

# Phosphorylation of Lipin 1 and Charge on the Phosphatidic Acid Head Group Control Its Phosphatidic Acid Phosphatase Activity and Membrane Association\*

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**Background:** Phosphorylation controls intracellular localization of lipin 1 and has been proposed to regulate activity.

**Results:** Lipin 1 preferentially binds di-anionic phosphatidic acid, and this is eliminated by phosphorylation.

**Conclusion:** Lipin 1 association with phosphatidic acid is regulated by phosphorylation and electrostatic charge of substrate.

**Significance:** Phosphorylation and the local membrane environment both significantly contribute to the regulation of lipin 1 PAP activity.

The lipin gene family encodes a class of  $Mg^{2+}$ -dependent phosphatidic acid phosphatases involved in the *de novo* synthesis of phospholipids and triglycerides. Unlike other enzymes in the Kennedy pathway, lipins are not integral membrane proteins, and they need to translocate from the cytosol to intracellular membranes to participate in glycerolipid synthesis. The movement of lipin 1 within the cell is closely associated with its phosphorylation status. Although cellular analyses have demonstrated that highly phosphorylated lipin 1 is enriched in the cytosol and dephosphorylated lipin 1 is found on membranes, the effects of phosphorylation on lipin 1 activity and binding to membranes has not been recapitulated *in vitro*. Herein we describe a new biochemical assay for lipin 1 using mixtures of phosphatidic acid (PA) and phosphatidylethanolamine that reflects its physiological activity and membrane interaction. This depends on our observation that lipin 1 binding to PA in membranes is highly responsive to the electrostatic charge of PA. The studies presented here demonstrate that phosphorylation regulates the ability of the polybasic domain of lipin 1 to recognize di-anionic PA and identify mTOR as a crucial upstream signaling component regulating lipin 1 phosphorylation. These results demonstrate how phosphorylation of lipin 1 together with pH and membrane phospholipid composition play important roles in the membrane association of lipin 1 and thus the regulation of its enzymatic activity.

Lipin proteins are a class of mammalian  $Mg^{2+}$ -dependent phosphatidic acid phosphatases that have dual functions in lipid synthesis and transcriptional regulation. Lipin family members catalyze the dephosphorylation of phosphatidic acid

(PA)<sup>2</sup> to diacylglycerol in the *de novo* pathway of neutral and phospholipid synthesis. There are three lipin family members, Lipins 1–3, and all contain a carboxyl-terminal region (CLIP) with a haloacid dehalogenase-like (HAD) domain as the catalytic core and an amino-terminal domain (NLIP) of unknown function (1, 2). There is also evidence that the lipins play a role in directly regulating gene transcription in the nucleus (3). Lipin 1 is capable of regulating cellular lipid status at multiple levels by controlling lipid synthesis directly through its PAP activity and indirectly through modulating the activity of transcription factors important for lipid biosynthesis and breakdown.

The lipins are highly phosphorylated with over 25 sites identified in the founding member, lipin 1 (4, 5). Although hormonal signaling can promote or inhibit lipin 1 phosphorylation, which appears to direct the localization of lipin within the cell to separate cellular compartments, the mechanisms by which phosphorylation controls lipin 1 translocation are not clear. It was shown that hyperphosphorylated lipin 1 associates with the cytosolic 14-3-3 proteins and proposed that this is required for lipin 1 localization to the cytosol (6). Although the role of phosphorylation on the intracellular localization would be predicted to alter lipin 1 PAP activity, there is little direct evidence supporting such a regulation of function. Highly phosphorylated lipin 1 from 3T3-L1 adipocytes showed no difference in PAP activity compared with the same lipin 1 dephosphorylated with recombinant PP1c when measured using Triton X-100/PA mixed micelles as a substrate (4). Furthermore, despite dramatically altering lipin 1 phosphorylation, hormonal signaling was also without effect on total lipin 1 phosphatase activity. In contrast, using similar assay conditions it has been reported that blocking cells in mitosis inhibits lipin 1 PAP activity (7). However, the lipin 1 examined under these conditions, either in lysates or immobilized as immune complexes on beads, was impure and prevented a detailed kinetic analysis. Three human

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<sup>2</sup> The abbreviations used are: PA, phosphatidic acid; mTOR, mammalian target of rapamycin; PAP, phosphatidic acid phosphatase; PE, phosphatidylethanolamine; TX, Triton X-100; PBD, polybasic domain; ER, endoplasmic reticulum; PC, phosphatidylcholine.

