for β-galactosidase activity. Each *data point* represents the average of triplicate enzyme determinations from two independent experiments ± S.D. (*error bars*).

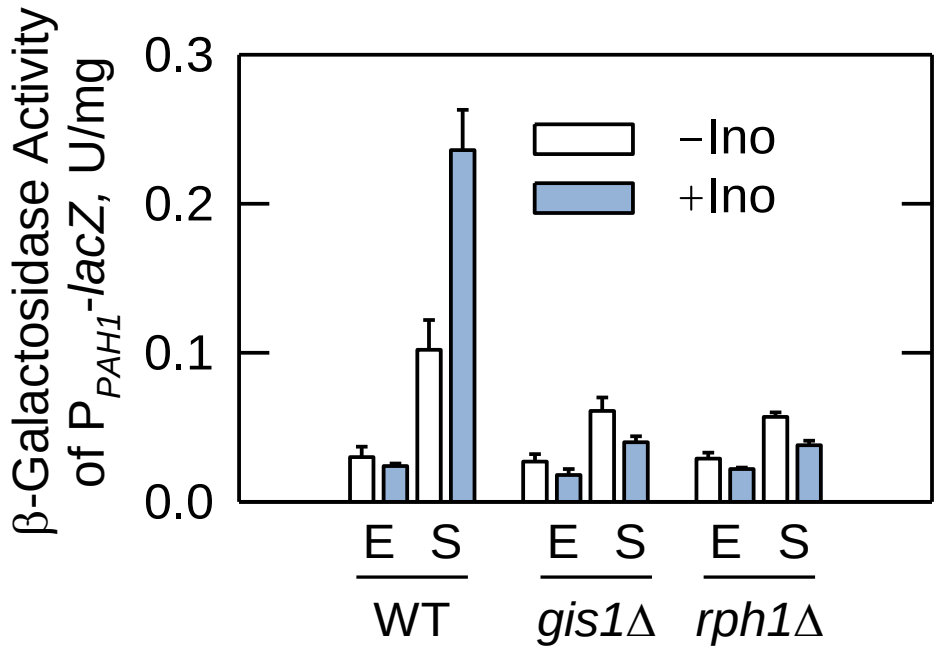

β-Galactosidase Activity
of P PAH1 -lacZ, U/mg
0.3
0.2
0.1
0.0
−Ino
+Ino
E S E S E S
WT
gis1Δ
rph1Δ

Figure 16. *PAH1* expression in P_{PAH1}-*lacZ* truncation mutants. *A*, the indicated P_{PAH1}-*lacZ* promoter truncation reporter genes were constructed as described under "Experimental Procedures." The putative Gis1p and Rph1p transcription factor binding sites in the *PAH1* promoter are indicated. The full-length promoter construct (*WT*) contains 1000 bases upstream of the ATG start site. The number of bases in each reporter gene construct is denoted at the *left side* of the figure. *B*, wild type cells bearing the indicated promoter truncation constructs were grown in the absence and presence of 75 μM inositol. Cells were harvested in exponential and stationary phases of growth and cell extracts prepared and assayed for β-galactosidase activity. Each *data point* represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. (*error bars*).

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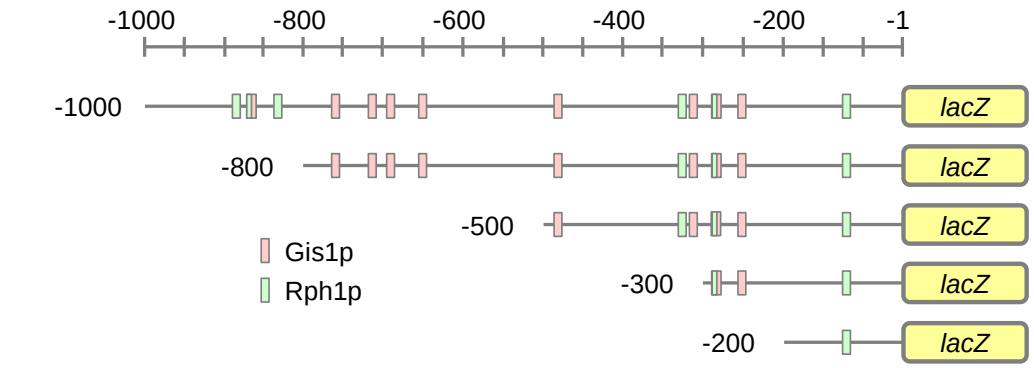
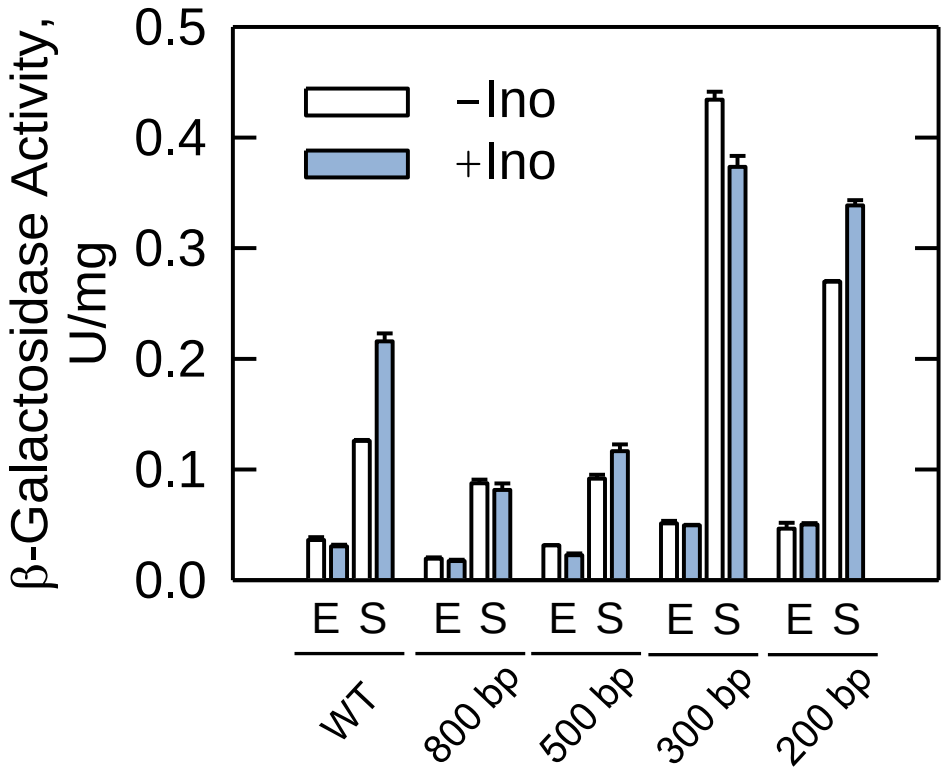
A


-1000
-800
-600
-400
-200
-1
-1000
-800
-500
-300
-200
lacZ
lacZ
lacZ
lacZ
lacZ
Gis1p
Rph1p

B

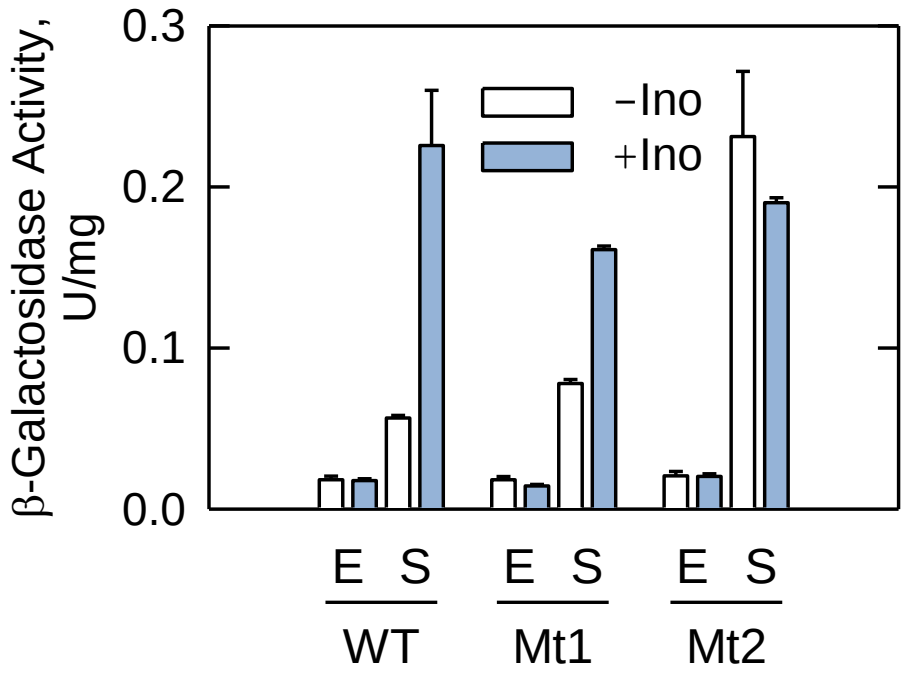

β-Galactosidase Activity, U/mg
0.5
0.4
0.3
0.2
0.1
0.0
−Ino
+Ino
E S
E S
E S
E S
E S
WT
800 bp
500 bp
300 bp
200 bp

cells bearing reporter genes with promoter truncations to -300 and -200 exhibited > 2-fold higher β-galactosidase activity levels compared to cells expressing the wild type promoter, indicating that a negative regulatory element is located between positions -500 to -200. The inositol-mediated induction of *PAH1* expression in stationary phase was also abolished in the -300 and -200 promoter deletion constructs.

Effects of Rph1p/Gis1p binding site mutations on the inositol- and growth phase-mediated regulation of *PAH1* expression

The -1000 to -800 region of the *PAH1* promoter contains two putative Rph1p binding sites at positions -866 (UAS_{PDS} 1, for *u*pstream *a*ctivating *s*equences during *p*ost-*d*iauxic *s*hift) and -847, and a shared Rph1p/Gis1p binding site at position -857 (UAS_{PDS} 2). Additionally, there is a single Gis1p putative binding site at position -488 in the -500 to -300 region of the *PAH1* promoter. We analyzed the role of the UAS_{PDS} 1 and UAS_{PDS} 2 sequences on the inositol- and growth phase-mediated regulation of *PAH1* (Fig. 17). Cells bearing wild type or mutant P_{PAH1}-*lacZ* reporter genes in which the UAS_{PDS} 1 and UAS_{PDS} 2 sequences were mutated to the nonconsensus sequence 5'-AAAAAAAA-3' were grown to exponential and stationary phases of growth in the absence and presence of inositol, and cell extracts prepared and assayed for β-galactosidase activity. The β-galactosidase activities of exponential phase cells expressing P_{PAH1}-*lacZ* with mutations in either UAS_{PDS} 1 or UAS_{PDS} 2 were comparable to that of cells expressing wild type reporter constructs. In stationary phase cells, mutations in UAS_{PDS} 1 resulted in a moderate attenuation of the inositol-mediated *PAH1* induction, in agreement with the results discussed above which pointed to the presence of an inositol-sensitive regulatory

Figure 17. Effects of mutations in the UAS_{PDS} 1 and UAS_{PDS} 2 sequences in the *PAH1* promoter on the expression of the P_{PAH1}-*lacZ* reporter gene in response to inositol and growth phase. Wild type cells bearing the P_{PAH1}-lacZ reporter plasmids pFP1 (*WT*), pFP2 (*Mt1*, mutation of UAS_{PDS} 1), and pFP3 (*Mt2*, mutation of UAS_{PDS} 2) were grown to exponential and stationary phase in the absence and presence of 75 μM inositol, and cell extracts prepared and assayed for β-galactosidase activity. Each *data point* represents the average of triplicate determinations from two independent experiments ± S.D. (*error bars*).


