JLR REVIEW

A review of phosphatidate phosphatase assays

Prabuddha Dey[1], **Gil-Soo Han**[1], **and George M. Carman**[1,*]

[1]Department of Food Science and the Rutgers Center for Lipid Research, New Jersey Institute for Food, Nutrition, and Health, Rutgers University, New Brunswick, NJ, USA

Abstract **Phosphatidate phosphatase (PAP) catalyzes the penultimate step in the synthesis of triacylglycerol and regulates the synthesis of membrane phospholipids. There is much interest in this enzyme because it controls the cellular levels of its substrate, phosphatidate (PA), and product, DAG; defects in the metabolism of these lipid intermediates are the basis for lipid-based diseases such as obesity, lipodystrophy, and inflammation. The measurement of PAP activity is required for studies aimed at understanding its mechanisms of action, how it is regulated, and for screening its activators and/or inhibitors. Enzyme activity is determined through the use of radioactive and nonradioactive assays that measure the product, DAG, or P_i. However, sensitivity and ease of use are variable across these methods. This review summarizes approaches to synthesize radioactive PA, to analyze radioactive and nonradioactive products, DAG and P_i, and discusses the advantages and disadvantages of each PAP assay.**

Supplementary key words Pah1 • lipin • enzyme assays • radioactive assays • nonradioactive assays • diacylglycerol • triacylglycerol • lipid metabolism

PAP IS AN IMPORTANT ENZYME IN LIPID METABOLISM

Phosphatidate phosphatase [PAP (3-*sn*-phosphatidate phosphohydrolase; EC 3.1.3.4)] catalyzes the Mg^{2+}-dependent dephosphorylation of phosphatidate (PA) to yield DAG (**Fig. 1**). The enzyme reaction was first described in 1957 from chicken liver extracts by Kennedy and coworkers (1). The purification of PAP to near homogeneity was achieved from yeast[2] in 1989 (2), but it was not until 2006 that the gene (i.e., *PAH1*) coding for the yeast enzyme was identified (3). The discovery of yeast *PAH1* revealed that the PAP-encoding gene is conserved in eukaryotes, including fungi (4), plants (5, 6), worms (7), flies (8, 9), mice (10, 11), and humans (3, 12). Whereas yeast PAP is also known as Pah1, the mouse and human forms of PAP, which are encoded by the *Lpin 1*, *2*, and *3* and *LPIN 1*, *2*, and *3* genes, respectively, are also known as lipin (10, 11). In organisms studied thus far, PAP activity is dependent on the D*X*D*X*(T/V) catalytic motif in the haloacid dehalogenase-like domain (10, 13, 14). The enzyme is extensively modified posttranslationally by phosphorylation, which acutely regulates its catalytic activity, subcellular localization, and protein stability (15–17).

PAP is a key lipid metabolic enzyme that is required for the synthesis of the neutral lipid triacylglycerol (TAG) and major membrane phospholipids (16–18) (Fig. 1). The enzyme product, DAG, is a direct precursor of TAG (16, 19–22), whereas its substrate, PA, is a direct precursor of the liponucleotide, CDP-DAG, a key intermediate in phospholipid synthesis that is converted in mammals to PI and cardiolipin (19) and in yeast to all major membrane phospholipids (16) (Fig. 1). In yeast and mammals (16, 19), the product, DAG, is also used for the synthesis of the phospholipids, PC and PE (Fig. 1). PA and DAG are also known to facilitate membrane fission/fusion events (23–28) and play roles in vesicular trafficking (29–33) and cell signaling (16, 34–39). Accordingly, altered levels of PAP activity confer a significant effect on lipid metabolism and cellular processes. For example, the lack of PAP in yeast causes drastic changes in lipid synthesis and cellular defects (e.g., reduced levels of TAG and lipid droplets, vacuole fragmentation, elevated levels of PA and membrane phospholipids, derepression of phospholipid synthesis genes, and aberrant nuclear/endoplasmic reticulum membrane expansion) (3, 13, 34, 40–52), and the enzyme deficiency in mammals causes lipid-based diseases (e.g., lipodystrophy, rhabdomyolysis, insulin resistance, hepatic steatosis, peripheral neuropathy, metabolic syndrome, chronic recurrent multifocal osteomyelitis congenital dyserythropoietic anemia, and type 2 diabetes) (10, 53–62).

Determination of PAP activity is essential for studying the enzyme function. In addition, the activity measurement is required to understand the mechanism of the enzyme action as well as its regulation. The PAP assay, optimized for high-throughput screening, would be valuable to discover the enzyme inhibitors and activators that are effective in ameliorating disease conditions such as lipodystrophy and obesity. In this review, we summarize PAP assays, which are conducted with radioactive and nonradioactive substrates, and discuss the advantages and disadvantages of each assay for its application to appropriate experimental conditions.

[2]The term yeast is used interchangeably with *Saccharomyces cerevisiae*.

*For correspondence: George M. Carman, gcarman@rutgers.edu.

DOI 10.1194/jlr.R120001092

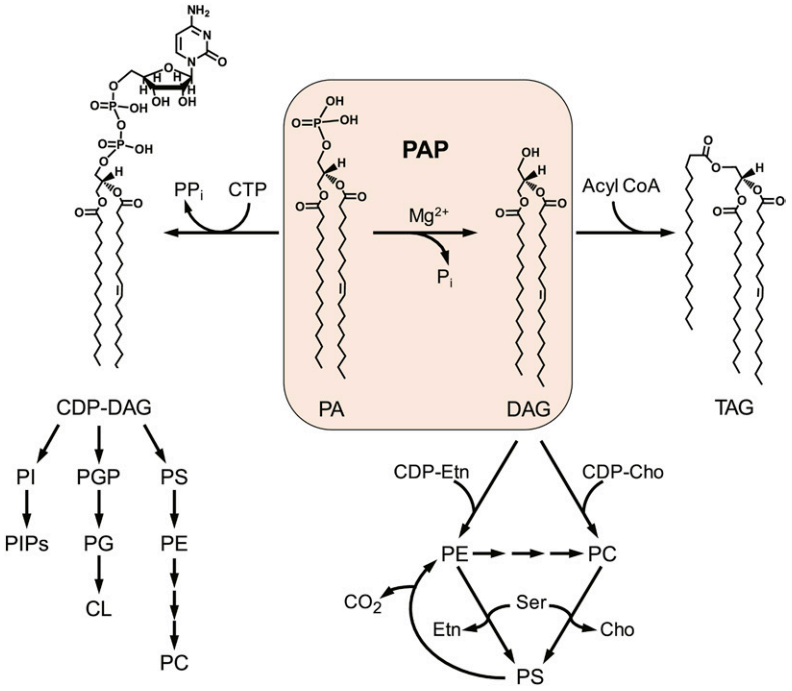

Fig. 1. Role of PAP in the synthesis of TAG and membrane phospholipids in yeast and mammals. The structures of CDP-DAG, PA, DAG, and TAG are shown with the C16:0 and C18:1 fatty acyl groups. PAP plays a major role in governing whether cells utilize PA for the synthesis of TAG via DAG or for the synthesis of membrane phospholipids via CDP-DAG. The DAG produced in the PAP reaction is also used for the synthesis of phospholipids. More detailed pathways for the synthesis of phospholipids in yeast (38) and mammals (19) are found elsewhere. Cho, choline; Etn, ethanolamine; PGP, hosphatidylglycerophosphate; PIPs, phosphoinositides; Ser, serine.

INCORPORATION OF PA INTO DETERGENT MICELLES AND LIPOSOMES

Natural PA, which contains long-chain fatty acyl groups on positions 1 and 2 of the glycerol backbone, is a water-insoluble substrate that should be presented to PAP in an aqueous environment that is buffered to the pH optimum (e.g., pH 7.5) for the enzyme reaction (2, 3, 12). The two most popular methods for delivery of PA to the assay system, which are discussed below, include its incorporation into detergent micelles and unilamellar phospholipid vesicles (liposomes). Water-soluble PA analogs containing short-chain (e.g., DiC8:0) fatty acyl groups are directly added to the aqueous assay system (63). Sodium, potassium, or ammonium salts of PA should be used in PAP assays; calcium should be avoided as it interferes with the reaction.