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lipin 1 alternatively spliced isoforms have been characterized biochemically and show considerable differences in enzymatic activity (8). At this time there have been no detailed studies of the effects of phosphorylation on mammalian lipin 1 that has been purified to homogeneity.

Much more is known about how phosphorylation regulates Pah1p, the yeast homolog of lipin 1. Several studies have reported the different kinases and phosphatases that control the phosphorylation of Pah1p (9–14). Of 16 Ser/Thr sites within yeast Pah1p to be identified, mutation of seven to Ala has been shown to be sufficient to affect Pah1p enzymatic activity and association with membranes (14). Although there are structural similarities between mammalian lipin proteins and yeast Pah1p, the kinases and phosphorylation sites are not highly conserved. Cdc28p, PKA, and Pho80p-Pho85p can phosphorylate Pah1p, and phosphorylation by PKA and Pho80p-Pho85p inhibits Pah1p activity when measured *in vitro* with Triton X-100/PA mixed micelles (9–12). The phosphatase responsible for dephosphorylating Pah1p is the Nem1p/Spo7p complex (15). The Nem1p/Spo7p complex is an integral membrane phosphatase with Nem1p as the catalytic component and Spo7p as a regulatory subunit. Loss of either Nem1p or Spo7p leads to accumulation of hyperphosphorylated Pah1p that compromises its function. The mammalian orthologs of Nem1p and Spo7p have been identified. Dullard, also known as CTDNEP1, is orthologous to the Nem1p phosphatase, and NEP1-R1 is the mammalian version of the Spo7p regulatory partner. Evidence exists that Dullard, or CTDNEP1, can dephosphorylate lipin 1 *in vitro* and *in vivo* (16–18).

The lipin family has a very high degree of specificity for phosphatidic acid (1, 8, 19). PA is a phosphomonoester and like other phosphomonoesters, such as ceramide-1-phosphate and sphingosine-1-phosphate, has a second  $pK_a$  in the physiologic pH range. Importantly, the second  $pK_a$  of phosphomonoesters within a phospholipid bilayer varies according to the composition of the bilayer (20, 21). This is from hydrogen bonds forming between hydrogen bond donors, such as the primary amine of phosphatidylethanolamine (PE), and the hydrogen bond-accepting oxygen atoms within PA. These hydrogen bonds allow deprotonation of the phosphate and create a di-anionic charge (charge of  $-2$ ) at the head group of PA, thus increasing its electrostatic interaction potential. All identified PA-binding proteins contain positively charged amino acids, such as lysine or arginine, that are responsible for binding to PA (22). Therefore, both intracellular pH and the composition of different intracellular membranes can affect the charge of PA found in those membranes and, potentially, the ability of PA to recruit PA-binding proteins.

Our previous work demonstrated phosphorylation of lipin 1 through the mTOR signaling pathway decreased the association of lipin 1 with membranes. While developing an *in vitro* assay to measure how phosphorylation might affect lipin 1 enzymatic activity, we found that lipin 1 association with membranes occurs more readily with deprotonated, di-anionic PA. This demonstrates that lipin 1 enzymatic activity and binding can be affected by membrane composition and pH. In addition, we find that phosphorylation impairs lipin 1 association with PA by inhibiting the action of the PA binding region located in

the polybasic domain. This suggests that lipin 1 binds to PA via the electrostatic/hydrogen bond switching mechanism (21). Thus, the ionization status of PA plays a key role in lipin 1 PAP activity and is the mechanism by which phosphorylation regulates binding of the polybasic domain to PA. These results provide new mechanistic insights explaining how mTOR-induced phosphorylation controls the physiological expression of lipin 1 and also provide a new *in vitro* assay that reflects this regulation.

## EXPERIMENTAL PROCEDURES

**Materials**—1,2-Dioleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycero-3-phosphate, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine were from Avanti Polar Lipids. Triton X-100, anti-FLAG M2 affinity gel, diacylglycerol kinase, dodecylamine, dodecyltrimethylammonium chloride, chlorpromazine, epinephrine, and most commonly used chemicals were from Sigma.