β-Galactosidase Activity, U/mg
0.3
0.2
0.1
0.0
−Ino
+Ino
E S
E S
E S
WT
Mt1
Mt2

element between positions -1000 and -300 (Fig. 16*B*), as well as the results suggesting that Gis1p and Rph1p are transcriptional activators of *PAH1* expression in response to inositol supplementation (Fig. 15). Mutations in UAS_{PDS} 2, however, led to the derepression of *PAH1* expression under inositol starvation conditions, suggesting that Rph1p/Gis1p may act as transcriptional repressors in response to growth phase when bound to this putative site. While these results are in contradiction with the notion that Rph1p and Gis1p are activators of *PAH1* expression, and that there is a positive regulatory element in the -1000 to -800 region of the *PAH1* promoter, there is an additional putative binding site in this region whose effects are yet to be investigated. Moreover, the promoter truncation analyses revealed the presence of additional regulatory elements in the -500 to -300 *PAH1* promoter region, the deletion of which resulted in a significantly stronger and opposite effect. Therefore, we cannot discount the possibility of detractive/additive effects of simultaneous binding of the different transcription factors involved, or even the existence of as yet unidentified transcriptional regulators of *PAH1* expression. These transcriptional studies were not carried out to completion due to conflicting results obtained with mRNA and protein analyses, which are described in the following sections. Given that PAP activity and *PAH1* expression are maximal under conditions of inositol supplementation, subsequent experiments were performed in the presence of inositol.

Regulation of *PAH1* mRNA abundance and stability by growth phase

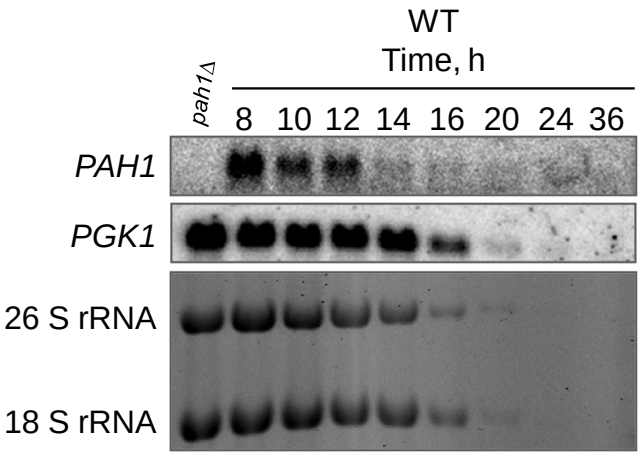
To confirm whether the induction in reporter gene activity correlated with gene expression, *PAH1* mRNA levels were examined. Total RNA was isolated from wild type

cells grown in complete synthetic medium at different times throughout growth and analyzed by Northern blotting (Fig. 18). Given the lipid labeling, PAP activity and reporter assay results, we expected to see an increase in *PAH1* mRNA levels, but found the opposite. *PAH1* mRNA appeared to be stable in exponentially growing cells, but could not be detected in later stages of growth (Fig. 18*A*). Moreover, the mRNA levels of the internal control *PGK1* also decreased in stationary phase, and therefore could not be used to normalize *PAH1* mRNA levels. During the course of these experiments, we also noted that the amount of total RNA derived from stationary phase cells was much lower than that of exponential cells, as evidenced by the decreased levels of rRNA in later time points. Thus, RNA extraction was maximized using a modified hot phenol method (274, 275), and total RNA analyzed by Northern blotting (Fig. 18*B*). This method allowed for the isolation of higher quantities of RNA at every time point, as is shown by the detection of rRNA out to 36 h of growth, and the enhanced detection of *PAH1* and *PGK1* mRNA. Despite these improvements, *PGK1* mRNA levels were still decreased in stationary phase, and thus the level of total rRNA was used as a loading control. The results showed *PAH1* mRNA decreases as cells progress throughout growth.

Taking into account the reporter assay results, which indicated *PAH1* expression was induced in stationary phase, we questioned whether this decrease in *PAH1* mRNA abundance was due to degradation of the transcript. Due to the inability to successfully isolate mRNA from stationary phase cells, we examined *PAH1* mRNA stability at those time points in which the transcript was readily detectable. For this purpose, transcription was arrested with thiolutin (15 μg/ml) (223) at 8, 10, 12, and 14 h of growth, and *PAH1* mRNA levels were analyzed by Northern blot (Fig. 19*A*). The *PAH1* transcript remained

Figure 18. Effect of growth phase on *PAH1* mRNA levels. *A,* total RNA was extracted from wild type cells harvested at the indicated time points, and the abundance of *PAH1* (top) and *PGK1* (loading control, middle) mRNA was determined by Northern blot analysis. The bottom panel depicts the agarose gels prior to transfer, and shows the decrease in rRNA as cells progress through the growth phases. *B,* RNA extraction was maximized using a modified method, and *PAH1* (top) and *PGK1* (loading control, middle) mRNA were analyzed by Northern blotting. The quantities of *PAH1* mRNA and rRNA were quantified using ImageQuant software. Signals were in the linear range of detectability.

A


WT
Time, h
pah1Δ
8 10 12 14 16 20 24 36
PAH1
PGK1
26 S rRNA
18 S rRNA

B

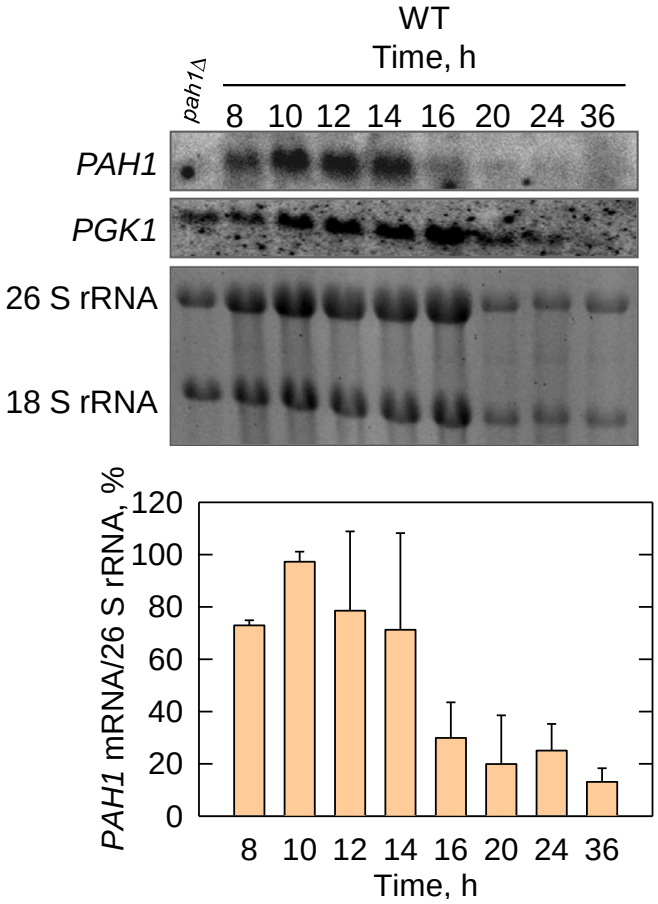
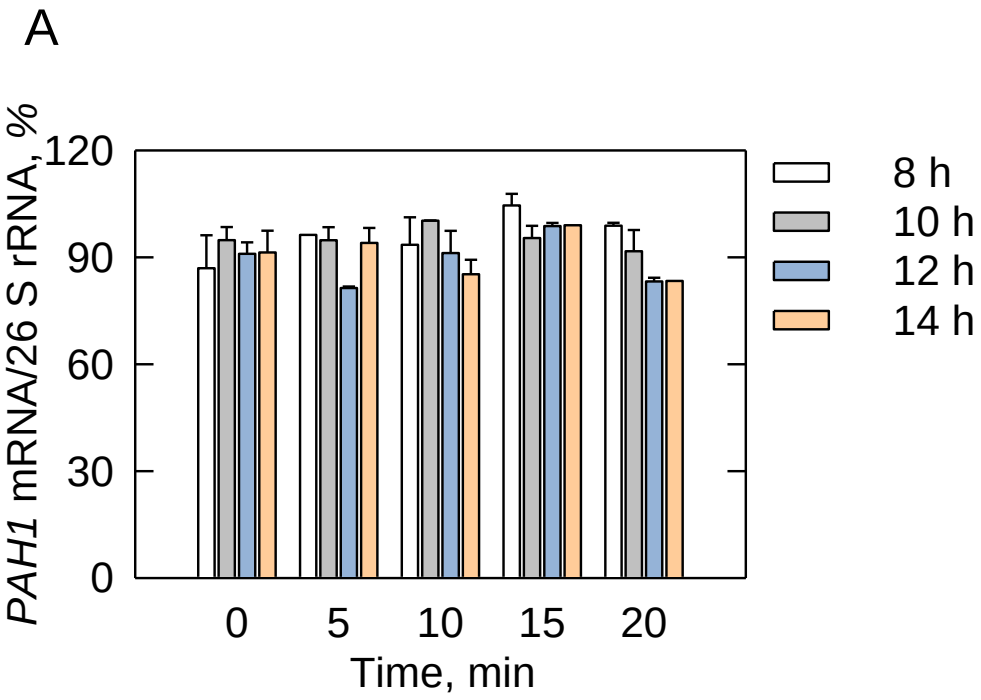
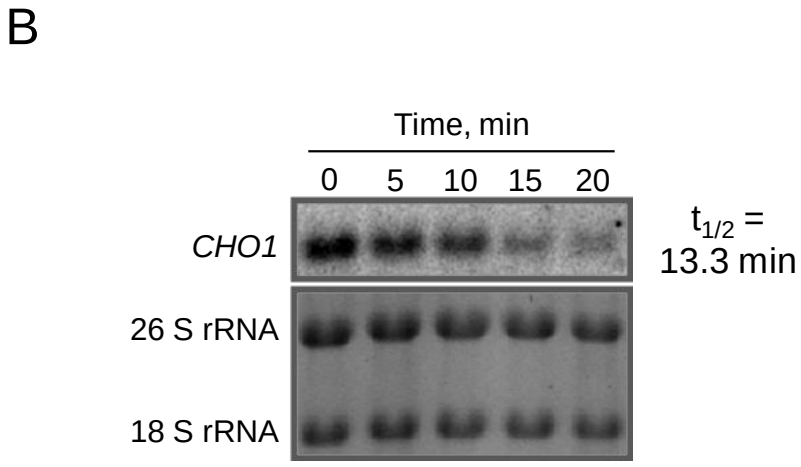

WT
Time, h
pah1Δ
8 10 12 14 16 20 24 36
PAH1
PGK1
26 S rRNA
18 S rRNA
PAH1 mRNA/26 S rRNA, %
120
100
80
60
40
20
0
8 10 12 14 16 20 24 36
Time, h

Figure 19. Effect of growth phase on *PAH1* mRNA stability. *A*, wild type cells were grown to the indicated time points. Following the arrest of transcription, 5-ml samples were taken every 5 min for 20 min, total RNA was extracted, and the levels of *PAH1* mRNA were determined by Northern blot analysis. The relative amounts of *PAH1* mRNA and rRNA were determined by ImageQuant analysis. The figure shows a plot of the relative amount of *PAH1* to rRNA *versus* time. The data shown in the figure are representative of two independent experiments ± S.D. (*error bars*). *B*, *CHO1* mRNA stability was used as a positive control. The level of *CHO1* mRNA derived from the 12 h sample depicted in panel *A* was analyzed by Northern blotting and normalized to rRNA. The half-life of the *CHO1* transcript was calculated from a plot of the log of the relative amount of *CHO1* to rRNA *versus* time.