Incorporation of PA into detergent micelles

In the original work (1), PA derived from egg lecithin is added to the assay system as an undefined emulsified form. Sonicated mixtures of PA and PC are also utilized to measure PAP activity (11, 64, 65), but the size and uniformity of the substrate-containing aggregates/vesicles are unknown. PAP activity from undefined substrates in the assay system is not conducive to meaningful data for understanding the mode of enzyme action and its kinetic properties. PAP, like other interfacial enzymes, acts on the substrate through a three-dimensional interaction in solution as well as through a two-dimensional surface interaction, and accordingly both of the enzyme-substrate interactions must be taken into account in the assay (66). Triton X-100 forms a mixed micelle with PA, providing a surface for catalysis (67). The use of detergent/phospholipid-mixed micelles makes it possible to analyze PAP activity that is dependent on the molar and surface concentrations of PA (3, 67, 68) according to the "surface dilution kinetics" model (66). The detergent/phospholipid-mixed micelle system is also useful in assessing the regulation of activity by phospholipids (69), sphingolipids (70), and nucleotides (71), as well as by the enzyme's posttranslational modification by phosphorylation (72–75).

Triton X-100 micelles containing PA are easily prepared by dissolving the dried phospholipid in the detergent solution (67), and the size and uniformity of Triton X-100/PA-mixed micelles are determined by gel filtration chromatography or by glycerol density gradient centrifugation (67, 69, 70). Additional lipids may be incorporated into the Triton X-100/PA-mixed micelles to examine their effects on PAP activity as activators or inhibitors (69, 70).

Incorporation of PA into liposomes

Although the Triton X-100/PA-mixed micelle is useful in measuring PAP activity to assess biochemical mechanisms of the enzyme regulation (3, 67, 69, 71–76), it does not simulate the membrane phospholipid bilayer. Liposomes are a widely accepted mimic of the biological membrane (77–79), and they have been used to measure PAP activity (11, 65, 80–83). In the assay, the phospholipid vesicles resembling the complex composition of the nuclear/endoplasmic reticulum membrane (e.g., PC/PE/PI/PS/PA) are shown to be better substrates than those composed of only PC (and/or PE) and PA (83). The size of liposomes also affects PAP activity; greater activity is elicited with larger liposomes (e.g., ⩾100 nm) when compared with smaller liposomes (e.g., 50 nm) (83). Of various methods to prepare the unilamellar vesicles (84), the most popular one is to extrude a phospholipid suspension through a membrane filter with a defined pore size (79). The size and distribution of the liposomes are assessed by light scattering (e.g., particle size analyzer) (85) or by gel filtration chromatography (86).

PAP activity on the PA-containing liposomes, like that on Triton X-100/PA-mixed micelles (3, 67, 68), is dependent on the molar and surface concentrations of PA when the substrate is incorporated into liposomes composed of complex phospholipid mixtures (83). The dependence of PAP activity on the molar concentration of PA indicates that the enzyme operates in the hopping mode (87). Kinetic analysis with these liposomes also demonstrates that PAP activity is dependent on the surface concentration of PA, and thus the enzyme also operates in the scooting mode (87).

PAP ASSAYS

The PAP assay typically measures the formation of reaction products (DAG and/or P_i) over time (65, 88, 89). To be in a linear range of activity, the enzyme assay is conducted such that less than 15% of the substrate PA is converted to products. The optimum reaction conditions of the PAP assay (e.g., pH, temperature, Mg^{2+}, and saturating concentrations of PA) need to be defined so that the catalytic activity is dependent on incubation time and the enzyme amount (i.e., zero order kinetics) (2, 3, 12). By convention (90), PAP activity is expressed as the unit of the enzyme amount that catalyzes the formation of 1 μmol of product per minute, and its specific activity is defined as the enzyme activity (i.e., units) per milligram of protein (65, 88). For low levels of PAP activity from cell extracts or crude enzyme preparations, it is often reported in nanomoles (or picomoles) per minure. The turnover number (molecular activity), which requires the molecular mass information of PAP, is defined as the mole product formed per mole of enzyme per second. PAP activity has been measured with different sizes and forms (e.g., native, radioactive, and fluorescent) of PA.

RADIOACTIVE ASSAYS

In the radioactive PAP assay, the substrate PA contains a radiolabel in the phosphate moiety (e.g., ^{32}P) or in the glycerol backbone or the fatty acyl moieties (e.g., ^{14}C). The radioactive PA molecules may not be readily available, and thus, we review their syntheses here (65, 88, 91, 92) (**Fig. 2**). [^{32}P]PA is synthesized from DAG and [γ-^{32}P]ATP by DAG kinase [Fig. 2, reaction 1 (88, 89)]. The ^{14}C-labeled PA in the glycerol backbone is synthesized from L-[^{14}C(U)]glycerol-3-P and fatty acyl CoA derivatives (e.g., 16:0 CoA or 18:1 CoA) by using rat liver (92) or yeast (88, 93) microsomes that contain glycerol-3-P acyltransferase and lyso-PA acyltransferase (Fig. 2, reaction 2). Likewise, PA radiolabeled in the fatty acyl moiety is synthesized from glycerol-3-P and the ^{14}C-labeled fatty acyl CoA derivative (e.g., 16:0 CoA or 18:1 CoA) (Fig. 2, reaction 3) (91). The radioactive PA may also be produced from ^{14}C-labeled PC by phospholipase D (Fig. 2, reaction 4) (94). The enzymatically synthesized radioactive PA needs to be purified, generally by TLC on silica gel (65, 88), and diluted with unlabeled PA to a desired specific radioactivity (e.g., 500–50,000 cpm/nmol).

In the PAP assay, to measure the release of $^{32}P_i$ from [^{32}P]PA, the enzyme reaction is terminated (e.g., with acid) and then subjected to a chloroform/methanol-water phase partition (95, 96), and a portion of the aqueous phase is measured for radioactivity by scintillation counting (88). In the PAP assay, to measure the production of the [^{14}C]DAG from [^{14}C]PA, the radioactive substrate and product are extracted in chloroform (e.g., chloroform/methanol-water phase partition) and then separated by TLC on silica gel. The silica gel spot containing the [^{14}C]DAG is scraped off from the TLC plate and measured for radioactivity by scintillation counting (65, 91, 92). Alternatively, [^{14}C]DAG on the TLC plate is quantified by phosphorimaging analysis with ^{14}C standards. Without the chromatographic separation, [^{14}C]PA in the chloroform extract is precipitated by aluminum oxide, and the supernatant containing the [^{14}C]DAG is measured for radioactivity by scintillation counting (65, 92).

Advantages of radioactive assays

The major advantages of the radioactive assays are specificity and sensitivity. Because of the radioactive nature of the substrate, the substrate-product relationship of the PAP reaction is defined even in a crude system (1). Moreover, studies to examine a reaction mechanism are facilitated by the use of radioactive substrates and products (97). The radioactive assay is particularly useful in measuring a low level of PAP activity. The sensitivity of the assay is limited only by the specific radioactivity of the precursor molecules used for the synthesis of radioactive PA. The measurement of the product $^{32}P_i$ from [^{32}P]PA is simpler and more sensitive when compared with the measurement of the product [^{14}C]DAG from [^{14}C]PA. In the former assay, the water-soluble $^{32}P_i$ is easily separated from the chloroform-soluble [^{32}P]PA by a chloroform/methanol-water phase partition. In contrast, the latter assay requires the extraction of both [^{14}C]DAG and [^{14}C]PA in chloroform, and then the separation of the radioactive substrate and product by TLC or by the precipitation of PA with aluminum oxide (65, 92). Compared with [^{14}C]PA, [^{32}P]PA can be synthesized to be more radioactive because of the high specific radioactivity of [γ-^{32}P]ATP.

