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Review

Phosphatidate phosphatase, a key regulator of lipid homeostasis ☆

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ABSTRACT

Yeast Pah1p phosphatidate phosphatase (PAP) catalyzes the penultimate step in the synthesis of triacylglycerol. PAP plays a crucial role in lipid homeostasis by controlling the relative proportions of its substrate phosphatidate and its product diacylglycerol. The cellular amounts of these lipid intermediates influence the synthesis of triacylglycerol and the pathways by which membrane phospholipids are synthesized. Physiological functions affected by PAP activity include phospholipid synthesis gene expression, nuclear/endoplasmic reticulum membrane growth, lipid droplet formation, and vacuole homeostasis and fusion. Yeast lacking Pah1p PAP activity are acutely sensitive to fatty acid-induced toxicity and exhibit respiratory deficiency. PAP is distinguished in its cellular location, catalytic mechanism, and physiological functions from Dpp1p and Lpp1p lipid phosphate phosphatases that utilize a variety of substrates that include phosphatidate. Phosphorylation/dephosphorylation is a major mechanism by which Pah1p PAP activity is regulated. Pah1p is phosphorylated by cytosolic-associated Pho85p–Pho80p, Cdc28p-cyclin B, and protein kinase A and is dephosphorylated by the endoplasmic reticulum-associated Nem1p–Spo7p phosphatase. The dephosphorylation of Pah1p stimulates PAP activity and facilitates the association with the membrane/phosphatidate allowing for its reaction and triacylglycerol synthesis. This article is part of a Special Issue entitled Phospholipids and Phospholipid Metabolism.

1. 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 triacylglycerol (TAG) molecule constitutes the most calorie-dense form of cellular energy storage, allowing organisms to withstand prolonged periods of nutrient deprivation [1]. Moreover, stores of TAG may provide a source of fatty acids and diacylglycerol (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 [2]. The regulation of TAG synthesis and storage is crucial in human health because both an excess and a defect in fat storage result in lipid-associated disorders such as obesity, lipodystrophy, insulin resistance, diabetes, hypertension, cardiovascular disease, and cancer [1].

Phosphatidate phosphatase (PAP), the enzyme involved in the penultimate step in TAG synthesis, catalyzes the dephosphorylation of phosphatidate (PA) to yield DAG and P_i [3] (Fig. 1). In *de novo* lipid synthesis in the yeast *Saccharomyces cerevisiae*, the DAG generated in the reaction is used for the synthesis of TAG as well as the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) via the Kennedy pathway [4–7], while the reaction substrate PA serves as a precursor for all major phospholipids via the CDP-DAG pathway [4–6] (Fig. 1). In mammalian cells, however, the phospholipids phosphatidylserine, PE and PC are derived from DAG [8]. 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 in higher eukaryotes [9–18]. 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 [1,7,19–31].

Abbreviations: TAG, triacylglycerol; DAG, diacylglycerol; PA, phosphatidate; PAP, PA phosphatase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CDP-DAG, CDP-diacylglycerol; CL, cardiolipin; DGPP, diacylglycerol pyrophosphate; ER, endoplasmic reticulum; NEM, *N*-ethylmaleimide; UAS_{INO}, inositol-responsive upstream activating sequence

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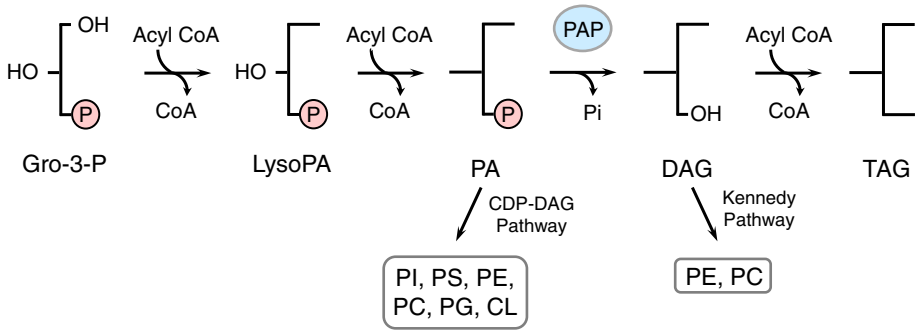