A
PAH1 mRNA/26 S rRNA, %
120
90
60
30
0
0
5
10
15
20
Time, min
8 h
10 h
12 h
14 h


B
Time, min
0
5
10
15
20
CHO1
26 S rRNA
18 S rRNA
t1/2 =
13.3 min

stable for 20 min after transcription arrest. The stability of *CHO1* mRNA was analyzed as a positive control for our experimental conditions (Fig. 19*B*). The *CHO1* transcript is relatively stable in respiratory sufficient cells, with a half-life of 12 min (226). Northern blot analysis of *CHO1* mRNA in our 12 h sample yielded the expected results. Therefore, due to the limitations of RNA extraction from stationary phase cells, we could only conclude from these experiments that the stability of the *PAH1* transcript is not affected by growth phase at those time points in which mRNA can be detected.

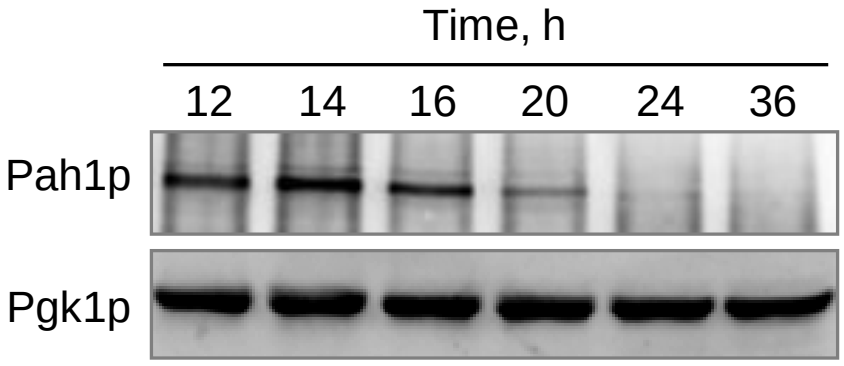
Effect of growth phase on Pah1p abundance and stability

To gain further insight into the mechanism of Pah1p PAP regulation by growth phase, we studied the abundance of Pah1p throughout growth by immunoblot analysis (Fig. 20). Pah1p levels were high in wild type exponential phase cells and decreased dramatically as cells entered stationary phase, while Pgk1p abundance was relatively constant throughout growth (Fig. 20*A*). Moreover, PAP activity in these cells increased during the transition from exponential to stationary phase, as expected (Fig. 20*B*). Surprisingly, Pah1p levels become virtually undetectable at the point of maximum PAP activity and cell growth.

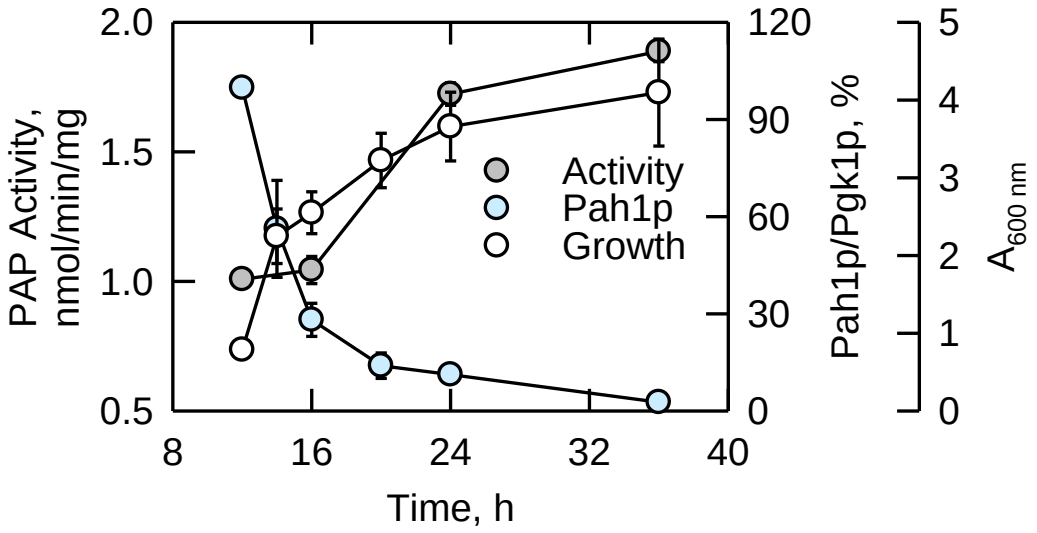
The immunoblot analyses described above were performed using polyclonal antibodies directed against the C-terminal portion of Pah1p (residues 778-794) (107). Studies (G.-S. Han and G.M. Carman, unpublished results) have shown that various C-terminal truncations of Pah1p retain PAP activity; therefore, it is possible that a truncated form of Pah1p may still be present and active in these cells, while escaping detection by the anti-Pah1p antibody used. To exclude this possibility, we analyzed Pah1p levels in

Figure 20. Effect of growth phase on Pah1p abundance. *A*, wild type cells were collected at the indicated times of growth. Cell extracts were prepared and subjected to immunoblot analysis using anti-Pah1p and anti-Pgk1p (loading control) antibodies. *B*, the figure shows a plot of the Pah1p levels, growth curve, and PAP activity of wild type cells used for immunoblot analysis in panel *A*. The levels of Pah1p and Pgk1p were determined by ImageQuant analysis, and the ratio of Pah1p/Pgk1p of the 12 h sample was arbitrarily set as 100% Pah1p. Growth was monitored by recording the optical density ($A_{600\ nm}$) of duplicate cultures at each time point, and the absorbance at 36 h was arbitrarily set at 100% culture saturation. PAP activity was measured in wild type cell extracts as described above. Each *data point* represents the average of two independent experiments ± S.D. (*error bars*).

A


Time, h
12
14
16
20
24
36
Pah1p
Pgk1p

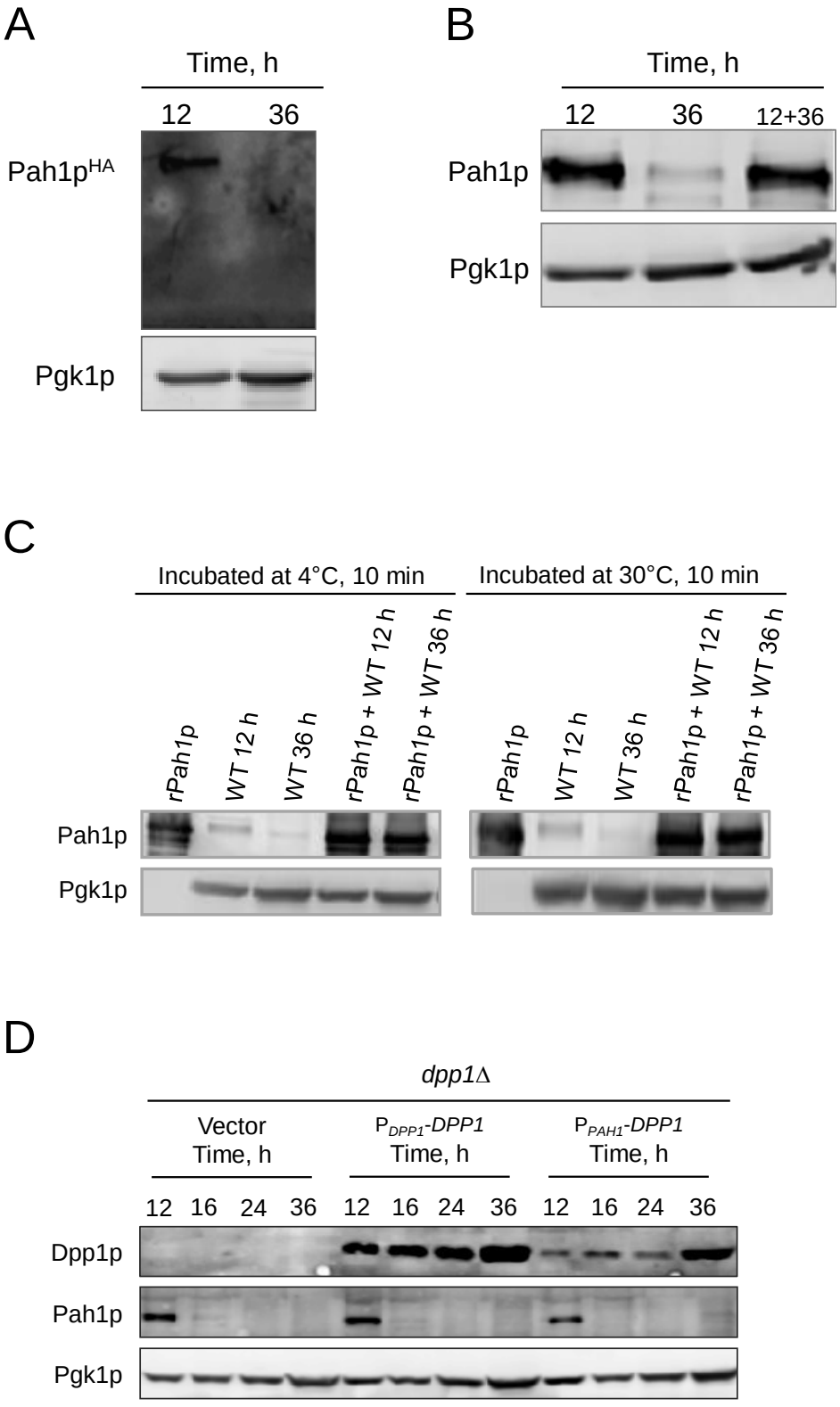
B


PAP Activity, nmol/min/mg
2.0
1.5
1.0
0.5
120
90
60
30
0
Pah1p/Pgk1p, %
5
4
3
2
1
0
A600 nm
Activity
Pah1p
Growth
8
16
24
32
40
Time, h

*pah1*Δ mutant cells bearing plasmid pGH312, which directs the expression of a functional N-terminal HA-tagged Pah1p (78). Cells were grown to exponential (12 h) and stationary (36 h) phases of growth, and cell extracts prepared and subjected to immunoblot analysis (Fig. 21*A*). The anti-HA antibodies detected Pah1p in cell extracts from exponential phase cells, but failed to do so in those derived from cells in stationary phase. We also considered the possibility that Pah1p could be degraded as a consequence of sample preparation for immunoblot analysis. The higher concentration of proteases in stationary phase (214, 292) coupled with the high temperature used to denature the proteins for SDS-PAGE could lead to increased degradation of Pah1p in extracts from stationary phase cells. To address this issue, we devised a simple experiment in which equal amounts (μg of protein) of exponential and stationary phase extracts were mixed prior to immunoblotting (Fig. 21*B*). As observed previously, Pah1p was present in exponential phase extracts and absent from those prepared from stationary phase cells, while the Pah1p signal detected in the mixed sample was equal in intensity to that of exponential phase extracts. In complementary experiments, recombinant Pah1p purified from *E. coli* was not degraded upon mixing with either exponential or stationary phase cell extracts, regardless of whether the samples were incubated at 4 °C or 30 °C prior to immunoblotting (Fig. 21*C*). Taken together, these results confirm that the observed decrease in Pah1p levels is growth phase-mediated and is not an artifact of our experimental conditions.