Disadvantages of radioactive assays

The major disadvantage of the radioactive PAP assay is that its substrate is not always readily available and must be synthesized and purified. The use of radioactive chemicals requires institute authorization and specialized equipment (e.g., scintillation counter and/or phosphorimager) that is not universally available. Of course, radioactive contamination (e.g., personal and laboratory space/equipment) is also a deterrent to performing radioactive assays. Additionally, the specific radioactivity of the ^{14}C-labeled precursors used for radiolabeling PA in the phosphatidyl moiety is lower when compared with that of the [γ-^{32}P]ATP used for radiolabeling the phosphate moiety. Accordingly, the sensitivity of the assay that measures [^{14}C]DAG is lower when compared with that of the assay measuring $^{32}P_i$. Moreover, PAP activity measured from crude extracts using [^{32}P]PA

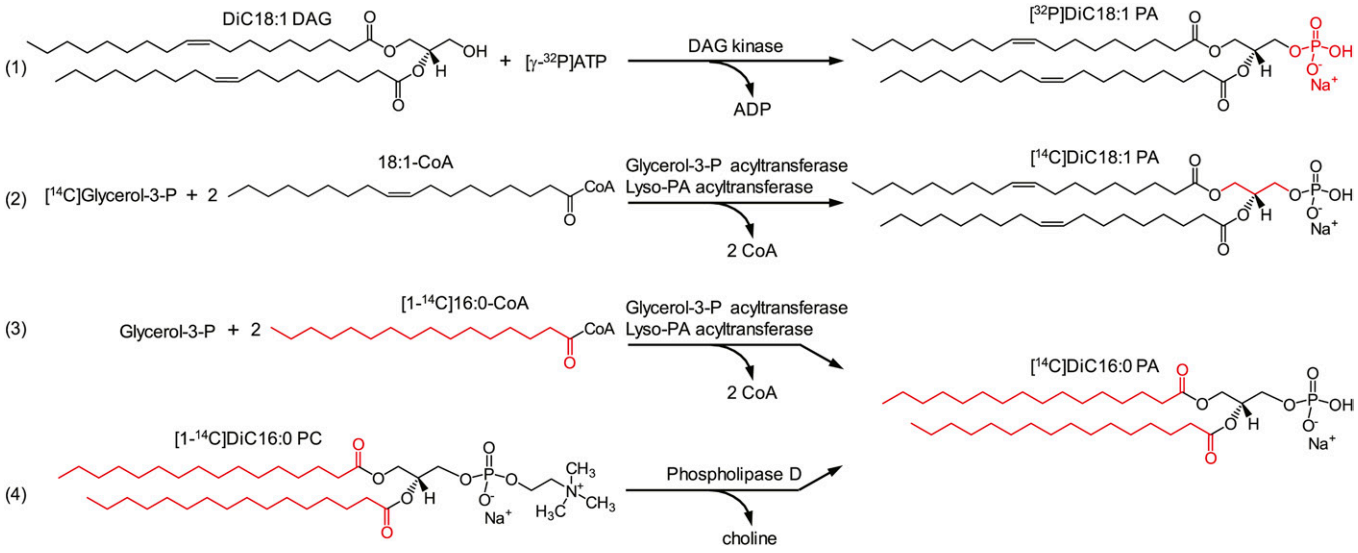

Fig. 2. Synthesis of radioactive PA. The figure outlines the enzymatic reactions used to synthesize PA with label in the phosphate moiety (reaction 1), glycerol backbone (reaction 2), and fatty acyl groups (reactions 3 and 4). The radioactive moieties in PA are depicted in red color for emphasis; the phosphorous atom is labeled in [^{32}P]PA and the first carbon atom in the acyl moiety is labeled in [^{14}C]PA.

or [^{14}C]PA may be compromised by the presence of phospholipase, lysophospholipase, neutral lipase, and alkaline phosphatase activities (65). These competing activities must be taken into account. For example, when glycerol-3-P is produced from PA, its conversion to $^{32}P_i$ may be minimized by the addition of racemic glycerol-3-P to the assay, and when DAG is hydrolyzed by lipases, their reactions may be prevented with DAG lipase inhibitors (65).

NONRADIOACTIVE ASSAYS

In the original work, Kennedy and coworkers (1) measured the production of P_i from PA by a colorimetric assay with the molybdate reagent first described in 1925 by Fiske and Subbarow (98). The sensitivity and stability of the molybdate reagent for measuring PAP activity has been improved by its combination with malachite green (12, 63, 99–102). The P_i produced in the PAP assay is measured after the removal of the enzyme by precipitation with trichloroacetic acid, which is also used to terminate the reaction (1). Alternatively, P_i is separated from PA by a chloroform/methanol-water phase partition (12, 95, 96). When water-soluble C8:0 PA is used as a substrate, the P_i released in the PAP reaction is directly complexed with the malachite green-molybdate reagent, which is also used to stop the enzyme reaction (63).

In the original work (1), the DAG produced in the PAP reaction is extracted with ether, purified by silica gel chromatography, and quantified by ester analysis (1); this method of DAG quantification is tedious and impractical for a routine PAP assay. As discussed above in the Radioactive Assays section, DAG is extracted from the reaction mixture by a simple chloroform/methanol-water phase partition (95, 96) and then separated from the coextracted PA by TLC or by the precipitation of PA with aluminum oxide (92). The lipids on the silica gel plate are detected by charring or by staining with iodine vapor (103, 104) or the fluorescent lipophilic dye, primulin (105). The chloroform-soluble DAG is also analyzed by HPLC with an evaporative light scattering detector (106) or by mass spectrometry (107).

The sensitivity of the nonradioactive assay in measuring PAP activity is enhanced by utilization of fluorescently labeled substrates such as NBD PA (108) and BODIPY PA (109). The fluorescent product (e.g., NBD DAG or BODIPY DAG) and substrate (NBD PA or BODIPY PA) are separated by TLC and then detected by fluorescence scanning (46) or analyzed by HPLC with a fluorescence detector (108, 109).

Advantages of nonradioactive assays

The obvious advantages of nonradioactive PAP assays are in the availability of PA and the reagents to analyze the reaction products. If short chain PA (e.g., DiC8:0) is used as substrate, there is no need to perform a chloroform/methanol-water phase partition after the reaction is completed (63). The sensitivities of the P_i measurement by the colorimetric assay and the DAG measurement by TLC or HPLC are in the nanomolar range. These assays are best suited for PAP samples with high activity (e.g., purified or overexpressed enzyme) that are devoid of, or less affected by, competing lipolytic and phosphatase activities. Fluorescently labeled PA is readily available, and the quantification of fluorescent DAG by TLC or HPLC is sensitive in the nanomolar to picomolar range. The fluorescence PAP assay is useful for measuring activity in crude extracts (46, 108, 109) or with purified enzyme (14) owing to the fluorescent nature of the substrates and products of the reaction.

Disadvantages of nonradioactive assays

The measurement of P_i by colorimetric assay with the malachite green-molybdate reagent may produce high background due to nonspecific phosphatase activities in crude enzyme samples and/or residual phosphate from cleaning detergents

left in test tubes. The colored complex is not stable for long periods of time, and although used in some systems (110), the assay is not conducive for measurement of crude enzyme preparations. Measurement of DAG from nonradioactive PA requires separation by TLC or HPLC, and is beset by nonspecific phospholipase, lysophospholipase, and neutral lipase activities in crude enzyme preparations. Short-chain PA, NBD PA, and BODIPY PA are not physiologically relevant substrates, and quantification of the fluorescent PAP enzyme products requires specialized equipment for detection. The advantages and disadvantages of radioactive and nonradioactive PAP assays are summarized in **Table 1**.