Fig. 1. Lipid synthesis in yeast. The pathways shown for the synthesis of TAG and phospholipids include the relevant steps discussed in this review. PAP (highlighted in blue) catalyzes the dephosphorylation of PA to form DAG in the penultimate step in TAG synthesis. The PAP substrate PA and product DAG are utilized for the synthesis of phospholipids via the CDP-DAG pathway and Kennedy pathway, respectively. Gro-3-P, glycerol-3-phosphate; LysoPA, lysophosphatidate; PA, phosphatidate; DAG, diacylglycerol; TAG, triacylglycerol; CDP-DAG, CDP-diacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

2. Discovery of 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 [3], providing a link between the neutral and phospholipid synthesis pathways in mammalian cells [6,32]. 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 [33], in contrast to other enzymes in the TAG and phospholipid synthesis pathways, which are integral membrane proteins [34–38]. Furthermore, PAP activity was found to translocate from the cytosol to the membrane fraction of cells treated with fatty acids [4,39,40]. 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* [33,41]. This simple eukaryote synthesizes lipids by pathways common to those of more complex organisms and is an attractive model system due to its molecular and genetic tractability [42–44]. The use of this model organism allowed for the purification of a 91-kDa PAP enzyme from the total membrane fraction in 1989 [45]. Additionally, 104-kDa, 75-kDa, and 45-kDa forms of PAP were isolated from microsomes, cytosol, and mitochondria, respectively [33,46]. The 91-kDa enzyme was later shown to be a degradation product of the 104-kDa form [46], while precursor–product relationships do not exist between the 75-kDa, 45-kDa, and 104-kDa proteins [46]. 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 [33,45,46]. 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 [47]. Characterization of the enzyme revealed that it catalyzes the removal of the β-phosphate from diacylglycerol pyrophosphate (DGPP) to form PA, and subsequently dephosphorylates PA to produce DAG; thus, the enzyme was termed DGPP phosphatase [47]. In contrast to the enzymes discussed above, this phosphatase enzyme does not require Mg^{2+} ions for catalysis [47]. Sufficient enzyme was purified to obtain amino acid sequence information, which was matched to a gene in the *S. cerevisiae* database and designated *DPP1* (for *d*iacylglycerol *p*yrophosphate *p*hosphatase) [48]. Dpp1p[2] is 289 amino acids in length and has a molecular mass of 33.5 kDa [48], which is in close agreement with the size of the purified DGPP phosphatase [47]. The identification of a conserved phosphatase sequence motif contained within several lipid phosphatases [49] revealed that DGPP phosphatase shares sequence homology with a mammalian Mg^{2+}-independent PAP (designated PAP2) believed to be involved in lipid signaling [50–53]. Shortly after this discovery, the *LPP1* (for *l*ipid *p*hosphate *p*hosphatase) gene was identified based on protein sequence homology with Dpp1p [54]. Lpp1p is 274 amino acids in length and has a predicted molecular mass of 31.6 kDa [54]. 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 [48,54,55]. 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 [56].

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 [21], 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 [57]. 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 endoplasmic reticulum (ER)-associated Nem1p–Spo7p phosphatase complex [20,58]. Upon identification of the molecular function as a PAP enzyme, the *SMP2* gene was renamed *PAH1* (for *p*hosphatidic *a*cid phospho*h*ydrolase) [21]. 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 [21]. While phosphorylation of Pah1p results in a shift in electrophoretic mobility to a position of a slightly higher molecular mass [20,22,25,27], Pah1p expressed in *E. coli* migrates as a 114-kDa protein product upon SDS-PAGE analysis [21]. 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 [21,59]. 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 [59]. *Lpin1* was identified as the gene whose mutation is responsible for the transient fatty liver dystrophy (*fld*) phenotype of mice [59,60]. 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 [59,61]. 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 [21,23], which is contained in the CLIP domain, lipin-1 was

[2] The protein product of a yeast gene is designated by the gene acronym followed by the letter p (e.g., Dpp1p, Lpp1p, and Pah1p).

characterized as a PAP enzyme [21,62]. 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 [62,63]. *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 [1,30,64]. 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 [65], 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 [21,63], mice [59,62], flies [66,67], worms [68], and plants [69,70]. All PAP enzymes have the HAD-like domain that contains a DXDX(T/V) catalytic motif and the NLIP domain of unknown function [21,23,59,71]. The reader is directed to recent reviews that summarize our current understanding of mammalian lipins [19,31,32,72,73].