**Recombinant Expression Plasmids**—The FLAG-tagged lipin 1b expression vector (pRK5-FLAG-lipin 1b) has been previously described (5). The FLAG-tagged polybasic domain (PBD) mutant was generated from this construct by PCR mutagenesis and designated pRK5-FLAG-lipin 1b-PBD. All amino acid numbering conforms to phosphatidic acid phosphatase LPIN1 isoform b (*Mus musculus*), accession number NP\_056578.

**Purification of Recombinant Lipin**—HEK293T cells were cultured in 15-cm plates in DMEM containing 5% fetal calf serum and penicillin/streptomycin (Invitrogen). Cells were transiently transfected with 30  $\mu$ g of plasmid/15-cm plate using Lipofectamine 2000 at a 2:1 ratio of DNA:Lipofectamine. 18–24 plates were used for each purification of lipin 1. Transfected HEK293T cells were harvested by centrifugation at  $16,000 \times g$  for 10 min, washed with ice cold phosphate-buffered saline, and either used directly or frozen at  $-80^\circ\text{C}$ . For radiolabeling experiments, 48 h after transfection the HEK293T cells were switched from culture medium to low phosphate buffer (145 mM NaCl, 5.4 mM KCl, 1.4 mM  $\text{CaCl}_2$ , 1.4 mM  $\text{MgSO}_4$ , 25 mM  $\text{NaHCO}_3$ , 5 mM glucose, 5 mg/ml BSA, 0.2 mM sodium phosphate, and 10 mM HEPES, pH 7.4) containing 10% serum and were radiolabeled with 0.02  $\mu\text{Ci/ml}$  [ $^{32}\text{P}$ ]ATP for 2 h before protein isolation. Cells were lysed in buffer A (150 mM NaCl, 20 mM HEPES, pH 7.2, 0.1% Brij 35), the lysates were cleared by centrifugation at  $16,000 \times g$  for 10 min, and the supernatant was incubated with anti-FLAG beads for 2–4 h at  $4^\circ\text{C}$ . Beads were isolated by centrifugation at  $2000 \times g$ , and the supernatant was removed. After washing, the slurry was incubated for 30 min at  $30^\circ\text{C}$  in phosphatase buffer (100 mM NaCl, 1 mM  $\text{MnCl}_2$ , 2 mM DTT, 50 mM HEPES, pH 7) with or without 2000 units of Lambda Protein Phosphatase (New England Biolabs) and subjected to gentle agitation. The slurry was packed onto a screening column, washed with buffer A, and lipin 1 was eluted by 5 successive additions of an equal volume of 0.5 mg/ml FLAG peptide (Lifetein) in 150 mM NaCl, 20 mM HEPES, pH 7.2. Elution fractions containing lipin 1 were pooled and dialyzed 3 times against 150 mM NaCl, 20 mM HEPES, pH 7.2, and 1% glycerol. Purified lipin 1 was quantitated using UV absorbance and comparison of bands of lipin 1 and bovine serum albumin standards on Coomassie Blue-stained SDS-PAGE gels.

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**Preparation of [<sup>32</sup>P]PA—**[<sup>32</sup>P]Phosphatidic acid was purified by thin layer chromatography after using [ $\gamma$ -<sup>32</sup>P] ATP and *E. coli* diacylglycerol kinase to phosphorylate 1,2-dioleoyl-*sn*-glycerol (23).

**Preparation of Triton X-100/PA Mixed Micelles—**PA was transferred to a glass tube, and solvent was eliminated *in vacuo* for 1 h. Triton X-100 (TX) was added to produce the appropriate concentration of TX/PA micelles. The mol % of lipid in a Triton X-100/lipid-mixed micelle was calculated using the formula, mol% = 100 × [lipid (M)]/([lipid (M)] + [Triton X-100 (M)]).