METHODS TO DIFFERENTIATE Mg^{2+}-DEPENDENT AND Mg^{2+}-INDEPENDNET PAP ACTIVITIES IN CRUDE CELL EXTRACTS

The PAP discussed above is a Mg^{2+}-dependent enzyme whose activity is based on the D*X*D*X*(T/V) catalytic motif in the haloacid dehalogenase-like domain (10, 13, 14). However, there is a nonspecific PAP [previously known as PAP2 and now known as lipid phosphate phosphatase (LPP) (111–113] that does not require Mg^{2+} or any other divalent cation (114–116), and its activity is directed by a three-domain catalytic motif consisting of the sequences KX_6RP, PSGH, and SRX_5HX_3D (117). Most of the PAP2/LPP activity in yeast is encoded by the *DPP1* (118) and *LPP1* (119) genes, whereas the activity is encoded by *LPP/Lpp* genes in higher eukaryotes (113, 120, 121). Unlike the yeast and mammalian PAP enzymes, which are specific for PA (3, 12), the LPP enzymes in yeast and mammalian cells have a broad substrate specificity. In addition to PA, other substrates of LPP enzymes include lysoPA, DAG pyrophosphate, sphingoid base phosphates, and isoprenoid phosphates (113, 118–127), which are known to play signaling roles in cell physiology. Thus, PAP activity involved with de novo lipid synthesis needs to be differentiated from PAP2/LPP activity involved in lipid signaling.

Mammalian PAP and PAP2/LPP enzymes are differentiated by their sensitivity to the thioreactive compound, *N*-ethylmaleimide (NEM) (65, 111, 112). However, in yeast, NEM sensitivity is not applicable to differentiate the Mg^{2+}-dependent and -independent PAP activities (3, 122, 123, 128, 129). Thus, NEM sensitivity cannot be used as a general approach to differentiate PAP and PAP2/LPP enzymes. To differentiate the two activities based on

TABLE 1. Advantages and disadvantages of PAP radioactive and nonradioactive assays

Substrate	Product Analyzed	Analysis	Advantages	Disadvantages
Radioactive assay				
[^{32}P]PA	$^{32}P_i$	Chloroform/methanol-water phase partition followed by scintillation counting	Sensitive in the picomolar range; PA synthesis reagents readily available; radioactivity measured directly after chloroform/methanol-water phase partition; facilitates mechanistic studies; conducive to crude enzyme preparations	[^{32}P]PA not commercially available and must be synthesized and purified and has a short half-life; requires radioactive authorization and scintillation counter
[^{14}C]PA	[^{14}C]DAG	Chloroform/methanol-water phase partition followed by TLC and scintillation counting or phosphorimaging; aluminum oxide precipitation of PA followed by scintillation counting	Sensitive in the nanomolar range; PA synthesis reagents readily available; facilitates mechanistic studies; conducive to crude enzyme preparations; [^{14}C]PA has a long half-life	[^{14}C]PA not readily available; [^{14}C] glycerol-3-P, [1-^{14}C]palmitoyl-CoA, and [1-^{14}C]dipalmitoyl-PC are expensive and radioactive specific activity low compared with [γ-^{32}P]ATP; requires separation of DAG from PA following phase partition; requires radioactive authorization, and scintillation counter or phosphorimager
Nonradioactive assay				
PA	P_i	Chloroform/methanol-water phase partition or TCA precipitation followed by colorimetric determination	Sensitive in the nanomolar to micromolar range; unlabeled PA and assay reagents readily available; spectrophotometric analysis of malachite green-molybdate-P_i measured directly after phase partition or TCA precipitation filtrates; phase partition not required with use of water-soluble DiC8 PA substrate	Not conducive for measurement of crude enzyme preparations due to high P_i background; malachite green-molybdate-P_i complex color unstable; detergents used to solubilize long chain PA give high background color; test tubes with phosphate residue from cleansers gives high background; DiC8 PA is not physiologically relevant
PA	DAG	Chloroform/methanol-water phase partition followed by TLC with charring or staining, HPLC with evaporative light scattering detection, or mass spectrometry	Sensitive in nanomolar range; unlabeled PA and assay reagents readily available	Requires separation of DAG from PA following phase partition; HPLC and/or mass spectrometry equipment required; not conducive for measurement of crude enzyme preparations due to phospholipase, lysophospholipase, neutral lipase, alkaline phosphatase activities that interfere with product analysis
NBD PA or BODIPY PA	NBD DAG or BODIPY DAG	Chloroform/methanol-water phase partition followed by TLC or HPLC with fluorescence detection	Sensitive in the nanomolar range; fluorescent-labeled PA substrates readily available; conducive to crude enzyme preparations	NBD PA and BODIPY PA are not physiologically relevant; requires separation of DAG from PA following phase partition; requires fluorescence detection equipment

cofactor requirement, the total PAP activity is measured in the presence of 1 mM of $MgCl_2$, and the Mg^{2+}-independent activity is separately measured in the absence of $MgCl_2$ but in the presence of 1 mM of EDTA, which is added to chelate the residual divalent cation in the cell extract (53, 58). The Mg^{2+}-dependent activity is then determined by subtracting the Mg^{2+}-independent activity from the total PAP activity (53, 58). In cases where the Mg^{2+}-dependent PAP is encoded by more than one gene, then the enzyme activity encoded by the gene of interest can be accurately measured in the absence of the unwanted activity [e.g., knockout or knockdown of the unwanted PAP-encoding gene(s)] (128).

PAP ASSAY RECOMMENDATIONS

For analysis of a pure PAP preparation with high specific activity, the most convenient is a nonradioactive assay that measures P_i with the malachite green-molybdate reagent. Our laboratory has used the colorimetric assay to characterize the PAP activity of purified human lipin 1 with respect to PA substrate specificity in detergent/PA-mixed micelles and to screen the enzyme inhibitors and activators (12). We have also used this assay to examine the hopping and scooting modes of purified yeast Pah1 PAP with liposomes (83). If the focus is to study the PAP reaction in a system that mimics the in vivo condition of a membrane phospholipid bilayer, then the incorporation of PA into liposomes is recommended. Otherwise, the detergent/PA-mixed micelle system is recommended. In either case, we recommend that saturating molar and surface concentrations of PA be used for routine assays (3, 12, 67) to ensure zero order kinetics. The colorimetric assay with purified PAP should be applicable to high throughput screening of activators/inhibitors in 96-well plates. Utilization of DiC8 PA in the assay obviates the need to separate the product P_i from the substrate PA following the reaction (63). That the short-chain PA is not physiologically relevant may not be an issue in a screen when a purified enzyme is utilized. To our knowledge, a high throughput PAP assay has not yet been developed but would be of great use in the search of enzyme effector molecules. For analysis of crude PAP preparations with low specific activity, a radioactive assay measuring the release of $^{32}P_i$ from [^{32}P]PA is recommended. This assay is very sensitive and limited only by the specific radioactivity of [γ-^{32}P]ATP used for the synthesis of [^{32}P]PA. If the radioactive assay is not available for crude PAP preparations, we recommend a nonradioactive assay with the fluorescent substrate NBD PA or BODIPY PA. As discussed above, however, the fluorescence assay requires the separation of the product from substrate by TLC or HPLC, which is a cumbersome component of the PAP assay that is shared by other nonradioactive and radioactive assays to measure the product DAG. For differentiating PAP and PAP2/LPP activities, we recommend the genetic approach to eliminate either form of the activity, and when not possible, utilize assays conducted with and without Mg^{2+} ions.

Acknowlegments

The authors thank Peter J. Carman, Joanna M. Kwiatek, and Geordan J. Stukey for helpful comments in the preparation of the manuscript.