3. Biochemical, enzymological and structural properties of PAP enzymes

Since the initial characterization of the PAP reaction in 1957 [3], both Mg^{2+}-dependent and -independent enzymes have been identified in yeast [21,48,54]. Besides the difference in their cofactor requirement, these enzymes are distinguished by several other properties (Figs. 2 and 3).

Dpp1p and Lpp1p are relatively small integral membrane proteins confined to the vacuole and Golgi membranes, respectively (Fig. 2). Both proteins possess six transmembrane domains distributed over their polypeptide sequences [48,54]. 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 membrane to access its substrate PA for catalysis [21] (Fig. 2). 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 [19,20]. Thus, the spatial differences in the subcellular localization of the PAP and LPP enzymes point to distinct cellular functions.

While mammalian Mg^{2+}-dependent PAP and Mg^{2+}-independent LPP enzymes are also differentiated based on their sensitivity to the thioreactive compound *N*-ethylmaleimide (NEM) [4,50], this distinction is not applicable to their yeast counterparts. In fact, Dpp1p activity is insensitive to NEM [47], whereas the activity of Lpp1p is potently inhibited by this compound [74]. Moreover, NEM has no effect on the PAP activity of Pah1p [21]. Similarly, the synthetic compound propranolol inhibits both Pah1p [46] as well as Lpp1p [74], even though it is thought to hinder activity by interacting with the Mg^{2+} binding site of enzymes [75]. Therefore, inhibition by NEM or propranolol should not be used to distinguish yeast PAP and LPP enzymes.

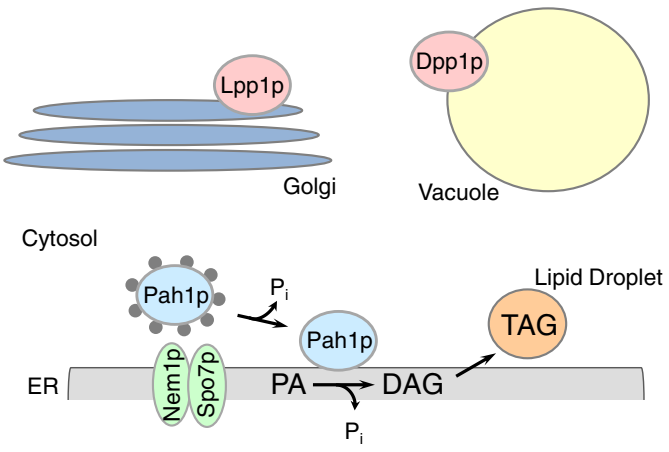

Fig. 2. PAP and LPP enzymes are localized in different cellular compartments. The cartoon shows the integral membrane enzymes Dpp1p and Lpp1p in the vacuole and Golgi, respectively. Pah1p in the cytosol is phosphorylated on multiple sites (symbols decorating enzyme). At the ER membrane, Pah1p is dephosphorylated 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.

Dpp1p and Lpp1p do not require divalent cations for activity [47,54,74], while maximum activity of Pah1p is dependent on Mg^{2+} ions [21]. 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. 3). The PAP activity of Pah1p is governed by a DXDX(T/V) motif found in members of a superfamily of Mg^{2+}-dependent phosphatase enzymes [76,77] that include mammalian lipin PAP enzymes [1,21]. Consistent with other Mg^{2+}-independent LPP enzymes [49,78,79], Dpp1p and Lpp1p contain a three-domain lipid phosphatase motif composed of the consensus sequences KXXXXXXRP (I), PSGH (II) and SRXXXXXHXXXD (III) [49], which confer these proteins their enzymatic activity [80].