While the decrease in Pah1p abundance in later stages of growth appears to be correlated with a reduction in *PAH1* mRNA levels, the P_{PAH1}-*lacZ* reporter assay results indicated *PAH1* expression is induced as cells progress throughout growth.

Figure 21. The decrease in Pah1p abundance is growth phase-mediated and Pah1p-specific. *A*, *pah1*Δ cells expressing HA-tagged Pah1p were grown to the exponential (*12 h*) and stationary (*36 h*) phases of growth, and cell extracts prepared and subjected to immunoblot analysis with anti-HA and anti-Pgk1p (loading control) antibodies. *B*, wild type exponential and stationary phase cell extracts were mixed in equal proportions and subjected to immunoblot analysis using anti-Pah1p and anti-Pgk1p antibodies. *C*, recombinant Pah1p (*rPah1p*) was mixed with wild type (*WT*) exponential (*12 h*) and stationary (*36 h*) phase cell extracts in equal proportions, incubated at either 4 °C or 30 °C for 10 min, and subjected to immunoblot analysis using anti-Pah1p and anti-Pgk1p antibodies. *D*, *dpp1*Δ cells expressing plasmids pRS415 (*vector*), pGH201 (P_{DPP1}-*DPP1*), and pGH339 (P_{PAH1}-*DPP1*) were grown to the indicated time points and cell extracts subjected to immunoblot analysis using anti-Pah1p, anti-Dpp1p, and anti-Pgk1p (loading control) antibodies.


A
Time, h
12
36
Pah1p^HA
Pgk1p
B
Time, h
12
36
12+36
Pah1p
Pgk1p
C
Incubated at 4°C, 10 min
Incubated at 30°C, 10 min
rPah1p
WT 12 h
WT 36 h
rPah1p + WT 12 h
rPah1p + WT 36 h
Pah1p
Pgk1p
D
dpp1Δ
Vector
Time, h
P_DPP1-DPP1
Time, h
P_PAH1-DPP1
Time, h
12 16 24 36 12 16 24 36 12 16 24 36
Dpp1p
Pah1p
Pgk1p

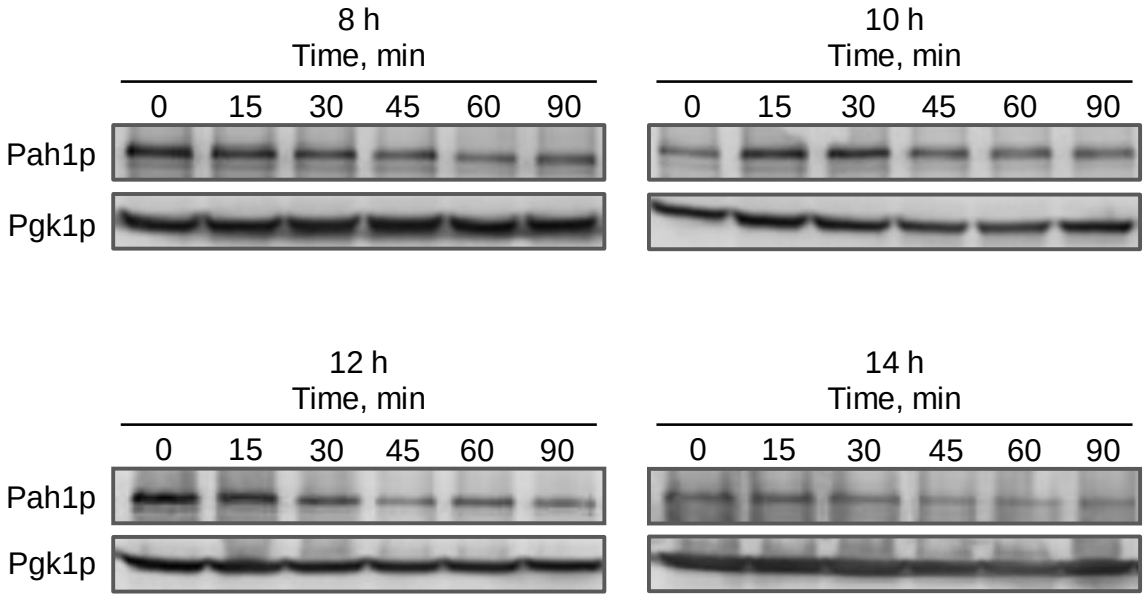
investigate this discrepancy in further detail, we analyzed the levels of Pah1p and Dpp1p in *dpp1*Δ cells bearing plasmid pGH339, which directs the expression of the *DPP1* gene driven by the *PAH1* promoter (Fig. 21*D*). As observed in previous experiments, Pah1p levels declined drastically upon transition into stationary phase, while the expression of Dpp1p driven by its endogenous promoter was greatly induced (186). The abundance of Dpp1p was lower when it was expressed under the *PAH1* promoter, but it was also significantly increased in stationary phase cells. These results confirmed those of the reporter assay, but more importantly, supported the conclusion that the growth phase-mediated regulation of Pah1p abundance occurs at the post-translational level and is Pah1p-specific.

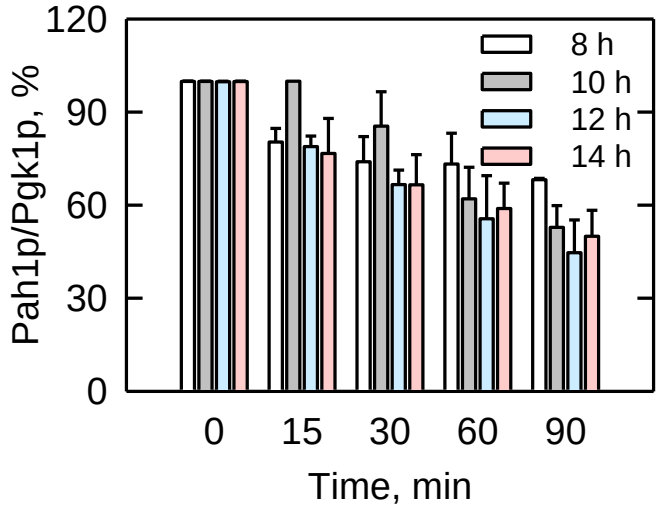
We examined the stability of Pah1p at the different time points in the growth phase in which the protein could be detected by immunoblotting. Wild type cells grown to 8, 10, 12 and 14 h were treated with cycloheximide (100 μg/mL), and Pah1p levels analyzed by Western blot at t = 0, 15, 30, 45, 60, and 90 min after translation arrest (Fig. 22). Despite the expected decrease in Pah1p abundance in later phases of growth, there was only a slight increase in the decay rate ($t_{1/2}$) of Pah1p with respect to growth phase. The half-life of Pah1p in cells grown for 10, 12 and 14 h was 10%, 14%, and 16% shorter, respectively, than that of cells grown for 8 h. These results suggest that the decrease in Pah1p abundance as cells transition into stationary phase occurs in a regulated manner.

Effect of proteasome inhibition on Pah1p abundance

Preliminary studies in our lab (A. Soto-Cardalda and G. M. Carman, unpublished

Figure 22. Effect of growth phase on Pah1p stability. Wild type cells were grown to the indicated time points. Following the arrest of translation, samples were taken at 15, 30, 45, 60, and 90 min. Cell extracts were prepared and analyzed by Western blot with anti-Pah1p and anti-Pgk1p (loading control) antibodies. The levels of Pah1p and Pgk1p were determined by ImageQuant analysis and the ratio of Pah1p/Pgk1p at t = 0 min was arbitrarily set as 100%. The data shown in the figure are representative of two independent experiments. The half-life of Pah1p at the different growth phases was calculated from plots of the log of Pah1p/Pgk1p *versus* time.





8 h, $t_{1/2}$ = 87.0 ± 5.1 min

10 h , $t_{1/2}$ = 78.2 ± 6.8 min

12 h , $t_{1/2}$ = 74.6 ± 5.3 min

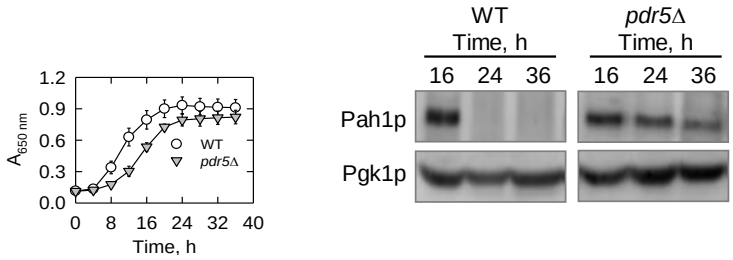
14 h , $t_{1/2}$ = 73.5 ± 3.3 min

results) showed that Pah1p may be a target of programmed proteolysis in the ubiquitin-proteasome pathway. To confirm these results, the abundance of Pah1p was examined in *pdr5*Δ mutant cells grown in the presence of the proteasome inhibitor MG132 (293, 294) (Fig. 23). Mutation of the *PDR5*-encoded multidrug transporter prevents the efflux of MG132, facilitating inhibition of the proteasome degradation pathway (293-295). Pah1p expression appeared to be higher in later phases of growth in the *pdr5*Δ mutant compared to wild type cells (Fig. 23*A*). In spite of this discrepancy among strains, Pah1p was degraded in a growth phase-dependent manner in the absence of MG132 in *pdr5*Δ mutant cells, whereas it was stabilized upon treatment with the inhibitor (Fig. 23*B*). Pah1p abundance decreased by 48% in the control cultures treated with vehicle (DMSO), and only 21% in those exposed to MG132. Studies have suggested that MG132 is not effective for periods of time longer than 4-6 hours in yeast cells (293). To eliminate this possibility, *pdr5*Δ mutant cells were grown to late-exponential phase (16 h) prior to treatment with vehicle or MG132, and Pah1p levels were examined at 0, 4, and 8 h after treatment. As expected, the levels of Pah1p were reduced by ~64% 8 h after treatment with vehicle in control cells, while cells treated with MG132 retained ~95% of the original levels of Pah1p.

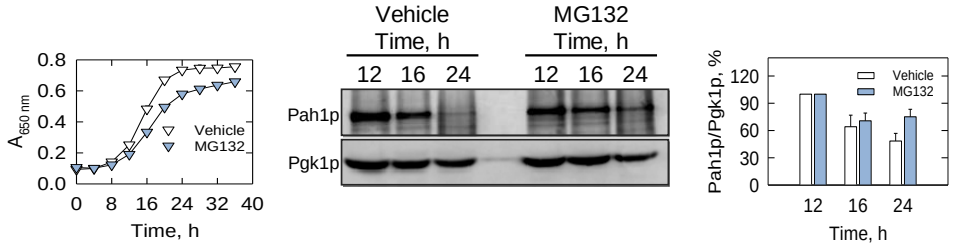
These results were corroborated in various mutants that exhibit a defect in proteasome function, including *rpn4*Δ, *blm10*Δ, *ump1*Δ, and *pre1-1 pre2-1* mutant strains (Fig. 24*A*). Mutation in Rpn4p, a transcription activator required for the upregulation of proteasome genes (296, 297), results in a 50% reduction in proteasome activity compared to wild type cells (298), and showed ~2-fold higher Pah1p levels in both exponential and stationary phases, suggesting Pah1p degradation occurs throughout growth. Moreover,

Figure 23. Pah1p is stabilized in the presence of the proteasome inhibitor MG132. *A*, the growth of wild type (*WT*) and *pdr5*Δ mutant cells was monitored for 36 h at 650 nm using a ThermoMax microplate reader ($A_{650\ nm}$). Each *data point* represents the average of duplicate cultures ± S.D. (*error bars*). Cells were harvested at the indicated times during growth, and Pah1p and Pgk1p (loading control) expression examined by immunoblotting with anti-Pah1p and anti-Pgk1p antibodies, respectively. *B*, the growth of *pdr5*Δ mutant cells treated with *vehicle* (DMSO) or *MG132* upon inoculation was monitored for 36 h at 650 nm using a ThermoMax microplate reader ($A_{650\ nm}$). Cells were collected at the indicated time intervals, and Pah1p and Pgk1p expression examined by immunoblotting. The levels of Pah1p and Pgk1p were determined by ImageQuant analysis and the ratio of Pah1p/Pgk1p at 12 h was arbitrarily set as 100%. The data shown in the figure are representative of two independent experiments ± S.D. (*error bars*). *C*, *pdr5*Δ mutant cells were grown for 16 h prior to treatment with *vehicle* (DMSO) or *MG132*. The effect of treatment on cell growth was monitored by recording the optical density ($A_{600\ nm}$) of the cultures at the indicated time points; the *arrow* indicates the point of treatment. Cells were collected at 0, 4 and 8 h after treatment, and Pah1p and Pgk1p expression examined by immunoblotting. The ratio of Pah1p/Pgk1p at 16 h was arbitrarily set as 100%. The data shown in the figure are representative of two independent experiments ± S.D. (*error bars*).