**Measurements of PAP Activities—**PAP activity was determined by using a modification of Han and Carman (23) to measure [<sup>32</sup>P]phosphate release from [<sup>32</sup>P]PA. Briefly, purified lipin 1 was added to reaction mixtures (100  $\mu$ l) containing Triton X-100/lipid-mixed micelles or liposomes, 10 mM  $\beta$ -mercaptoethanol, [<sup>32</sup>P]PA (10,000 cpm/nmol), 0.5 mM MgCl<sub>2</sub>, and 50 mM Tris-HCl, pH 7.5 or 8.0, where indicated. For assays performed at pH 7.0, 50 mM Tris-maleate was used instead. After 20 min at 30 °C, reactions were terminated by adding 0.5 ml of 0.1 N HCl in methanol. [<sup>32</sup>P]Phosphate was extracted by vigorous mixing after adding 1 ml of chloroform and 1 ml of 1 M MgCl<sub>2</sub>. The [<sup>32</sup>P]phosphate in 0.5 ml of the aqueous phase was determined by scintillation counting. One unit of PAP activity is defined as the amount required to convert 1  $\mu$ mol of PA to diacylglycerol/min. All enzyme activity assays were performed in triplicate, and error is shown as  $\pm$ S.D. The kinetic data were analyzed using GraphPad Prism 5 using Michaelis-Menten or Allosteric Sigmoidal equations where appropriate.  $k_{cat}$  was calculated according to the equation  $k_{cat} = V_{max}/E_t$ , where  $E_t$  = enzyme catalytic site concentration. Each assay was confirmed with an independent enzyme purification preparation.

**Preparation of Liposomes—**Unilamellar phospholipid vesicles were prepared by the lipid extrusion method of MacDonald *et al.* (24). Briefly, the indicated concentrations of lipids and amphiphilic amines were dried *in vacuo* overnight to form a thin film that was suspended in buffer B consisting of 20 mM Tris-HCl, 1 mM EDTA, and pH 7.2 unless otherwise indicated. The lipid suspensions were extruded 11 times using a mini-extruder (Avanti) through a 100-nm diameter polycarbonate filter after five freeze/thaw cycles. Liposome production and diameter was verified using a Dynamic Light Scattering instrument from DynaPro. Liposomes were used immediately after generation. For PAP assays utilizing liposomes, [<sup>32</sup>P]PA (10,000 cpm/nmol) was added to the lipids before drying to a film. The subsequent steps for liposome production were performed in a 1/2-inch acrylic ultra-clear glove box to minimize exposure.

**Measurement of Membrane Binding—**Binding of lipin 1 to large unilamellar vesicles was measured following a slightly modified version of Höfer *et al.* (25). Briefly, lipin 1 was incubated for 20 min at 30 °C with liposomes containing the indicated phospholipid concentrations, and then an equal volume of 80% (w/v) sucrose in buffer B was added. This mixture was placed in 5 × 41-mm Beckman tubes and carefully overlaid with 150  $\mu$ l of 20% (w/v) sucrose in buffer B and 50  $\mu$ l of buffer B without sucrose and centrifuged in a SW 55Ti swinging bucket rotor containing nylon inserts at 240,000 × *g* for 1 h. The top 50- $\mu$ l fraction was collected with a Hamilton syringe and

analyzed by SDS-PAGE. All binding assays were performed at least three times. The average relative binding compared with PC:PA (20 mol%) liposomes is shown  $\pm$  S.D. Student's *t* test was used to determine statistical significance.