Finally, the PAP and LPP enzymes in yeast differ with respect to their substrate specificities. Dpp1p and Lpp1p utilize a variety of lipid phosphate substrates, including PA, DGPP, lysoPA, sphingoid base phosphates, and isoprenoid phosphates [47,48,54,55,74,81]; however, only DGPP and PA have been shown to be substrates in vivo [54]. 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 [47]. Conversely, PA is the preferred substrate for Lpp1p, followed by DGPP and lysoPA [54,74]. Moreover, the affinity of Lpp1p for PA, DGPP, and lysoPA as substrates is greater than the affinity of Dpp1p for these substrates [74]. In contrast, Pah1p is specific for PA [33,45–47]. Additionally, 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 [6,48,54]. In fact, *dpp1*Δ *lpp1*Δ mutations do not affect lipid synthesis, whereas, the *pah1*Δ mutation affects both the synthesis of TAG as well as the synthesis of phospholipids [21,26]. Thus, the synthesis of the DAG required for phospholipid and TAG synthesis in *S. cerevisiae* is attributed to the Pah1p enzyme [21,26,82].

4. 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 [20,22,23,55]. Consistent with a lack of enzyme activity, this mutant shows elevated levels of PA and decreased levels of DAG and TAG [21,23,26]. Additionally, the amounts of phospholipids, fatty acids, and sterol esters are also elevated in response to the *pah1*Δ mutation [21,23,26], indicating that PAP regulates overall lipid synthesis. The effects on TAG (>90% decrease) are most pronounced in the stationary phase of growth [21,23], where the synthesis of TAG is predominant over the synthesis of membrane phospholipids [83].

In addition to the alteration in lipid metabolism, phenotypes of the *pah1*Δ mutant include slow growth [20], aberrant expansion of the nuclear/ER membrane [20], respiratory deficiency [21], defects in lipid droplet formation [28] and morphology [84], vacuole homeostasis and fusion [29], fatty-acid induced lipotoxicity [26], and a growth sensitivity to elevated temperature [20,21]. 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 [23,26,28,29].

A contributing factor for the increased amounts of phospholipids and fatty acids in the *pah1*Δ mutant is the derepression of UAS_{INO}-containing lipid synthesis genes in response to elevated PA levels [20,21,23,85–89]. The UAS_{INO} element contains the binding site for the Ino2p-Ino4p complex that stimulates expression of lipid synthesis