Author ORCIDs

Gil-Soo Han https://orcid.org/0000-0002-8170-854X; George M. Carman https://orcid.org/0000-0003-4951-8233

Funding and additional information

This work was supported, in whole or in part, by National Institutes of Health United States Public Health Service Grants GM028140, GM050679, and GM136128. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

LPP, lipid phosphate phosphatase; NBD PA, 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4yl)amino]hexanoyl}-*sn*-glycero-3-phosphate; NEM, *N*-ethylmaleimide; PA, phosphatidate; PAP, phosphatidate phosphatase TAG, triacylglycerol.

Manuscript received August 21, 2020, and in revised form September 9, 2020. Published, JLR Papers in Press, September 22, 2020, DOI 10.1194/jlr.R120001092.

REFERENCES

1. Smith, S. W., S. B. Weiss, and E. P. Kennedy. 1957. The enzymatic dephosphorylation of phosphatidic acids. *J. Biol. Chem.* **228:** 915–922.
2. Lin, Y-P., and G. M. Carman. 1989. Purification and characterization of phosphatidate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264:** 8641–8645.
3. Han, G-S., W-I. Wu, and G. M. Carman. 2006. The *Saccharomyces cerevisiae* lipin homolog is a Mg^{2+}-dependent phosphatidate phosphatase enzyme. *J. Biol. Chem.* **281:** 9210–9218.
4. Liu, N., Y. Yun, Y. Yin, M. Hahn, Z. Ma, and Y. Chen. 2019. Lipid droplet biogenesis regulated by the FgNem1/Spo7-FgPah1 phosphatase cascade plays critical roles in fungal development and virulence in Fusarium graminearum. *New Phytol.* **223:** 412–429.
5. Nakamura, Y., R. Koizumi, G. Shui, M. Shimojima, M. R. Wenk, T. Ito, and H. Ohta. 2009. Arabidopsis lipins mediate eukaryotic pathway of lipid metabolism and cope critically with phosphate starvation. *Proc. Natl. Acad. Sci. USA.* **106:** 20978–20983.
6. Eastmond, P. J., A. L. Quettier, J. T. Kroon, C. Craddock, N. Adams, and A. R. Slabas. 2010. Phosphatidic acid phosphohydrolase 1 and 2 regulate phospholipid synthesis at the endoplasmic reticulum in Arabidopsis. *Plant Cell.* **22:** 2796–2811.
7. Golden, A., J. Liu, and O. Cohen-Fix. 2009. Inactivation of the C. elegans lipin homolog leads to ER disorganization and to defects in the breakdown and reassembly of the nuclear envelope. *J. Cell Sci.* **122:** 1970–1978.
8. Valente, V., R. M. Maia, M. C. Vianna, and M. L. Paco-Larson. 2010. Drosophila melanogaster lipins are tissue-regulated and developmentally regulated and present specific subcellular distributions. *FEBS J.* **277:** 4775–4788.
9. Ugrankar, R., Y. Liu, J. Provaznik, S. Schmitt, and M. Lehmann. 2011. Lipin is a central regulator of adipose tissue development and function in *Drosophila*. *Mol. Cell. Biol.* **31:** 1646–1656.