## RESULTS

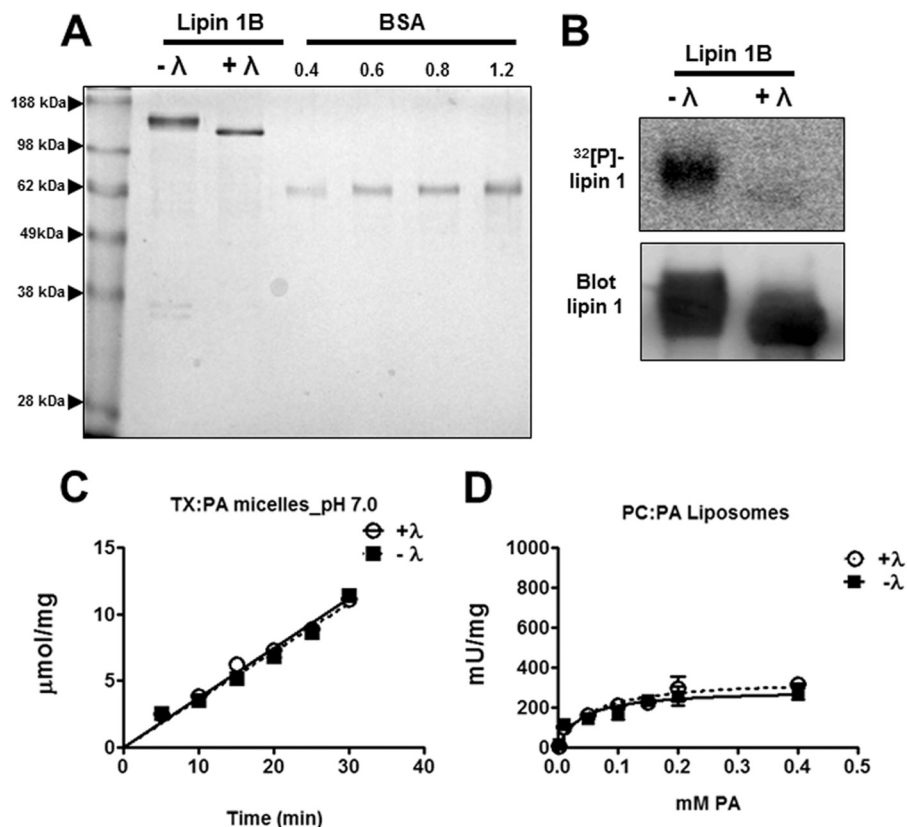
**Phosphorylation of Lipin 1 Does Not Affect Enzymatic Activity under Standard Conditions—**We sought to develop an *in vitro* assay that accurately reflects how phosphorylation is thought to affect lipin 1 PAP activity within cells. We transfected HEK293T cells with FLAG-tagged lipin 1b (termed lipin 1 in the remainder of the manuscript). Two days later we purified highly phosphorylated lipin 1 using FLAG affinity chromatography. While still bound to affinity beads, the samples were split into parallel reactions and treated with or without  $\lambda$ -phosphatase to dephosphorylate lipin 1. Treatment with phosphatase increased the mobility of the dephosphorylated form from ~140 to ~120 kDa (Fig. 1A). Note that 120-kDa is still a rather aberrant mobility for a protein with a predicted molecular mass of ~95 kDa. To more precisely define the extent of phosphate removal by  $\lambda$ -phosphatase, in a parallel series of experiments we isolated FLAG-lipin 1 from cells radiolabeled with <sup>32</sup>P. Fig. 1B shows that  $\lambda$ -phosphatase treatment removed >95% of the phosphate from lipin 1. Under certain conditions lipin 1 has also been reported to be sumoylated (26). However, we found no evidence for lipin 1 sumoylation in HEK293T cells under these conditions.

Phosphorylated ( $-\lambda$ ) and dephosphorylated ( $+\lambda$ ) lipin 1 was then eluted with FLAG peptide and dialyzed. PAP activity was measured in TX/PA mixed micelles (9.1 mol%) according to previous protocols (4, 23). Under these conditions there is no apparent difference in the rate of product formation between the phosphorylated and dephosphorylated forms (Fig. 1C). Note that this is in stark contrast to the reports for the lipin 1 ortholog from yeast, where phosphorylation of Pah1p decreases PAP activity (9, 12, 13). Consistent with previous findings using immune complex-bound lipin 1, Fig. 1C suggests that the purification state of lipin 1 is not the critical factor in determining whether phosphorylation will affect enzymatic activity *in vitro*.

We reasoned that perhaps the lack of effect of phosphorylation on lipin 1 enzymatic activity was due to the use of Triton X-100 mixed micelles for substrate presentation. We hypothesized that perhaps when PA in micelles was presented to lipin 1, the effect of phosphorylation on enzymatic activity might be masked. Although TX-100 mixed micelles provide a convenient assay system for determining the kinetics of PA phosphatase activity, the micelles may not accurately reflect the presentation of PA on the surface of internal membranes. For example, the relatively small size of the micelles implies that they have an extreme surface curvature. Alternatively, the phospholipids that make up internal membranes may themselves influence the interaction of lipin 1 with membranes and, hence, unmask the effects of lipin 1 phosphorylation on the dephosphorylation of PA within these membranes. For example, mixtures of PC with PA were previously shown to provide favorable substrates for measuring the PAP reaction of the lipins *versus* that of the lipid phosphate phosphatases (27). To more closely approximate a membrane surface, we generated