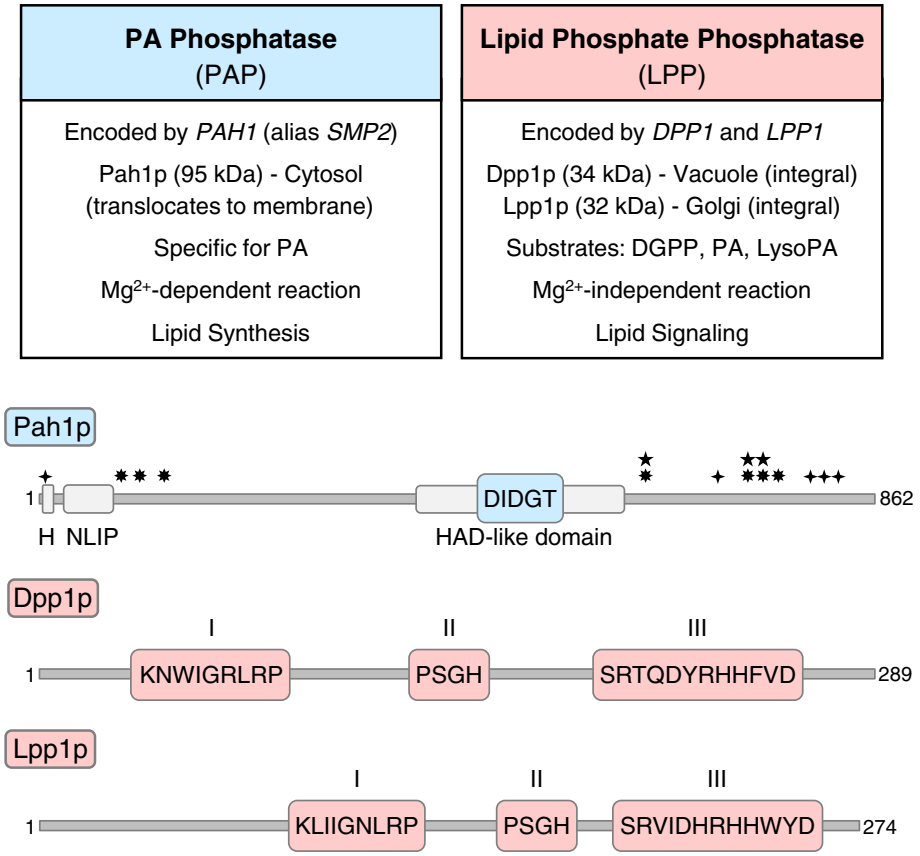

Fig. 3. Distinguishing characteristics of PAP and LPP enzymes. The basic characteristics of the yeast PAP (Pah1p) and LPP (Dpp1p and Lpp1p) enzymes, including their catalytic motifs and phosphorylation sites, are summarized in the figure. *H*, amphipathic helix; *HAD*, haloacid dehalogenase; ✱, approximate 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 protein kinase A. The diagrams of each protein are not drawn to scale.

genes [85]. The PA-mediated regulation of these genes is controlled by the transcriptional repressor Opi1p [90], 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 [91,92]. A 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 [92,93]. 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 be a factor for the susceptibility of the *pah1*Δ mutant to fatty acid-induced toxicity [26].

The yeast *DGK1*-encoded DAG kinase (Dgk1p) has recently been identified as an enzyme whose function counterbalances that of the Pah1p PAP [94] (Fig. 4). This nuclear/ER integral membrane enzyme is unique in that, in contrast to other DAG kinases in bacteria, plants, and animals [13,95–98], it utilizes CTP rather than ATP as the phosphate donor in the phosphorylation reaction [94] (Fig. 4). Cells bearing a *dgk1*Δ mutation do not exhibit any remarkable phenotypes under standard growth conditions [94]. 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 [94], providing evidence that Dgk1p activity antagonizes that of the Pah1p enzyme by regulating the cellular levels of PA (Fig. 4).

The aberrant membrane expansion phenotype of the *pah1*Δ mutant has been attributed to the abnormal increase in phospholipid synthesis associated with this mutation [20]; however, recent reports have shown that increased expression of phospholipid biosynthetic genes alone is not sufficient for nuclear/ER membrane expansion [22,94]. In addition, *DGK1* overexpression results in anomalous nuclear/ER membrane morphology [94], 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 [94]. 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 [26,28], 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 [84]. 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 [28]. 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 [94], increase in fatty acid content [94], fatty acid-induced toxicity [26], and temperature sensitivity[3] 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 [29]. While this phenotype is due to the loss of Pah1p PAP activity [29], 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.

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

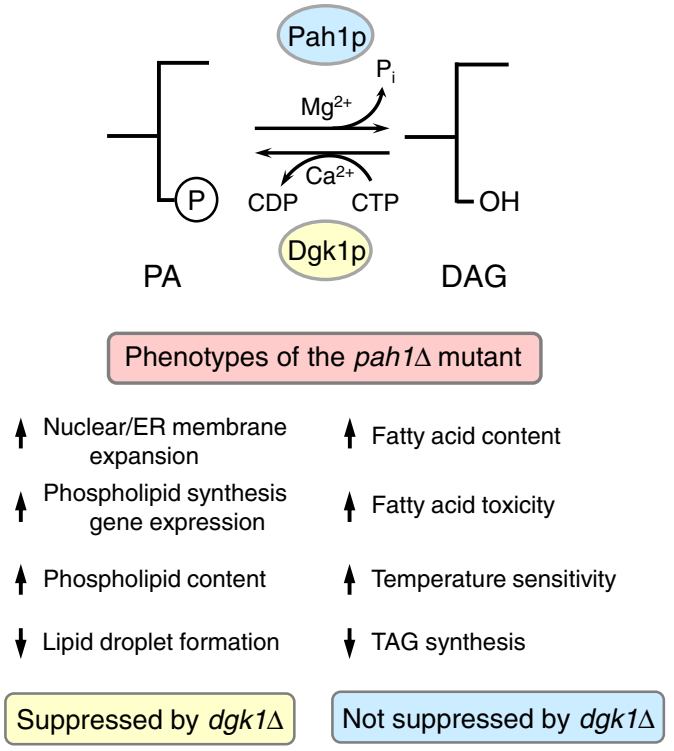

Fig. 4. Summary of *pah1*Δ mutant phenotypes. The reactions catalyzed by Pah1p PAP and Dgk1p DAG kinase control the balance of PA and DAG. The *pah1*Δ phenotypes suppressed and not suppressed by the *dgk1*Δ mutation are listed in the figure.