A


1.2
0.9
0.6
0.3
0.0
$A_{650\ nm}$
WT
pdr5Δ
0 8 16 24 32 40
Time, h
WT
Time, h
16 24 36
pdr5Δ
Time, h
16 24 36
Pah1p
Pgk1p

B


0.8
0.6
0.4
0.2
0.0
$A_{650\ nm}$
Vehicle
MG132
0 8 16 24 32 40
Time, h
Vehicle
Time, h
12 16 24
MG132
Time, h
12 16 24
Pah1p
Pgk1p
Pah1p/Pgk1p, %
120
90
60
30
0
Vehicle
MG132
12 16 24
Time, h

C

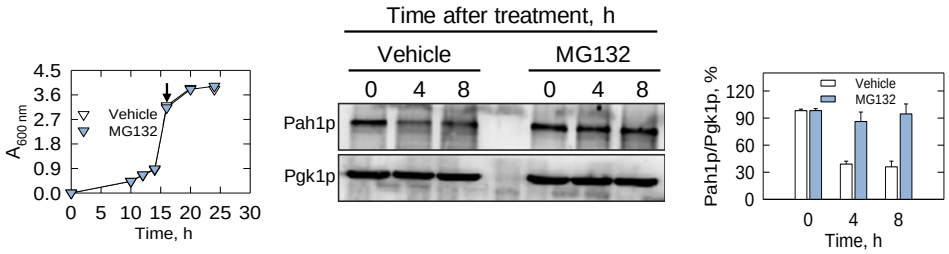
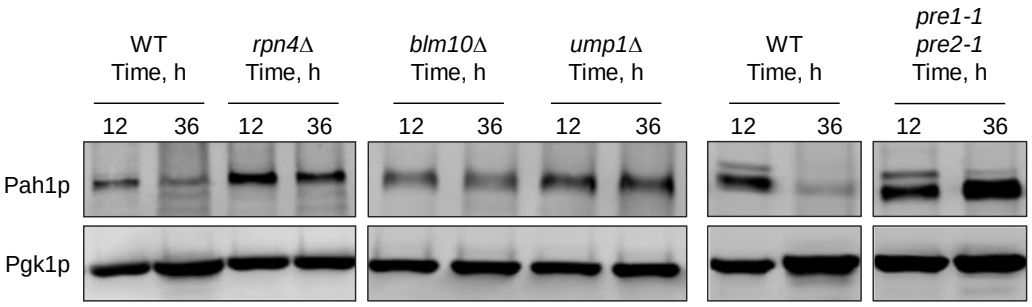
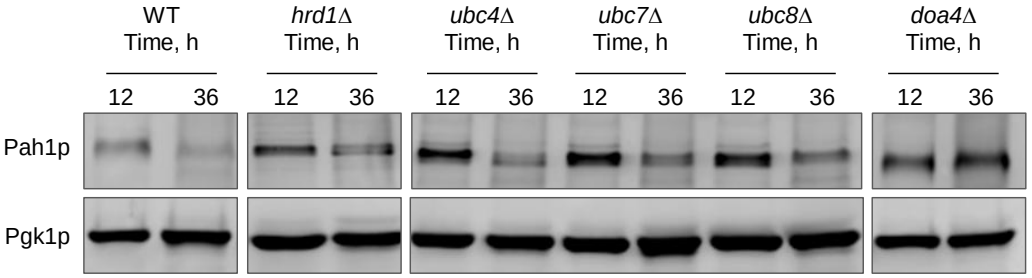

4.5
3.6
2.7
1.8
0.9
0.0
$A_{600\ nm}$
Vehicle
MG132
0 5 10 15 20 25 30
Time, h
Time after treatment, h
Vehicle
0 4 8
MG132
0 4 8
Pah1p
Pgk1p
Pah1p/Pgk1p, %
120
90
60
30
0
Vehicle
MG132
0 4 8
Time, h

Figure 24. Pah1p is stabilized in mutants with impaired proteasome function and ubiquitination, but not in mutants defective in vacuolar degradation. *A*, wild type and *rpn4*Δ, *blm10*Δ, *ump1*Δ, and *pre1-1 pre2-1* mutant cells were collected at the indicated times of growth. Cell extracts were prepared and subjected to immunoblot analysis using anti-Pah1p and anti-Pgk1p (loading control) antibodies. *B*, wild type and *hrd1*Δ, *ubc4*Δ, *ubc7*Δ, *ubc8*Δ and *doa4*Δ mutant cells were grown to the indicated time points, and cell extracts prepared and subjected to immunoblot analysis using anti-Pah1p and anti-Pgk1p (loading control) antibodies. C, wild type and *pep4*Δ mutant cells were harvested at 12 and 36 h of growth and cell extracts prepared and subjected to immunoblot analysis using anti-Pah1p and anti-Pgk1p (loading control) antibodies. The blots shown are representative of three independent experiments.

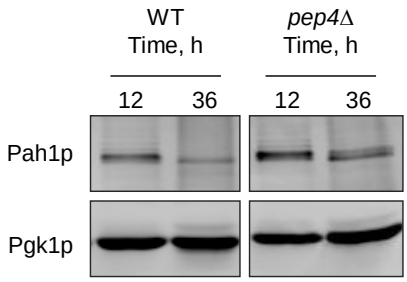
A


WT
Time, h
12
36
rpn4Δ
Time, h
12
36
blm10Δ
Time, h
12
36
ump1Δ
Time, h
12
36
WT
Time, h
12
36
pre1-1
pre2-1
Time, h
12
36
Pah1p
Pgk1p

B


WT
Time, h
12
36
hrd1Δ
Time, h
12
36
ubc4Δ
Time, h
12
36
ubc7Δ
Time, h
12
36
ubc8Δ
Time, h
12
36
doa4Δ
Time, h
12
36
Pah1p
Pgk1p

C


WT
Time, h
12
36
pep4Δ
Time, h
12
36
Pah1p
Pgk1p

Pah1p levels decreased only by ~40% in *rpn4*Δ mutant cells in stationary phase, whereas wild type cells showed an ~80% decrease in Pah1p in stationary phase. Blm10p is an activator of the enzymatic activity of the core particle of the proteasome and is required for its maturation (299-302). While *blm10*Δ mutant cells show modest deficiency in proteasome function (~20%) (300), Pah1p levels were significantly stabilized in these mutants in stationary phase. Cells lacking Ump1p, a maturation factor required for coordination of the proteasome assembly and enzymatic activation, exhibit reduced proteasome activity and a dramatic stabilization of ubiquitinated substrates (298, 303). The abundance of Pah1p was significantly increased in stationary phase cells of *ump1*Δ mutants when compared to wild type cells. Finally, *pre1-1 pre2-1* mutants are defective in the chymotrypsin-like activity of the 20S proteasome, resulting in stabilization of defined proteasomal substrates and accumulation of ubiquitinated proteins (304). These mutants also showed increased Pah1p abundance in stationary phase compared to wild type cells.

To further investigate the role of ubiquitination on Pah1p degradation, we examined Pah1p levels in cells lacking the ubiquitin ligases Hrd1p, Ubc4p, Ubc7p, and Ubc8p, as well as the deubiquitinase Doa4p (Fig. 24*B*). Hrd1p and Ubc7p are ER-resident ubiquitin ligases which function in ER-associated degradation (305-309); Ubc4p is an ubiquitin ligase which resides in the proteasome (310), while Ubc8p resides in the cytosol (311, 312). Pah1p levels were stabilized in these mutants, but to a lesser degree compared to the mutants deficient in proteasome function discussed above, perhaps due to the redundancy of these ligases. A clearer indication of the role of ubiquitination on Pah1p degradation was obtained through the analysis of *doa4*Δ mutants, which due to the

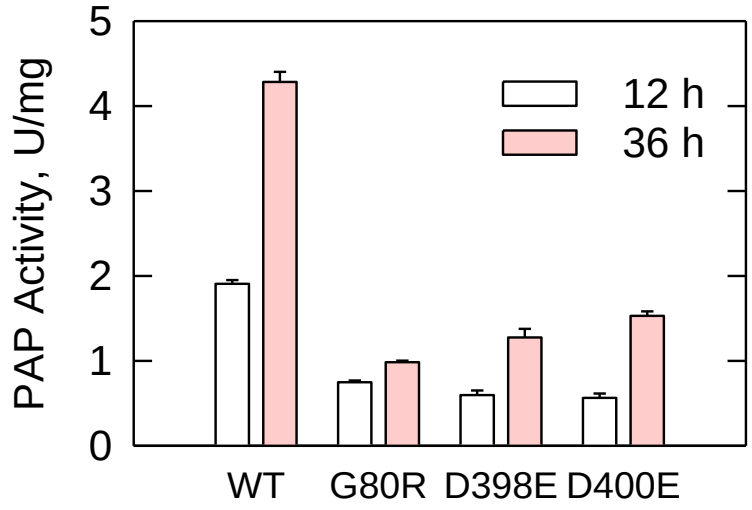
lack of Doa4p, an ubiquitin isopeptidase required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates, are unable to maintain normal levels of ubiquitin (298, 313, 314). Pah1p levels were again stabilized in this mutant in stationary phase, suggesting Pah1p may undergo ubiquitin-dependent proteasomal degradation.

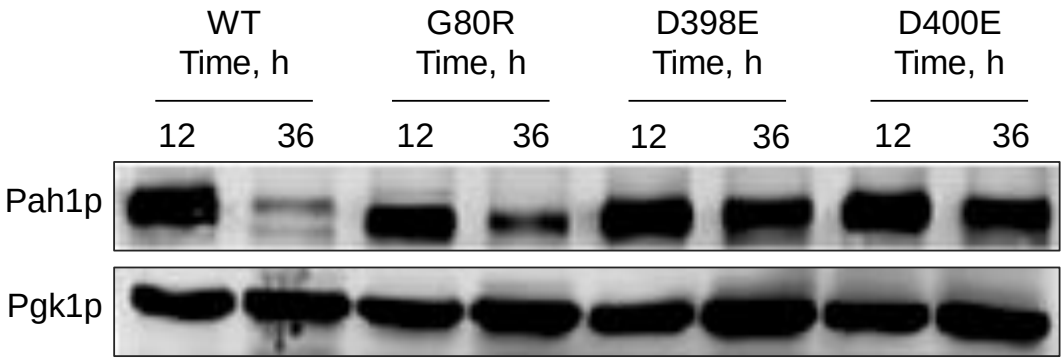
Finally, to assess whether Pah1p is also subject to vacuolar degradation upon its growth phase-mediated induction, the abundance of Pah1p was examined in *pep4*Δ mutants (Fig. 24*C*). Pep4p catalyzes the committed step in vacuolar proteolysis and is required for the maturation of other vacuolar proteases (315, 316). Pah1p levels were stabilized only moderately in stationary phase in the *pep4*Δ mutant compared to wild type cells, suggesting proteolysis of Pah1p in the vacuole is not the major mechanism for its degradation.