10. Péterfy, M., J. Phan, P. Xu, and K. Reue. 2001. Lipodystrophy in the *fld* mouse results from mutation of a new gene encoding a nuclear protein, lipin. *Nat. Genet.* **27:** 121–124.
11. Donkor, J., M. Sariahmetoglu, J. Dewald, D. N. Brindley, and K. Reue. 2007. Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. *J. Biol. Chem.* **282:** 3450–3457.
12. Han, G-S., and G. M. Carman. 2010. Characterization of the human *LPIN1*-encoded phosphatidate phosphatase isoforms. *J. Biol. Chem.* **285:** 14628–14638.
13. Han, G-S., S. Siniossoglou, and G. M. Carman. 2007. The cellular functions of the yeast lipin homolog Pah1p are dependent on its phosphatidate phosphatase activity. *J. Biol. Chem.* **282:** 37026–37035.
14. Khayyo, V. I., R. M. Hoffmann, H. Wang, J. A. Bell, J. E. Burke, K. Reue, and M. V. Airola. 2020. Crystal structure of a lipin/Pah phosphatidic acid phosphatase. *Nat. Commun.* **11:** 1309.
15. Carman, G. M. 2011. The discovery of the fat-regulating phosphatidic acid phosphatase gene. *Front. Biol. (Beijing).* **6:** 172–176.
16. Kwiatek, J. M., G. S. Han, and G. M. Carman. 2020. Phosphatidate-mediated regulation of lipid synthesis at the nuclear/endoplasmic reticulum membrane. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids.* **1865:** 158434.
17. Reue, K., and H. Wang. 2019. Mammalian lipin phosphatidic acid phosphatases in lipid synthesis and beyond: metabolic and inflammatory disorders. *J. Lipid Res.* **60:** 728–733.
18. Carman, G. M., and G. S. Han. 2019. Fat-regulating phosphatidic acid phosphatase: a review of its roles and regulation in lipid homeostasis. *J. Lipid Res.* **60:** 2–6.
19. Vance, D. E. 2004. Glycerolipid biosynthesis in eukaryotes. *In* Biochemistry of Lipids, Lipoproteins and Membranes. 5th edition. D. E. Vance and J. Vance, editors. Elsevier Science Publishers B.V., Amsterdam. 153–181.
20. Weiss, S. B., E. P. Kennedy, and J. Y. Kiyasu. 1960. The enzymatic synthesis of triglycerides. *J. Biol. Chem.* **235:** 40–44.
21. Czabany, T., K. Athenstaedt, and G. Daum. 2007. Synthesis, storage and degradation of neutral lipids in yeast. *Biochim. Biophys. Acta.* **1771:** 299–309.
22. Sorger, D., and G. Daum. 2003. Triacylglycerol biosynthesis in yeast. *Appl. Microbiol. Biotechnol.* **61:** 289–299.
23. Liao, M. J., and J. H. Prestegard. 1979. Fusion of phosphatidic acid-phosphatidylcholine mixed lipid vesicles. *Biochim. Biophys. Acta.* **550:** 157–173.
24. Koter M., B. de Kruijiff, and L. L. van Deenen. 1978. Calcium-induced aggregation and fusion of mixed phosphatidylcholine-phosphatidic acid vesicles as studied by 31P NMR. *Biochim. Biophys. Acta.* **514:** 255–263.
25. Blackwood, R. A., J. E. Smolen, A. Transue, R. J. Hessler, D. M. Harsh, R. C. Brower, and S. French. 1997. Phospholipase D activity facilitates Ca2+-induced aggregation and fusion of complex liposomes. *Am. J. Physiol.* **272:** C1279–C1285.
26. Weigert, R., M. G. Silletta, S. Spano, G. Turacchio, C. Cericola, A. Colanzi, S. Senatore, R. Mancini, E. V. Polishchuk, M. Salmona, et al. 1999. CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. *Nature.* **402:** 429–433.
27. Goñi, F. M., and A. Alonso. 1999. Structure and functional properties of diacylglycerols in membranes. *Prog. Lipid Res.* **38:** 1–48.
28. Chernomordik, L., M. M. Kozlov, and J. Zimmerberg. 1995. Lipids in biological membrane fusion. *J. Membr. Biol.* **146:** 1–14.
29. Roth, M. G. 2008. Molecular mechanisms of PLD function in membrane traffic. *Traffic.* **9:** 1233–1239.
30. Morris, A. J. 2007. Regulation of phospholipase D activity, membrane targeting and intracellular trafficking by phosphoinositides. *Biochem. Soc. Symp.* **74:** 247–257.
31. Maissel, A., M. Marom, M. Shtutman, G. Shahaf, and E. Livneh. 2006. PKCeta is localized in the Golgi, ER and nuclear envelope and translocates to the nuclear envelope upon PMA activation and serum-starvation: C1b domain and the pseudosubstrate containing fragment target PKCeta to the Golgi and the nuclear envelope. *Cell. Signal.* **18:** 1127–1139.
32. Lehel, C., Z. Olah, G. Jakab, Z. Szallasi, G. Petrovics, G. Harta, P. M. Blumberg, and W. B. Anderson. 1995. Protein kinase C epsilon subcellular localization domains and proteolytic degradation sites. A model for protein kinase C conformational changes. *J. Biol. Chem.* **270:** 19651–19658.
33. Baron, C. L., and V. Malhotra. 2002. Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. *Science.* **295:** 325–328.
34. Santos-Rosa, H., J. Leung, N. Grimsey, S. Peak-Chew, and S. Siniossoglou. 2005. The yeast lipin Smp2 couples phospholipid biosynthesis to nuclear membrane growth. *EMBO J.* **24:** 1931–1941.
35. Han, G-S., and G. M. Carman. 2017. Yeast *PAH1*-encoded phosphatidate phosphatase controls the expression of *CHO1*-encoded phosphatidylserine synthase for membrane phospholipid synthesis. *J. Biol. Chem.* **292:** 13230–13242.
36. Kudo, S., H. Shiino, S. Furuta, and Y. Tamura. 2020. Yeast transformation stress, together with loss of Pah1, phosphatidic acid phosphatase, leads to Ty1 retrotransposon insertion into the INO4 gene. *FASEB J.* **34:** 4749–4763.
37. Carman, G. M., and S. A. Henry. 2007. Phosphatidic acid plays a central role in the transcriptional regulation of glycerophospholipid synthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **282:** 37293–37297.
38. Henry, S. A., S. Kohlwein, and G. M. Carman. 2012. Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. *Genetics.* **190:** 317–349.
39. Dey, P., W. M. Su, G. S. Han, and G. M. Carman. 2017. Phosphorylation of lipid metabolic enzymes by yeast Pkc1 protein kinase C requires phosphatidylserine and diacylglycerol. *J. Lipid Res.* **58:** 742–751.
40. Pascual, F., A. Soto-Cardalda, and G. M. Carman. 2013. *PAH1*-encoded phosphatidate phosphatase plays a role in the growth phase- and inositol-mediated regulation of lipid synthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **288:** 35781–35792.
41. Adeyo, O., P. J. Horn, S. Lee, D. D. Binns, A. Chandrahas, K. D. Chapman, and J. M. Goodman. 2011. The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. *J. Cell Biol.* **192:** 1043–1055.
42. Fakas, S., Y. Qiu, J. L. Dixon, G-S. Han, K. V. Ruggles, J. Garbarino, S. L. Sturley, and G. M. Carman. 2011. Phosphatidate phosphatase activity plays a key role in protection against fatty acid-induced toxicity in yeast. *J. Biol. Chem.* **286:** 29074–29085.
43. Park, Y., G. S. Han, E. Mileykovskaya, T. A. Garrett, and G. M. Carman. 2015. Altered lipid synthesis by lack of yeast Pah1 phosphatidate phosphatase reduces chronological life span. *J. Biol. Chem.* **290:** 25382–25394.
44. Siniossoglou, S., H. Santos-Rosa, J. Rappsilber, M. Mann, and E. Hurt. 1998. A novel complex of membrane proteins required for formation of a spherical nucleus. *EMBO J.* **17:** 6449–6464.
45. Hassaninasab, A., G-S. Han, and G. M. Carman. 2017. Tips on the analysis of phosphatidic acid by the fluorometric coupled enzyme assay. *Anal. Biochem.* **526:** 69–70.
46. Sasser, T., Q. S. Qiu, S. Karunakaran, M. Padolina, A. Reyes, B. Flood, S. Smith, C. Gonzales, and R. A. Fratti. 2012. The yeast lipin 1 orthologue Pah1p regulates vacuole homeostasis and membrane fusion. *J. Biol. Chem.* **287:** 2221–2236.
47. Xu, X., and K. Okamoto. 2018. The Nem1-Spo7 protein phosphatase complex is required for efficient mitophagy in yeast. *Biochem. Biophys. Res. Commun.* **496:** 51–57.
48. Rahman, M. A., M. G. Mostofa, and T. Ushimaru. 2018. The Nem1/Spo7-Pah1/lipin axis is required for autophagy induction after TORC1 inactivation. *FEBS J.* **285:** 1840–1860.
49. Lussier M., A. M. White, J. Sheraton, T. di Paolo, J. Treadwell, S. B. Southard, C. I. Horenstein, J. Chen-Weiner, A. F. Ram, J. C. Kapteyn, et al. 1997. Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics.* **147:** 435–450.
50. Ruiz, C., V. J. Cid, M. Lussier, M. Molina, and C. Nombela. 1999. A large-scale sonication assay for cell wall mutant analysis in yeast. *Yeast.* **15:** 1001–1008.
51. Irie, K., M. Takase, H. Araki, and Y. Oshima. 1993. A gene, *SMP2*, involved in plasmid maintenance and respiration in *Saccharomyces cerevisiae* encodes a highly charged protein. *Mol. Gen. Genet.* **236:** 283–288.
52. Córcoles-Sáez, I., M. L. Hernandez, J. M. Martinez-Rivas, J. A. Prieto, and F. Randez-Gil. 2016. Characterization of the *S. cerevisiae* inp51 mutant links phosphatidylinositol 4,5-bisphosphate levels with lipid content, membrane fluidity and cold growth. *Biochim. Biophys. Acta.* **1861:** 213–226.
53. Nadra, K., A-S. De Preux Charles, J-J. Medard, W. T. Hendriks, G-S. Han, S. Gres, G. M. Carman, J-S. Saulnier-Blache, M. H. G.