5. PAP activity 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 previously, overexpression of a phosphorylation-deficient form of Pah1p (7A, discussed in later sections) in *S. cerevisiae* is also detrimental to cell growth [22,25,94]. 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.

5.1. Regulation of PAP expression

Lpin1/2-encoded 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) [99–101], sterol response element binding protein-1 (SREBP-1) [102], and cAMP response element binding protein (CREBP) [103]. In contrast, *Lpin2* is known to be induced by fasting [100,104], but this regulation is not affected by cAMP signaling or glucocorticoids [100]. Compared with its mammalian orthologs, relatively little is known about the transcriptional regulation of the yeast Pah1p PAP enzyme. Microarray data has indicated that *PAH1* expression is induced upon transition from glucose-based fermentative growth to glycerol- and ethanol-based respiratory growth [105]. Furthermore, despite the observed increase in Mg^{2+}-dependent PAP activity in stationary phase cells [41], reports suggest that *PAH1* is repressed during the diauxic shift [106]. Additional studies are needed to determine the effect of growth phase on *PAH1* expression.

Recent work by Soto-Cardalda and colleagues has shown the expression of *PAH1*-encoded PAP activity is affected by intracellular levels of zinc [107]. This essential nutrient in *S. cerevisiae* and higher eukaryotes [108,109] serves as a cofactor for numerous enzymes, and is a structural component of many proteins [108,110,111]. 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 [111–120]. 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 [107,109,121–126]. *PAH1* expression is induced in response to zinc depletion in a Zap1p-dependent manner through its interaction with zinc-responsive upstream activating (UAS_{ZRE}) sequences in the *PAH1* promoter [107]. This induction correlates with an increase in Pah1p PAP activity and elevated TAG levels [107].

5.2. Biochemical regulation of PAP

5.2.1. 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 [127]. On the other hand, PAP activity is inhibited by the sphingoid bases sphingosine, phytosphingosine, and sphinganine [127] 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 [42], 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 [128]. Moreover, cellular ATP and CTP levels correlate with synthesis of phospholipids and TAG [128]: 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 [129].

5.2.2. Regulation by phosphorylation/dephosphorylation

At 3910 molecules per cell [130], Pah1p is a relatively abundant enzyme in *S. cerevisiae* [38]. 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 [21,24,25,27]. Recent studies have demonstrated phosphorylation/dephosphorylation of Pah1p governs it subcellular localization, thus serving as the major regulator of PAP activity [22,24,25,27]. Phosphorylated Pah1p resides in the cytosol, while its dephosphorylated form is associated with the membrane [25] (Fig. 2). 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 [20,24,25,58] (Fig. 2).

The nuclear/ER membrane-associated Nem1p–Spo7p complex was identified in studies that showed that it is essential for the formation of a spherical nucleus [20]. Further work revealed the protein phosphatase activity of the complex is dependent on the catalytic motif DXDX(T/V) in Nem1p, as well as binding of the regulatory subunit Spo7p to Nem1p [20]. 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 [20,58]. Overexpression of the Nem1p–Spo7p complex is lethal only in the presence of its substrate Pah1p [20], 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 [130], 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* [131,132] and *CDC28* [133]. In *S. cerevisiae*, Cdc28p is essential and sufficient for cell cycle progression, while the non-essential Pho85p supports many additional functions [134]. Cross-talk between these two kinases allows for regulation of cell morphology, gene expression, macromolecular metabolism, and signaling in response to environmental stimuli [134–137]. 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 [22]. In fact, all seven sites are phosphorylated by Pho85p–Pho80p [27], and three of the sites (Ser^{602}, Thr^{723}, and Ser^{744}) are also targets of Cdc28p-cyclin B [25] (Fig. 3). The phosphorylation efficiency of the three common sites is much greater for Pho85p–Pho80p when compared with Cdc28p-cyclin B [27]. Phosphorylation by Pho85p–Pho80p results in a 6-fold reduction in the catalytic efficiency (V_{max}/K_m) of PAP [27], whereas activity is not affected by phosphorylation via Cdc28p-cyclin B [25]. That these kinases phosphorylate Pah1p in vivo is supported by the analysis of Pah1p phosphorylation in mutants lacking Pho85p or functional Cdc28p [20,27].