Role of PA/DAG in Pah1p degradation

To test whether the alteration in the PA/DAG balance is the signal for Pah1p degradation, we examined Pah1p levels in *pah1*Δ cells expressing catalytically inactive forms of the enzyme (Fig. 25). Mutations in Gly^{80}, Asp^{398} and Asp^{400} to residues that minimize changes in protein structure result in the abrogation of PAP activity (79). As observed in previous studies, *pah1*Δ cells expressing the G80R, D398E and D400E mutant Pah1p enzymes displayed a drastic reduction in PAP activity when compared to cells expressing wild type Pah1p; the residual activity in these cells is attributed to Dpp1p, Lpp1p and App1p. The ablation of Pah1p PAP activity in the G80R, D398E and D400E mutants resulted in a dramatic stabilization of Pah1p levels in stationary phase, suggesting PAP activity and the reduction of PA is essential for Pah1p degradation. We

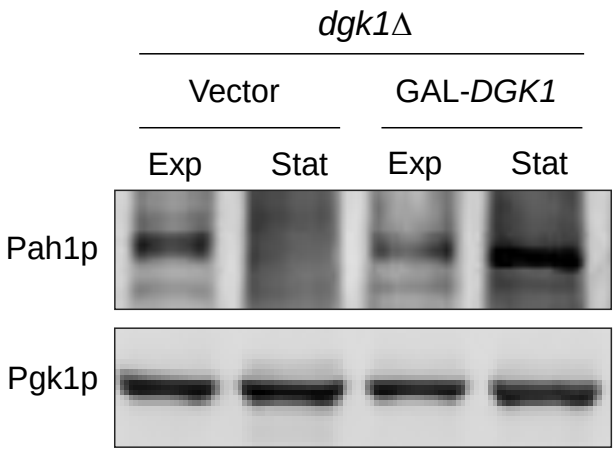
Figure 25. Role of PAP activity in Pah1p degradation. *pah1*Δ cells expressing wild type (*WT*) and catalytic site mutant (*G80R*, *D398E*, and *D400E*) *PAH1* alleles were grown to the indicated growth phase. Cell extracts were prepared and assayed for PAP activity as described above. Each *data point* represents the average of triplicate enzyme determinations from two independent experiments ± S.D. (*error bars*). The expression of the wild type and mutant enzymes was examined by immunoblot analysis using anti-Pah1p and anti-Pgk1p (loading control) antibodies. The blot shown is representative of three independent experiments.


PAP Activity, U/mg
0
1
2
3
4
5
12 h
36 h
WT
G80R
D398E
D400E


WT
Time, h
12
36
G80R
Time, h
12
36
D398E
Time, h
12
36
D400E
Time, h
12
36
Pah1p
Pgk1p

further addressed this question by examining the levels of Pah1p in *dgk1*Δ cells overexpressing *DGK1*, the enzyme whose function counterbalances that of Pah1p by controlling cellular PA levels (Fig. 26). Galactose-inducible overexpression of *DGK1* results in increased production of PA which accumulates on intracellular membranes surrounding the nucleus (87). Immunoblot analysis of *dgk1*Δ cells revealed Pah1p abundance is dramatically increased in cells expressing *GAL-DGK1* compared to cells carrying a vector control, indicating that alterations in the cellular PA/DAG balance regulate Pah1p degradation.

Figure 26. Alterations in the PA/DAG balance affect Pah1p stability. The *dgk1*Δ strain bearing a centromeric plasmid expressing *DGK1* under the control of a galactose-inducible promoter (*GAL-DGK1*) or the empty *vector* control were grown to exponential phase, transferred from raffinose to galactose-containing medium, and harvested immediately after transfer (*Exp*) and 36 h later (*Stat*). Cell extracts were prepared and subjected to immunoblot analysis using anti-Pah1p and anti-Pgk1p (loading control) antibodies. The blot shown is representative of three independent experiments.


*dgk1*Δ
Vector
GAL-*DGK1*
Exp
Stat
Exp
Stat
Pah1p
Pgk1p

DISCUSSION

In this work, we advanced the understanding of the involvement of Pah1p PAP activity on lipid synthesis and its regulation by growth phase, and uncovered a novel mechanism for the modulation of Pah1p PAP function.

Yeast Pah1p PAP catalyzes the penultimate step in the synthesis of TAG, and plays a crucial role in lipid homeostasis by controlling the relative proportions of its substrate PA and its product DAG (70, 78). The cellular amounts of these lipid intermediates influence the synthesis of TAG and the pathways by which membrane phospholipids are synthesized (89-92). Analyses of *pah1*Δ mutant cells have revealed that Pah1p PAP is essential for the synthesis of phospholipids and TAG in exponentially growing cells, and particularly in the stationary phase of growth (70, 78) when the decreased availability of essential nutrients results in a shift in lipid metabolism from phospholipids towards TAG synthesis (122, 164). Moreover, the accumulation of TAG in stationary phase cells has been correlated to an increase in PAP activity as cells progress throughout growth (122). Thus, we hypothesized that a growth phase-mediated induction in Pah1p function is directly responsible for the accumulation of TAG in stationary phase cells.

We investigated whether Pah1p function is regulated by growth phase at the transcriptional level. P_{PAH1}-*lacZ* reporter gene assays indicated that *PAH1* expression increased as cells progress throughout growth. Moreover, the growth phase-mediated increase in *PAH1* expression was further induced by inositol supplementation, in agreement with the early studies by Morlock et al. (124, 287) in which Mg^{2+}-dependent PAP activity was shown to increase upon addition of inositol via an indirect mechanism.

The inositol-mediated regulation of *PAH1* is opposite to that observed for the CDP-DAG pathway enzymes that are repressed upon inositol supplementation and in stationary phase (12, 119), suggesting that *PAH1* expression is not regulated in this manner. The induction of *PAH1* in response to inositol, however, agrees with the model proposed in Fig. 6, and confirms the role of Pah1p PAP in the regulation of other UAS_{INO}-containing phospholipid biosynthetic genes. By this model, supplementation with inositol results in higher expression of *PAH1*, which is translated into higher Pah1p PAP activity and lower PA levels, and thus allows for the release of Opi1p from the ER membrane and its entry into the nucleus for the repression of UAS_{INO}-containing genes (166, 171, 173, 174). The inositol- or growth phase-dependent repression of these genes is mediated by a UAS_{INO} *cis*-acting element in their promoters, which contains the binding site for the Ino2p-Ino4p activating complex, and requires Opi1p (166, 171, 173, 174) (Fig. 6).

The *PAH1* promoter contains three putative UAS_{INO} elements, which display high similarity to the consensus sequence, and thus led us to question whether the *PAH1* gene is subject to inositol-mediated transcriptional regulation via the Ino2p, Ino4p, and Opi1p transcription factors. Surprisingly, while the expression of inositol-regulated phospholipid biosynthetic enzymes is repressed in the *ino2*Δ and *ino4*Δ mutant strains and is not affected by inositol supplementation (2, 10, 12, 289), *PAH1* expression was induced in the absence of inositol in both mutant backgrounds, and particularly in stationary *ino4*Δ mutant cells. Moreover, the lack of Opi1p resulted in a dramatic repression of *PAH1* expression in exponential phase cells, an attenuation of the growth-phase dependent *PAH1* induction, and the loss of the inositol-mediated *PAH1* regulation in stationary phase. Studies by Klig et al. (317) have shown that *opi1*Δ cells grown in the

absence of inositol contain higher levels of PA, consistent with lower Pah1p PAP activity which could result from decreased *PAH1* expression. Additionally, Morlock et al. (287) showed that the induction in PAP activity in exponential phase cells supplemented with inositol is abrogated in the *opi1*Δ mutant, again in agreement with the results observed here.

PAH1 expression is clearly controlled by Ino2p, Ino4p, and Opi1p, albeit in an inverse manner than that of other UAS_{INO}-containing genes (12, 119). One potential explanation for this different mode of regulation is that the Ino2p-Ino4p-Opi1p transcriptional circuit controls a repressor of *PAH1* expression. Alternatively, it is possible that the regulation by Ino2p, Ino4p, and Opi1p does not occur via UAS_{INO}. In support of this hypothesis, we observed that the UAS_{INO} elements contained in the *PAH1* promoter display specific changes in the consensus motif, which are known to completely eliminate or severely reduce UAS_{INO} function (290). Yet a third possibility is that there are other binding partners affecting this regulation. Ino2p and Ino4p are members of the basic helix-loop-helix (bHLH) family of transcription factors that regulate important cellular processes including glycolysis, phosphate utilization, and phospholipid synthesis (179, 318). An important feature of the bHLH proteins is their capacity to form multiple dimer combinations via their HLH domains, conferring them with different DNA binding specificities and thus the ability to target different sets of genes (179). Neither Ino2p nor Ino4p are capable of forming homodimers and must therefore form heterodimers in order to bind DNA (319). In particular, Ino4p is known to bind all other yeast bHLH proteins (320), making it a central player in the regulation of diverse biological processes. Our analyses showed that *PAH1* expression was altered in both the *ino2*Δ and *ino4*Δ mutants,

suggesting that an Ino2p-Ino4p complex may be required for proper *PAH1* regulation. However, it is also possible that the lack of Ino2p in this mutant altered the amount of Ino4p that is available to interact with other bHLH proteins, thereby indirectly affecting *PAH1* expression. Ino4p has been shown to interact with Pho4p (320), a bHLH regulator involved in the activation of transcription in response to phosphate limitation (318, 321). In addition, Pho4p has been implicated in the inositol-dependent regulation of the *GIT1* gene encoding for a glycerophosphoinositol transporter, and it was postulated that this regulation occurred via the interaction of Ino4p and Pho4p (322). Interestingly, the transcriptional activity of Pho4p depends on its phosphorylation by Pho85p-Pho80p, providing a possible link with the regulation of Pah1p function. Phosphorylation of Pho4p by Pho85p-Pho80p results in its translocation to the cytoplasm, where it is rendered unable to regulate transcription in the nucleus (323). Thus, we can speculate that under phosphate limiting conditions in stationary phase, Pho4p is activated through decreased Pho85p-Pho80p phosphorylation and via dimerization with Ino4p represses *PAH1* expression. Thus, the higher amounts of Ino4p available for interaction with Pho4p in *ino2*Δ mutant cells would result in the repression of *PAH1* expression we observed in this mutant. The role of Ino4p-Pho4p on *PAH1* regulation warrants further investigation, and can be confirmed through analyses of *PAH1* expression in *pho4*Δ and *pho4*Δ *ino4*Δ mutant cells. Additionally, the *PAH1* promoter contains a putative Pho4p binding site immediately adjacent to a Pho2p binding site, both of which show >90% homology to their respective consensus sequences. Pho2p is a homebox transcription factor, whose binding to DNA is thought to disrupt intra-molecular interactions within a repression domain of Pho4p, which in turn results in enhanced access to the

transcriptional activation domain and subsequent induction of phosphatase genes in response to phosphate limiting conditions (324, 325). This suggests the possibility of an Ino4p-independent regulation of *PAH1* expression via the Pho4p-Pho2p complex, and should be studied in further detail.