Verheijen, and R. Chrast. 2008. Phosphatidic acid mediates demyelination in *Lpin1* mutant mice. *Genes Dev.* **22:** 1647–1661.
54. Zeharia A., A. Shaag, R. H. Houtkooper, T. Hindi, P. de Lonlay, G. Erez, L. Hubert, A. Saada, Y. de Keyzer, G. Eshel, et al. 2008. Mutations in *LPIN1* cause recurrent acute myoglobinuria in childhood. *Am. J. Hum. Genet.* **83:** 489–494.
55. Donkor, J., P. Zhang, S. Wong, L. O'Loughlin, J. Dewald, B. P. C. Kok, D. N. Brindley, and K. Reue. 2009. A conserved serine residue is required for the phosphatidate phosphatase activity but not transcriptional coactivator functions of lipin-1 and lipin-2. *J. Biol. Chem.* **284:** 29968–29978.
56. Zhang, P., M. A. Verity, and K. Reue. 2014. Lipin-1 regulates autophagy clearance and intersects with statin drug effects in skeletal muscle. *Cell Metab.* **20:** 267–279.
57. Wiedmann, S., M. Fischer, M. Koehler, K. Neureuther, G. Riegger, A. Doering, H. Schunkert, C. Hengstenberg, and A. Baessler. 2008. Genetic variants within the *LPIN1* gene, encoding lipin, are influencing phenotypes of the metabolic syndrome in humans. *Diabetes.* **57:** 209–217.
58. Mul, J. D., K. Nadra, N. B. Jagalur, I. J. Nijman, P. W. Toonen, J-J. Medard, S. Gres, A. de Bruin, G-S. Han, J. F. Browers, et al. 2011. A hypomorphic mutation in *Lpin1* induces progressively improving neuropathy and lipodystrophy in the rat. *J. Biol. Chem.* **286:** 26781–26793.
59. Ferguson, P. J., and H. I. El-Shanti. 2007. Autoinflammatory bone disorders. *Curr. Opin. Rheumatol.* **19:** 492–498.
60. Ferguson, P. J., S. Chen, M. K. Tayeh, L. Ochoa, S. M. Leal, A. Pelet, A. Munnich, S. Lyonnet, H. A. Majeed, and H. El-Shanti. 2005. Homozygous mutations in *LPIN2* are responsible for the syndrome of chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anaemia (Majeed syndrome). *J. Med. Genet.* **42:** 551–557.
61. Aulchenko, Y. S., J. Pullen, W. P. Kloosterman, M. Yazdanpanah, A. Hofman, N. Vaessen, P. J. L. M. Snijders, D. Zubakov, I. Mackay, M. Olavesen, et al. 2007. *LPIN2* is associated with type 2 diabetes, glucose metabolism and body composition. *Diabetes.* **56:** 3020–3026.
62. Zhang, P., L. S. Csaki, E. Ronquillo, L. J. Baufeld, J. Y. Lin, A. Gutierrez, J. R. Dwyer, D. N. Brindley, L. G. Fong, P. Tontonoz, et al. 2019. Lipin 2/3 phosphatidic acid phosphatases maintain phospholipid homeostasis to regulate chylomicron synthesis. *J. Clin. Invest.* **129:** 281–295.
63. Havriluk, T., F. Lozy, S. Siniossoglou, and G. M. Carman. 2008. Colorimetric determination of pure Mg^{2+}-dependent phosphatidate phosphatase activity. *Anal. Biochem.* **373:** 392–394.
64. Butterwith, S. C., R. Hopewell, and D. N. Brindley. 1984. Partial purification of soluble phosphatidate phosphatase from rat liver. *Biochem. J.* **220:** 825–833.
65. Martin, A., A. Gomez-Munoz, Z. Jamal, and D. N. Brindley. 1991. Characterization and assay of phosphatidate phosphatase. *Methods Enzymol.* **197:** 553–563.
66. Carman, G. M., R. A. Deems, and E. A. Dennis. 1995. Lipid signaling enzymes and surface dilution kinetics. *J. Biol. Chem.* **270:** 18711–18714.
67. Lin, Y-P., and G. M. Carman. 1990. Kinetic analysis of yeast phosphatidate phosphatase toward Triton X-100/phosphatidate mixed micelles. *J. Biol. Chem.* **265:** 166–170.
68. Karanasios, E., G-S. Han, Z. Xu, G. M. Carman, and S. Siniossoglou. 2010. A phosphorylation-regulated amphipathic helix controls the membrane translocation and function of the yeast phosphatidate phosphatase. *Proc. Natl. Acad. Sci. USA.* **107:** 17539–17544.
69. Wu, W-I., and G. M. Carman. 1996. Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by phospholipids. *Biochemistry.* **35:** 3790–3796.
70. Wu, W-I., Y-P. Lin, E. Wang, A. H. Merrill, Jr., and G. M. Carman. 1993. Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by sphingoid bases. *J. Biol. Chem.* **268:** 13830–13837.
71. Wu, W-I., and G. M. Carman. 1994. Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by nucleotides. *J. Biol. Chem.* **269:** 29495–29501.
72. Choi, H-S., W-M. Su, G-S. Han, D. Plote, Z. Xu, and G. M. Carman. 2012. Pho85p-Pho80p phosphorylation of yeast Pah1p phosphatidate phosphatase regulates its activity, location, abundance, and function in lipid metabolism. *J. Biol. Chem.* **287:** 11290–11301.
73. Su, W-M., G-S. Han, J. Casciano, and G. M. Carman. 2012. Protein kinase A-mediated phosphorylation of Pah1p phosphatidate phosphatase functions in conjunction with the Pho85p-Pho80p and Cdc28p-cyclin B kinases to regulate lipid synthesis in yeast. *J. Biol. Chem.* **287:** 33364–33376.
74. Su, W-M., G-S. Han, and G. M. Carman. 2014. Cross-talk phosphorylations by protein kinase C and Pho85p-Pho80p protein kinase regulate Pah1p phosphatidate phosphatase abundance in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **289:** 18818–18830.
75. Hassaninasab, A., L. S. Hsieh, W. M. Su, G. S. Han, and G. M. Carman. 2019. Yck1 casein kinase I regulates the activity and phosphorylation of Pah1 phosphatidate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **294:** 18256–18268.
76. Wu, W-I., and G. M. Carman. 2000. Kinetic analysis of sphingoid base inhibition of yeast phosphatidate phosphatase. *Methods Enzymol.* **312:** 373–380.
77. Enoch, H. G., and P. Stritmatter. 1979. Formation and properties of 1000 A diameter, single-bilayer phospholipid vesicles. *Proc. Natl. Acad. Sci. USA.* **76:** 145–149.
78. Szoka, F., and D. Papahadjopoulos. 1980. Comparative properties and methods of preparation of lipid vesicles (liposomes). *Annu. Rev. Biophys. Bioeng.* **9:** 467–508.
79. MacDonald, R. C., R. I. MacDonald, B. P. Menco, K. Takeshita, N. K. Subbarao, and L. R. Hu. 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta.* **1061:** 297–303.
80. Xu, Z., W-M. Su, and G. M. Carman. 2012. Fluorescence spectroscopy measures yeast *PAH1*-encoded phosphatidate phosphatase interaction with liposome membranes. *J. Lipid Res.* **53:** 522–528.
81. Eaton, J. M., G. R. Mullins, D. N. Brindley, and T. E. Harris. 2013. Phosphorylation of lipin 1 and charge on the phosphatidic acid head group control its phosphatidic acid phosphatase activity and membrane association. *J. Biol. Chem.* **288:** 9933–9945.
82. Boroda, S., S. Takkellapati, R. T. Lawrence, S. W. Entwisle, J. M. Pearson, M. E. Granade, G. R. Mullins, J. M. Eaton, J. Villen, and T. E. Harris. 2017. The phosphatidic acid-binding, polybasic domain is responsible for the differences in the phosphoregulation of lipins 1 and 3. *J. Biol. Chem.* **292:** 20481–20493.
83. Kwiatek, J. M., and G. M. Carman. 2020. Yeast phosphatidic acid phosphatase Pah1 hops and scoots along the membrane phospholipid bilayer. *J. Lipid Res.* **61:** 1232–1243.
84. Ostro, M. J., editor. 1983. Liposomes. Marcel Dekker, Inc., New York.
85. Pencer, J., and F. R. Hallett. 2003. Effects of vesicle size and shape on static and dynamic light scattering measurements. *Langmuir.* **19:** 7488–7497.
86. Reynolds, J. A., Y. Nozaki, and C. Tanford. 1983. Gel-exclusion chromatography on S1000 Sephacryl: application to phospholipid vesicles. *Anal. Biochem.* **130:** 471–474.
87. Jain, M. K., and O. G. Berg. 1989. The kinetics of interfacial catalysis by phospholipase A2 and regulation of interfacial activation: hopping versus scooting. *Biochim. Biophys. Acta.* **1002:** 127–156.
88. Carman, G. M., and Y-P. Lin. 1991. Phosphatidate phosphatase from yeast. *Methods Enzymol.* **197:** 548–553.
89. Han, G-S., and G. M. Carman. 2004. Assaying lipid phosphate phosphatase activities. *Methods Mol. Biol.* **284:** 209–216.
90. Webb, E. C. 1992. Enzyme Nomenclature: Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. Academic Press, Inc., San Diego.
91. Brindley, D. N., and M. Bowley. 1975. Drugs affecting the synthesis of glycerides and phospholipids in rat liver. The effects of clofibrate, halofenate, fenfluramine, amphetamine, cinchocaine, chlorpromazine, demethylimipramine, mepyramine and some of their derivatives. *Biochem. J.* **148:** 461–469.
92. Martin, A., P. Hales, and D. N. Brindley. 1987. A rapid assay for measuring the activity and the Mg2+ and Ca2+ requirements of phosphatidate phosphohydrolase in cytosolic and microsomal fractions of rat liver. *Biochem. J.* **245:** 347–355.
93. Tillman, T. S., and R. M. Bell. 1986. Mutants of *Saccharomyces cerevisiae* defective in sn-glycerol-3-phosphate acyltransferase. *J. Biol. Chem.* **261:** 9144–9149.
94. Kates, M. 1955. Hydrolysis of lecithin by plant plastid enzymes. *Can. J. Biochem.* **33:** 575–589.
95. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
96. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
97. Cleland, W. W. 1970. Steady state kinetics. *In* The Enzymes. P. D. Boyer, editor. Academic Press, New York. 1–65.

98. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:** 375–400.
99. Itaya, K., and M. Ui. 1966. A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta.* **14:** 361–366.
100. Van Veldhoven, P. P., and G. P. Mannaerts. 1987. Inorganic and organic phosphate measurements in the nanomolar range. *Anal. Biochem.* **161:** 45–48.
101. Mahuren, J. D., S. P. Coburn, A. Slominski, and J. Wortsman. 2001. Microassay of phosphate provides a general method for measuring the activity of phosphatases using physiological, nonchromogenic substrates such as lysophosphatidic acid. *Anal. Biochem.* **298:** 241–245.
102. Martínez Gache, S. A., A. A. Recoulat Angelini, M. L. Sabeckis, and F. L. Gonzalez Flecha. 2020. Improving the stability of the malachite green method for the determination of phosphate using Pluronic F68. *Anal. Biochem.* **597:** 113681.
103. Kates, M. 1986. Separation of Lipid Mixtures. *In* Techniques of Lipidology: Isolation, Analysis and Identification of Lipids. Elsevier, New York. 186–278.
104. Henderson, R. J., and D. R. Tocher. 1992. Thin-layer chromatography. *In* Lipid Analysis. R. J. Hamilton and S. Hamilton, editors. IRL Press, New York. 65–111.
105. White, T., S. Bursten, D. Federighi, R. A. Lewis, and E. Nudelman. 1998. High-resolution separation and quantification of neutral lipid and phospholipid species in mammalian cells and sera by multi-one-dimensional thin-layer chromatography. *Anal. Biochem.* **258:** 109–117.
106. Silversand, C., and C. Haux. 1997. Improved high-performance liquid chromatographic method for the separation and quantification of lipid classes: application to fish lipids. *J. Chromatogr. B Biomed. Sci. Appl.* **703:** 7–14.
107. Comba, S., S. Menendez-Bravo, A. Arabolaza, and H. Gramajo. 2013. Identification and physiological characterization of phosphatidic acid phosphatase enzymes involved in triacylglycerol biosynthesis in *Streptomyces coelicolor*. *Microb. Cell Fact.* **12:** 9.
108. Burgdorf, C., L. Hansel, M. Heidbreder, O. Johren, F. Schutte, H. Schunkert, and T. Kurz. 2009. Suppression of cardiac phosphatidate phosphohydrolase 1 activity and lipin mRNA expression in Zucker diabetic fatty rats and humans with type 2 diabetes mellitus. *Biochem. Biophys. Res. Commun.* **390:** 165–170.
109. Burgdorf, C., A. Prey, G. Richardt, and T. Kurz. 2008. A HPLC-fluorescence detection method for determination of phosphatidic acid phosphohydrolase activity: application in human myocardium. *Anal. Biochem.* **374:** 291–297.
110. Ullah, A. H. J., K. Sethumadhavan, and J. Shockey. 2012. Measuring phosphatidic acid phosphohydrolase (EC 3.1.3.4) activity using two phosphomolybdate-based colorimetric methods. *Adv. Biol. Chem.* **2:** 416–421.
111. Jamal, Z., A. Martin, A. Gomez-Munoz, and D. N. Brindley. 1991. Plasma membrane fractions from rat liver contain a phosphatidate phosphohydrolase distinct from that in the endoplasmic reticulum and cytosol. *J. Biol. Chem.* **266:** 2988–2996.
112. Brindley, D. N., D. English, C. Pilquil, K. Buri, and Z. C. Ling. 2002. Lipid phosphate phosphatases regulate signal transduction through glycerolipids and sphingolipids. *Biochim. Biophys. Acta.* **1582:** 33–44.
113. Tang, X., and D. N. Brindley. 2020. Lipid phosphate phosphatases and cancer. *Biomolecules.* **10:** E1263.
114. Carman, G. M., and G-S. Han. 2006. Roles of phosphatidate phosphatase enzymes in lipid metabolism. *Trends Biochem. Sci.* **31:** 694–699.
115. Carman, G. M., and G-S. Han. 2009. Phosphatidic acid phosphatase, a key enzyme in the regulation of lipid synthesis. *J. Biol. Chem.* **284:** 2593–2597.
116. Yu, H., P. Braun, M. A. Yildirim, I. Lemmens, K. Venkatesan, J. Sahalie, T. Hirozane-Kishikawa, F. Gebreab, N. Li, N. Simonis, et al. 2008. High-quality binary protein interaction map of the yeast interactome network. *Science.* **322:** 104–110.
117. Stukey, J., and G. M. Carman. 1997. Identification of a novel phosphatase sequence motif. *Protein Sci.* **6:** 469–472.
118. Toke, D. A., W. L. Bennett, D. A. Dillon, W-I. Wu, X. Chen, D. B. Ostrander, J. Oshiro, A. Cremesti, D. R. Voelker, A. S. Fischl, et al. 1998. Isolation and characterization of the *Saccharomyces cerevisiae DPP1* gene encoding for diacylglycerol pyrophosphate phosphatase. *J. Biol. Chem.* **273:** 3278–3284.
119. Toke, D. A., W. L. Bennett, J. Oshiro, W-I. Wu, D. R. Voelker, and G. M. Carman. 1998. Isolation and characterization of the *Saccharomyces cerevisiae LPP1* gene encoding a Mg^{2+}-independent phosphatidate phosphatase. *J. Biol. Chem.* **273:** 14331–14338.
120. Brindley, D. N., and D. W. Waggoner. 1998. Mammalian lipid phosphate phosphohydrolases. *J. Biol. Chem.* **273:** 24281–24284.
121. Brindley, D. N., and C. Pilquil. 2009. Lipid phosphate phosphatases and signaling. *J. Lipid Res.* **50(Suppl):** S225–S230.
122. Wu, W-I., Y. Liu, B. Riedel, J. B. Wissing, A. S. Fischl, and G. M. Carman. 1996. Purification and characterization of diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271:** 1868–1876.
123. Furneisen, J. M., and G. M. Carman. 2000. Enzymological properties of the *LPP1*-encoded lipid phosphatase from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta.* **1484:** 71–82.
124. Faulkner, A., X. Chen, J. Rush, B. Horazdovsky, C. J. Waechter, G. M. Carman, and P. C. Sternweis. 1999. The *LPP1* and *DPP1* gene products account for most of the isoprenoid phosphatase activities in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **274:** 14831–14837.
125. Roberts, R., V. A. Sciorra, and A. J. Morris. 1998. Human type 2 phosphatidic acid phosphohydrolases - Substrate specificity of the type 2a, 2b, and 2c enzymes and cell surface activity of the 2a isoform. *J. Biol. Chem.* **273:** 22059–22067.
126. Smyth, S. S., V. A. Sciorra, Y. J. Sigal, Z. Pamulkar, Z. Wang, Y. Xu, G. D. Prestwich, and A. J. Morris. 2003. Lipid phosphate phosphatases regulate lysophosphatidic acid production and signaling in platelets: Studies using chemical inhibitors of lipid phosphate phosphatase activity. *J. Biol. Chem.* **278:** 43214–43223.
127. Sciorra, V. A., and A. J. Morris. 2002. Roles for lipid phosphate phosphatases in regulation of cellular signaling. *Biochim. Biophys. Acta.* **1582:** 45–51.
128. Chae, M., G-S. Han, and G. M. Carman. 2012. The *Saccharomyces cerevisiae* actin patch protein App1p is a phosphatidate phosphatase enzyme. *J. Biol. Chem.* **287:** 40186–40196.
129. Chae, M., and G. M. Carman. 2013. Characterization of the yeast actin patch protein App1p phosphatidate phosphatase. *J. Biol. Chem.* **288:** 6427–6437.