Pah1p is also a substrate for protein kinase A [138,139], the principal mediator of signals transmitted through the *RAS*/cAMP pathway in *S. cerevisiae* [140,141]. Protein kinase A 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 [139]. The protein kinase A-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 [27,139]. The inhibitory effect of protein kinase A on PAP activity is primarily conferred by phosphorylation at Ser^{10} [139].

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 protein kinase A [22,25,27,139]. 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 [22,25]. In vivo, expression of the 7A mutant enzyme complements the *pah1*Δ *nem1*Δ double mutant phenotypes that include temperature sensitivity, nuclear/ER membrane expansion, and derepression of phospholipid synthesis genes [22,25]. 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 [24,25]. Cells lacking the Nem1p–Spo7p complex exhibit reduced TAG due to loss of PAP function, thus indicating that the lack of phosphorylation of the seven sites renders Pah1p capable of bypassing the Nem1p–Spo7p requirement for in vivo function [25]. Simultaneous mutation of the three Cdc28p-cyclin B phosphorylation sites (3A) only partially mimics the physiological consequences of the 7A mutations [25].

Analysis of the S10A and S10D mutations (mimicking dephosphorylation and phosphorylation, respectively, by protein kinase A), alone or in combination with the 7A mutations, indicates that phosphorylation at Ser^{10} inhibits its association with membranes, PAP activity, and TAG synthesis [139]. 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 [139]. Thus, the protein kinase A-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 [139].

In addition to Pho85p–Pho80p, Cdc28p-cyclin B, and protein kinase A, Pah1p is phosphorylated by protein kinase C and casein kinase II [138]. These protein kinases are also known to regulate phospholipid synthesis in yeast [142]. Phosphorylation of Pah1p by protein kinase C and casein kinase II decreases its interaction with model membranes [138], suggesting an inhibitory effect of phosphorylation on PAP activity in vivo. Further studies identifying the protein kinase C 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 [143]. 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 [144], 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 phosphorylation[65], 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) [144,145]. 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 [145].

6. Regulation by protein abundance

Stationary phase is the stage of growth where the synthesis of TAG predominates over the synthesis of phospholipids [83], and this change in lipid metabolism correlates with an increase in PAP activity [41,83]. That the *pah1*Δ mutation results in elevated phospholipids and a dramatic decrease in TAG in stationary phase [21,26] 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 causes dramatic reductions (50–60%) in Pah1p abundance [25,27]. 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 [27]. 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 [22,25,94], as well as the overexpression of Nem1p–Spo7p [20], is deleterious to growth [20,22,25,94]. 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.

7. Perspectives/future developments

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.

Phosphorylation is clearly a key modulator of PAP function. This process is mediated by multiple kinases, the action of which will likely result in different physiological outcomes. The effect of phosphorylation by the cell-cycle regulated kinases Pho85p–Pho80p and Cdc28p-cyclin B and by protein kinase A has been examined. Although other kinases have been shown to phosphorylate Pah1p, their individual effects on PAP activity have yet to be elucidated. While each of these kinases can phosphorylate Pah1p in the absence of prior phosphorylations by other kinases, it is not known whether phosphorylation at a particular site by one kinase might stimulate or inhibit the phosphorylation of other sites by the same kinase, or by different kinases. Thus, studies examining the interdependencies of the various phosphorylations will advance our understanding of the mechanisms that fine-tune PAP function.

Dephosphorylation of Pah1p by the Nem1p–Spo7p complex governs its subcellular localization, thereby affecting PAP physiological activity. Additional knowledge of the factors regulating Nem1p–Spo7p function will further our understanding of the mechanism by which Pah1p translocates to the membrane and thus becomes activated in vivo. In this regard, structural information of Pah1p will also shed light on the mechanisms underlying its interaction with the membrane.

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