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**FIGURE 1. Phosphorylation status of lipin 1 does not change PAP activity using the conventional Triton X-100 mixed micelle assay.** *A*, HEK293T cells were transfected with an expression vector for FLAG-tagged lipin 1b. Two days after transfection the cells were homogenized in buffer A, and the lipin 1 contained in the extracts was bound to FLAG beads. After washing in buffer B, the beads were suspended in phosphatase buffer and treated with (+λ) or without (-λ) λ-phosphatase for 30 min. After further washing, the lipin 1 was eluted with 0.5 mg/ml FLAG peptide and quickly dialyzed. A representative 8.75% acrylamide SDS-PAGE gel stained with Coomassie is shown. μg of BSA are indicated. *B*, HEK293T cells were transfected as in *A* and radiolabeled with 0.02 μCi/ml [<sup>32</sup>P]ATP in low phosphate buffer containing 10% serum for 2 h before protein isolation. The radiolabeled lipin 1 contained within the protein extracts was immunoprecipitated with FLAG beads, washed, then treated or not with λ-phosphatase as in *A*. After phosphatase treatment, lipin 1 was eluted by boiling in SDS load buffer, resolved by SDS-PAGE, and transferred to membrane. Radiolabeled lipin 1 was visualized by autoradiography, and the membrane was subjected to immunoblotting to detect total lipin 1. *C*, PAP enzymatic activity from 50 ng of phosphorylated (-λ) and dephosphorylated lipin 1 (+λ) was measured as a function of time in Tris-maleate buffer, pH 7.0, with 9.1 mol% PA in Triton X-100 micelles at a final concentration of 0.2 mM PA. *D*, phosphorylated (-λ) and dephosphorylated (+λ) lipin 1 PAP activity was measured as a function of PA concentration in 100 nm PC:PA liposomes at pH 7.5. The surface concentration of PA was 10 mol%.

100-nm liposomes composed of PC and PA. Liposome sizes were verified by dynamic light scattering (not shown). We measured the enzymatic activities of phosphorylated and dephosphorylated lipin using increasing concentrations of liposomes (Fig. 1*D*), with PA at a final surface concentration of 10 mol%, as has been previously described for yeast Pah1p (11). As reported for yeast Pah1p, lipin 1 showed activity against PA in PC:PA liposomes. Under these conditions both phosphorylated and dephosphorylated lipin 1 again showed similar activity when measured as a function of PA concentration.

**Phosphatidylethanolamine Increases Activity and the Binding of Lipin 1 to PA-containing Liposomes**—Intracellular membranes contain other phospholipids besides PC and PA, with the next most abundant being PE and phosphatidylserine. To more accurately represent a model membrane, we prepared 100-nm PC liposomes containing PA at 10 mol% and PE at an increasing molar ratio. The inclusion of increasing concentrations of PE in the liposomes uncovered rather dramatic differences in the enzymatic activity of lipin 1 that was dependent on its phosphorylation state (Fig. 2, *A–D*). In the presence of PE, the  $k_{cat}$  of dephosphorylated lipin 1 was significantly greater

than that of phosphorylated lipin 1 (Table 1). Note that although phosphorylated and dephosphorylated lipin 1 both show an increase in  $k_{cat}$  with PC:PE:PA liposomes compared with PC:PA liposomes, at equal mol% PE and PC, the overall increase for dephosphorylated lipin 1 was 8-fold compared with a <2-fold increase with phosphorylated lipin 1 (45 mol% PE, Table 1). This suggests that PE enhances lipin 1 catalytic efficiency preferentially for dephosphorylated lipin 1. To visualize the effect of PE on lipin 1 PAP activity, the turnover number was graphed as a function of the mol% PE (Fig. 2*E*). Dephosphorylated, but not phosphorylated, lipin 1 displayed a sigmoidal curve, suggesting a cooperative effect of the PE concentration on lipin 1 PAP activity. In addition, an examination of the kinetic data from Table 1 indicates that increasing PE concentrations decrease the apparent  $K_m$  of dephosphorylated lipin 1 but not phosphorylated lipin 1 (Table 1). This suggests that PE enhances the affinity of lipin 1 for substrate but only for dephosphorylated lipin 1.