Pah1p PAP is not the first example of an enzyme that is regulated by inositol in this seemingly opposite mechanism to that of most phospholipid biosynthetic genes (7). Of particular relevance to this work is the regulation of the *DPP1* gene encoding DGPP phosphatase, which is induced in stationary phase cells and upon supplementation with inositol (186, 281). Like *PAH1*, *DPP1* expression is also repressed in *opi1*Δ mutant cells and significantly induced in *ino4*Δ mutant cells in exponential phase (186). Moreover, this regulation occurs in the absence of a UAS_{INO} element (186), in parallel with *PAH1*, the promoter of which contains non-functional UAS_{INO} elements. The expression of *DPP1* is repressed in stationary phase by the transcription factors Gis1p and Rph1p (281), which share low overall sequence homology, but contain finger regions which are 100% identical in the amino acids believed to contact DNA(265). Examination of the *gis1*Δ mutant has revealed alterations in phospholipid levels which cannot be explained solely by the Gis1p-mediated regulation of *DPP1* expression (281), thereby suggesting that this transcription factor might also regulate the expression of other genes involved in lipid metabolism. Specifically, *gis1*Δ mutants display a 24% increase in PC and a 39% reduction in PE levels (281). PC content is also increased in *pah1*Δ cells, in agreement with a positive regulatory role of Gis1p on *PAH1* expression (70, 78, 79). Analysis of *PAH1* expression in *gis1*Δ and *rph1*Δ mutant cells indicated that these transcription factors may be involved in the growth phase- and inositol-mediated regulation of the

PAH1 gene. Moreover, *PAH1* expression was affected in cells expressing reporter genes with promoter truncations or Gis1p/Rph1p binding site mutations, supporting this notion; however, further studies are necessary to address this hypothesis. In complementary experiments, *PAH1* expression can be analyzed in cells overexpressing Gis1p (281) and Rph1p, in which elevated levels of *PAH1* expression compared to that of the wild type cells would be expected. A possible caveat to these experiments is that *GIS1* overexpression appears to have lethal consequences, as *GAL*-inducible overexpression of *GIS1* has been shown to completely inhibit growth in wild type cells, while its constitutive expression through an *ADH1* promoter results in 50% growth reduction (291). Additionally, work by Oshiro et al. showed that multicopy overexpression of *GIS1* in *gis1*Δ mutant cells results in very low transformation efficiency and slow growth, in agreement with the toxic effect of *GIS1* overexpression previously observed (281). Whether this toxicity is due to excessive induction or repression of Gis1p target genes is not known. Expression of the P_{PAH1}-*lacZ* promoter truncation mutants in *gis1*Δ and *rph1*Δ mutant cells could aid in the identification of additional Gis1p/Rph1p binding sites responsible for the growth phase- and/or inositol-mediated regulation of *PAH1* expression. Analyses of *PAH1* expression in cells bearing P_{PAH1}-*lacZ* reporter genes with mutations in these sites will assist in corroborating the role of Gis1p/Rph1p in *PAH1* regulation. Binding of recombinant His_6-tagged versions of Gis1p or Rph1p to cis-elements in labeled oligonucleotides containing the putative Gis1p/Rph1p binding sites in the *PAH1* promoter can be further confirmed in electrophoretic mobility shift assays (EMSA) (262, 281). Alternatively, the involvement of Gis1p and Rph1p in the regulation of *PAH1* can also be analyzed through chromatin

immunoprecipitation (ChIP) analysis (104, 326).

In the event that the regulation by Ino4p-Pho4p and/or Gis1p/Rph1p does not fully explain the growth phase- and inositol-dependent effects on *PAH1* expression, further studies will be required to identify the additional transcription factors involved. As discussed above, Ino4p has the ability to bind all other members of the bHLH protein family (320), and thus there exists the possibility that partnership with other such transcription factors will result in different modalities of *PAH1* regulation. Additionally, protein interactions that do not occur via HLH domains are also possible. For instance, Ume6p has been shown to function as a positive regulator of several phospholipid biosynthetic genes, including *CHO1*, *CHO2*, *OPI3*, *PIS1*, and the *INO2* regulatory gene (327, 328). The recently revised model postulates that induction by Ume6p occurs through the ablation of Ume6p repressor function via association with Cdc20p (329, 330). Thus, in glucose-replete conditions PKA phosphorylates Cdc20p and prevents its interaction with Ume6p, leading to stabilization of Ume6p and repression of its target genes. Conversely, glucose depletion causes PKA inactivation and results in Cdc20p-Ume6p association, Ume6p degradation and increased target gene expression (330). We can therefore speculate that Ume6p may repress *PAH1* expression in exponentially growing cells, while the decrease in glucose levels in stationary phase would lead to its degradation and subsequent derepression of *PAH1*.

The observed growth phase-dependent induction in *PAH1* expression was not translated into increased *PAH1* mRNA or Pah1p levels as expected. The reason for this phenomenon is two-fold. First, the reporter gene product is a non-native protein in yeast, and is therefore not subjected to degradation or turnover in the same manner as mRNA or

Pah1p. Secondly, the decreased mRNA extractability of stationary phase cells prevented us from gathering conclusive evidence as to the effect of growth phase on *PAH1* mRNA abundance. Due to the conflicting results obtained from *PAH1* expression studies and subsequent mRNA and protein abundance analyses, our transcriptional studies were relegated in favor of elucidating the mechanism responsible for these inconsistencies.

While Northern blot analysis suggested that *PAH1* mRNA levels decreased as cells progressed to stationary phase, the accompanying decrease in the total RNA and *PGK1* mRNA detected indicated that extraction of RNA from stationary phase cells was incomplete. Yeast cells are surrounded by a rigid cell wall that varies in thickness and composition depending upon growth conditions and that may impede extraction of cellular contents, particularly in stationary phase (292, 331). Despite the use of a modified RNA extraction method to take these changes in cell morphology into account, both the mRNA levels of the internal control *PGK1* and the rRNA levels were very low in stationary phase. Moreover, subsequent Western blot analyses of Pgk1p revealed its levels remain constant throughout growth, further confirming the limitations of RNA extraction from stationary phase cells. Thus, we could only conclude that, in agreement with our reporter assay results, *PAH1* expression increased in early time points in the growth phase, and that the stability of the *PAH1* transcript was not affected by growth phase at least up to those time points in which *PAH1* mRNA could be detected by the methods employed here. It is important to note, however, that reduced detection of *PAH1* mRNA does not necessarily correlate with decreased Pah1p expression. For instance, the *RAS2* transcript is not readily detectable by Northern blot analysis in stationary phase, yet Ras2p is synthesized at comparable rates during this stage of growth

to those of exponentially growing cells (271, 332), suggesting that the translatability of the transcript might be affected (332).

Studies in *dpp1*Δ *lpp1*Δ, *pah1*Δ *dpp1*Δ *lpp1*Δ, and *app1*Δ *dpp1*Δ *lpp1*Δ mutants confirmed that Pah1p PAP activity was induced as cells progressed throughout growth and that this activity translocated to the membrane fraction, in correlation with the observed Pah1p-mediated increase in TAG content and synthesis in stationary wild type cells. In addition, TAG levels and synthesis rates were dramatically reduced in *pah1*Δ mutant cells lacking Pah1p PAP activity, confirming that Pah1p is essential for the synthesis of TAG and that its accumulation in stationary phase cells is due in part to a Pah1p-mediated increase in the rate of synthesis. Due to the important role of phosphorylation/dephosphorylation on the modulation of Pah1p PAP function, we examined the effect of the *nem1*Δ mutation on lipid synthesis throughout growth. This work provided the first indication that Nem1p-Spo7p-mediated dephosphorylation of Pah1p is crucial for the synthesis of TAG throughout growth, as cells lacking Nem1p exhibited TAG levels and synthesis rates comparable to those of the *pah1*Δ mutant.

Surprisingly, Pah1p abundance was reduced to undetectable levels during the transition from exponential to stationary phase of growth. Given that lipid and Pah1p PAP activity analyses had indicated that the enzyme is present in stationary phase cells, we questioned whether C-terminal truncations or post-lysis proteolysis events were responsible for the observed decrease in protein levels; however, control experiments demonstrated that Pah1p was degraded *in vivo*. Analysis of the stable Dpp1p under the control of the *PAH1* promoter provided confirmation for our reporter assay results, and supported the notion that the observed decrease in protein levels in stationary phase cells

was Pah1p-specific, pointing to a mechanism of programmed protein degradation. This hypothesis was confirmed with the use of the proteasome inhibitor MG132 as well as in various mutant cells deficient in proteasome function. Treatment with MG132 or defects in proteasome function resulted in stabilization of Pah1p in both exponential and stationary phases, but particularly in stationary phase cells, suggesting Pah1p is subject to proteasome-mediated degradation throughout growth. In addition, it is predicted that the Pah1p sequence contains multiple ubiquitination sites, and thus Pah1p expression was examined in mutant strains lacking specific enzymes involved in the ubiquitination/deubiquitination of proteasome-bound substrates. These analyses showed that Pah1p degradation is significantly reduced in stationary phase mutant cells lacking ubiquitinating/deubiquitinating enzymes, suggesting Pah1p may undergo ubiquitin-dependent proteasomal degradation.

Modulation of Pah1p PAP activity is crucial for normal cell physiology, as overexpression of either the unregulated and hyperactive phosphorylation-deficient 7A-Pah1p or Nem1p result in lethal phenotypes (87, 104, 105, 107). Moreover, our current study has highlighted the importance of phosphorylation/dephosphorylation on the regulation of Pah1p function. Pah1p is dephosphorylated exclusively by Nem1p-Spo7p *in vivo*; however, it is a target for phosphorylation by multiple kinases including Pho85p-Pho80p, Cdc28p-cyclin B, and PKA (107, 108, 244, 333). That these kinases are required for normal growth and cell cycle progression suggests that Pah1p phosphorylation may occur primarily during exponential phase. We considered the possibility that the decrease in Pah1p phosphorylation resulting from reduced kinase activity in stationary phase could be the basis for the induction in Pah1p PAP activity and

function observed at this stage of growth. Moreover, the translocation of Pah1p PAP activity to the membrane fraction in stationary phase cells supported this theory. The current work has shown, however, that TAG synthesis is not increased in *nem1*Δ mutant stationary phase cells, indicating that Pah1p cannot circumvent the Nem1p-Spo7p requirement for *in vivo* function during this growth phase and is therefore still phosphorylated in stationary phase cells.

Additional work is required to determine the effect of growth phase and inositol supplementation on the phosphorylation status of the enzyme, and the significance of this phosphorylation on Pah1p *in vivo* function. The extent of Pah1p phosphorylation throughout growth and under inositol supplementation conditions can be examined via *in vivo* $^{32}P_i$ labeling assays. Given the growth phase-mediated degradation of Pah1p we reported here, these assays should be conducted on Pah1p catalytic site mutants to circumvent this limitation. The phosphorylation sites affected can be identified by peptide mapping, phospho amino acid, and mass spectrometry analyses (105, 334, 335), and their sequences matched to the kinases involved through the yeast database. Once the potential kinases are identified, their effect on the growth phase-mediated phosphorylation of Pah1p can be corroborated through analyses of kinase-deficient and phosphorylation site mutants. In addition, recent studies have indicated that the phosphorylations by the different kinases are interconnected, and thus phosphorylation by one kinase may stimulate or inhibit the phosphorylation and regulation by the same kinase, or by different kinases. The interdependencies of the various phosphorylations elevate the regulation of Pah1p to a higher level of complexity, and therefore warrant further investigation. In this regard, additional knowledge of the factors regulating

Nem1p–Spo7p function, particularly with respect to growth phase or inositol supplementation, will further our understanding of the process by which Pah1p is dephosphorylated and thereby stabilized *in vivo*.

Pah1p phosphorylation is mediated by numerous kinases, the action of which results in different physiological outcomes. For instance, the effect of Pah1p phosphorylation on its catalytic activity varies depending on the kinase involved. Phosphorylation by Cdc28p-cyclin B has no direct effect on Pah1p PAP activity (107), while phosphorylation by PKA and Pho85p-Pho80p result in a 2- and 6-fold reduction in catalytic efficiency, respectively (108, 244). Additionally, the 7A (e.g., Pho85p-Pho80p) phosphorylation-deficient Pah1p mutant also exhibits elevated PAP activity (105). However, the direct effect of phosphorylation on Pah1p enzymatic activity is not sufficient to explain the dramatic induction we observed in PAP activity and TAG synthesis, suggesting the regulation of other processes via Pah1p phosphorylation have a more significant effect on Pah1p function. Accordingly, phosphorylation is known to inhibit Pah1p association with membranes, thus preventing access to the substrate PA and thereby indirectly controlling Pah1p PAP activity (105-108). Furthermore, the regulation of Pah1p stability via phosphorylation has emerged as a key mechanism for the control of Pah1p function (107, 108, 244). Studies of phosphorylation deficient mutants have shown that decreased Pah1p phosphorylation results in dramatic reductions in Pah1p abundance compared to wild type Pah1p levels, and that this effect can be attenuated in the absence of Nem1p (107, 108).

We postulate that an alteration in the phosphorylation status of Pah1p in response to growth phase is directly involved in the control of Pah1p stability; however, the exact

mechanism through which this regulation occurs remains to be elucidated. One possibility is that crosstalk between phosphorylation and ubiquitination results in the inhibition of Pah1p degradation. For instance, phosphorylation of Pah1p may induce a conformational change in its tertiary structure that inhibits ubiquitin ligase substrate recognition (336). Alternatively, the predominantly cytosolic localization of phosphorylated Pah1p may provide spatial separation from the ubiquitin ligases involved in its degradation (336). In addition, phosphorylation may promote or suppress association of Pah1p with other proteins, thereby inhibiting Pah1p degradation. Interestingly, lipin 1 has been shown to interact with 14-3-3 proteins, a family of highly conserved proteins that interact with a variety of phosphorylated proteins (337). Lipin 1 interaction with 14-3-3 proteins occurs in the cytoplasm and is promoted by insulin, which stimulates lipin 1 phosphorylation in an mTOR-dependent manner (245, 338), resulting in the cytoplasmic retention of lipin 1 (339). In yeast, 14-3-3 proteins participate in many different cellular processes through physical interactions with diverse binding partners, and appear to have a major role in the subcellular localization of proteins (337). Moreover, 14-3-3 activity itself is regulated at the transcriptional level by exposure to several stresses, including prolonged stationary phase (340). *S. cerevisiae* has two genes encoding 14-3-3 proteins, *BMH1* and *BMH2* (337). Disruption of either gene has no significant effects on cell viability, whereas simultaneous mutation of both genes results in a lethal phenotype (337). Thus, it would be interesting to examine whether 14-3-3 proteins play a role in the modulation of Pah1p function via its compartmentalization and/or stability. This can be achieved through the analysis of Pah1p subcellular localization and abundance in *bmh1*Δ and *bmh2*Δ mutants.

The transition from the exponential to the stationary phase of growth resulted in Pah1p-dependent increases in TAG content/synthesis and Pah1p PAP activity, which coincided with the proteasomal degradation of Pah1p. Therefore, we questioned whether Pah1p PAP activity and the balance of PA/DAG mediate the signal for the degradation of the enzyme. Pah1p catalytic site mutants that exhibit no Pah1p PAP activity and increased PA levels (79) were stable in stationary phase cells compared to wild type, suggesting that the balance of PA/DAG is essential for Pah1p degradation. These results were further confirmed in *dgk1*Δ cells overexpressing *DGK1*, which have been shown to accumulate PA on perinuclear membranes (87), and in which the alterations in the PA/DAG balance resulted in Pah1p stabilization. Analysis of additional mutants could aid in distinguishing the effects of microsomal PA vs DAG pools on Pah1p degradation. For instance, Pah1p stability can be analyzed in mutants defective in the ER-resident *GPT2* and *SCT1* redundant acyltransferase genes that are required for PA production (Fig. 2) (73, 341), and in which we expect to see a destabilization of Pah1p. Examination of PA levels and Pah1p abundance in cells overexpressing these acyltransferases can further corroborate our observations (73). In a similar manner, disruption or overexpression of *SPO14*/*PLD1*, the gene encoding phospholipase D (PLD) and thus responsible for the generation of PA from PC or PE (342-344) (Fig. 2), may also affect PA levels and thereby promote Pah1p degradation. Additionally, overexpression of *CDS1*, an essential gene encoding a CDP-DAG synthase localized to both mitochondria and microsomal fractions (18, 345), results in reduced PA levels (18); therefore, we speculate that Pah1p stability would be decreased under these conditions.

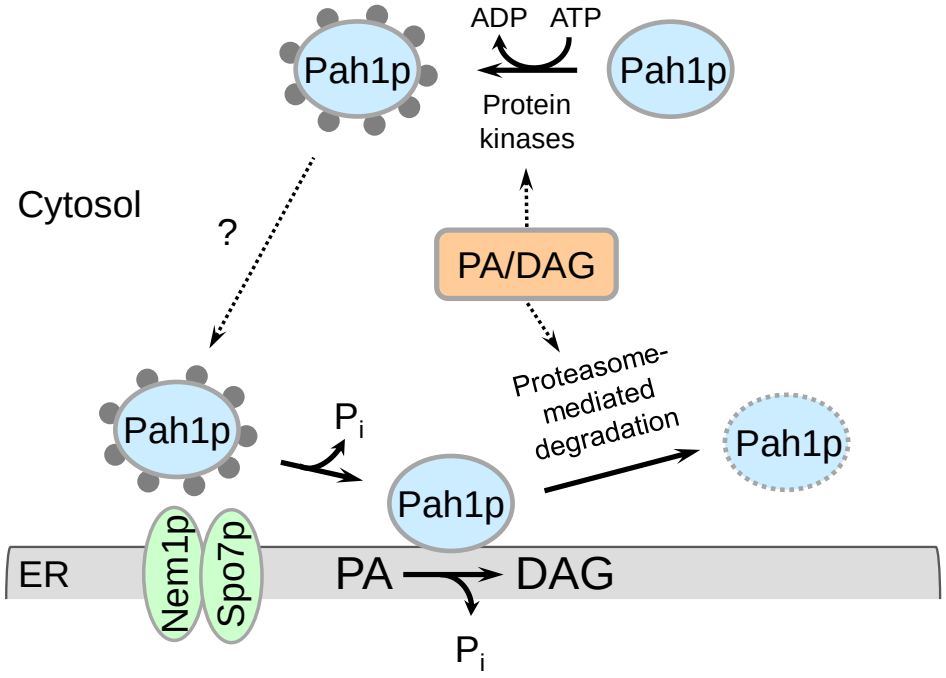
Finally, the underlying mechanism mediating the signal for Pah1p degradation via

changes in PA/DAG levels remains to be characterized. While Pah1p function is primarily implicated in lipid synthesis, PA and DAG are important lipid signaling molecules that regulate a variety of cellular processes. In this context, Pah1p PAP is unique in that it catalyzes the conversion of one lipid messenger into another. The product of Pah1p PAP activity, DAG, is a potent intracellular mediator involved in the allosteric activation of PKC and other non-kinase effectors in animal systems (93-95, 346). In relation to human disorders associated with imbalances in PAP function, the DAG-dependent activation of serine/threonine kinase cascades has been correlated with defects in insulin resistance (102). Additionally, the Pah1p substrate PA is also a lipid messenger in its own right, mediating a range of cellular functions related to survival, proliferation and reproduction (96, 97, 97, 98). PA exerts its signaling functions via diverse mechanisms, such as direct binding and modulation of enzymatic activity, membrane tethering, and through changes in membrane structure and metabolism (97, 98). A variety of PA targets have been identified in mammalian cells, including PKC, phosphatidylinositol 4-phosphate 5-kinase, sphingosine kinase, and phospholipase C (97). In yeast, PA targets the SNARE protein Spo20p and the inositol-regulated transcriptional repressor Opi1p (97). PA has also been shown to bind and activate mTOR, a master regulator of cell growth (101). While available data indicates that signaling PA is primarily produced via PLD and DGK, other PA-generating metabolic pathways contribute to the activation of mTOR (98, 101). For instance, LPA acyltransferase suppression has been reported to repress mTOR activation (347) and PA produced by DGK has been shown to activate mTOR (348). Moreover, the PA utilized by lipin 1 also appears to have signaling functions, as recent studies have shown that

elevated PA levels due to loss of lipin 1 lead to diminished lipolysis and PKA activity, and these observations are in agreement with the correlation between lipin 1 expression, lipolytic rates and PKA signaling in adipose tissue of obese human subjects (349). Interestingly, the PKA and TOR pathways are downregulated in stationary phase yeast cells, while the inactivation of TOR transiently activates PKC signaling during the diauxic shift (292). The correlation between PA levels and TOR activity may provide an explanation for the observed decrease in Pah1p stability in stationary phase cells. We propose that through the growth phase-mediated induction in its enzymatic activity, Pah1p may serve to remove PA and effectively terminate its signaling action. This results in the attenuation of the signaling cascades described above, which in turn may affect phosphorylation and activation of ubiquitin ligases (336), or activation/inhibition of known and/or unidentified kinases responsible for Pah1p phosphorylation, thereby regulating Pah1p degradation (Fig. 27).

Clearly, the novel mechanisms for the regulation of Pah1p function uncovered in this work have opened new avenues of study that warrant further investigation. The importance of understanding these mechanisms is underscored by the involvement of this enzyme in lipid-based disorders in human physiology. Mutations in the *LPIN1* gene have been linked to insulin resistance and diabetic phenotypes (113, 350), suggesting lipin 1 plays a crucial role in the maintenance of metabolic homeostasis in humans. Thus, additional studies in model organisms are required to elucidate the mechanistic basis for these observations to fully understand the effects of lipin 1 on human health.

Figure 27. Model for Pah1p regulation by phosphorylation/dephosphorylation, localization and degradation. The model describes the mechanism by which Pah1p phosphorylation/dephosphorylation affects its localization and *in vivo* function, and how alterations in the PA/DAG balance may play a role in the regulation of Pah1p stability. *DAG*, diacylglycerol; *ER*, endoplasmic reticulum; *PA*, phosphatidate.


Pah1p
ADP
ATP
Pah1p
Protein
kinases
Cytosol
?
PA/DAG
Proteasome-
mediated
degradation
Pah1p
Pi
Pah1p
Pah1p
ER
Nem1p
Spo7p
PA
DAG
Pi

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