The enhanced lipin 1 activity in the presence of PE could be due to either an increase in the binding affinity of lipin 1 for PA, an increase in the active site efficiency, or both. However, *in*

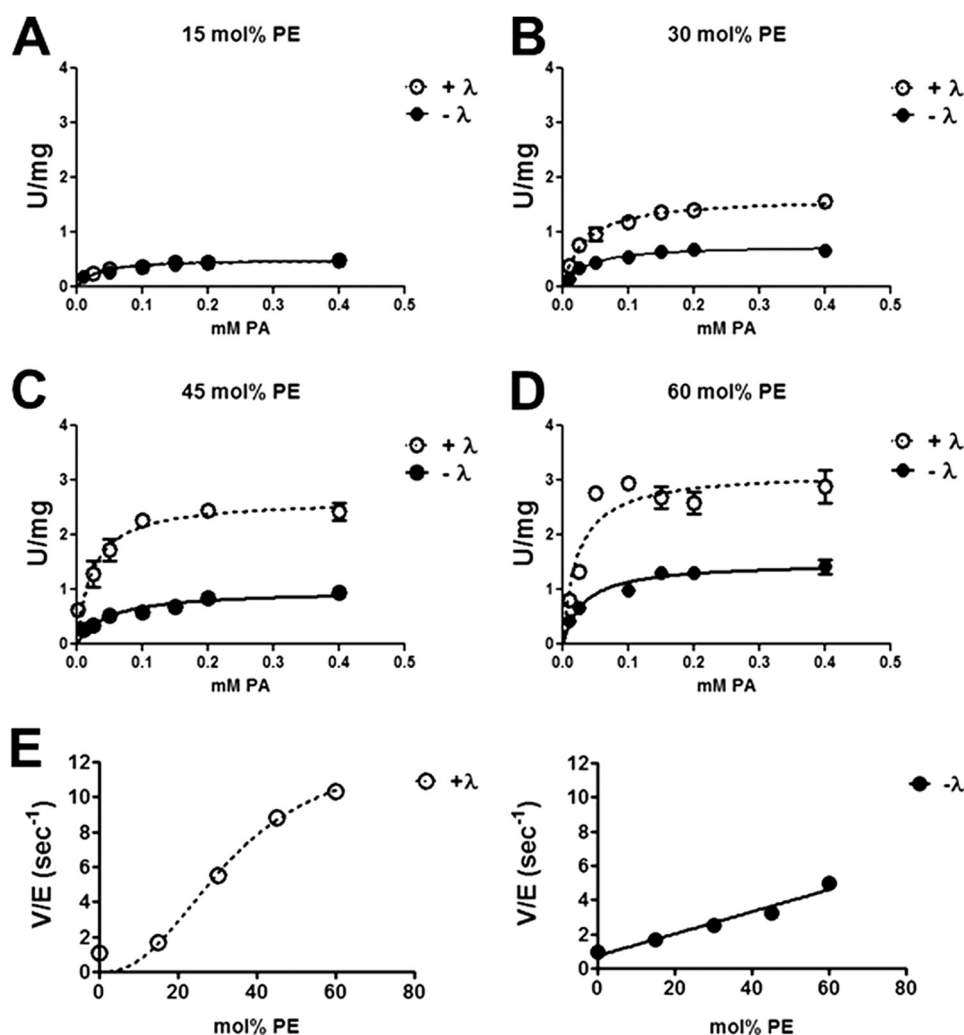


FIGURE 2. PAP activity of phosphorylated and dephosphorylated lipin 1 in liposomes. A, phosphorylated ( $-\lambda$ ) and dephosphorylated ( $+\lambda$ ) lipin 1 PAP activity was measured as a function of the molar concentration of PA in 100 nm PC:PE:PA (75:15:10) mol% liposomes at pH 7.5. B, lipin 1 PAP activity was measured as in A but with liposomes made with PC:PE:PA (60:30:10 mol%). C, lipin 1 PAP activity was measured as in A but with liposomes made with PC:PE:PA (45:45:10 mol%). D, lipin 1 PAP activity was measured as in A but with liposomes made with PC:PE:PA (30:60:10 mol%). E, shown is graph of  $k_{\text{cat}}$  values from Table 1 plotted as a function of the mol% PE for Dephosphorylated lipin 1 (left) and phosphorylated lipin 1 (right). U, units.

TABLE 1

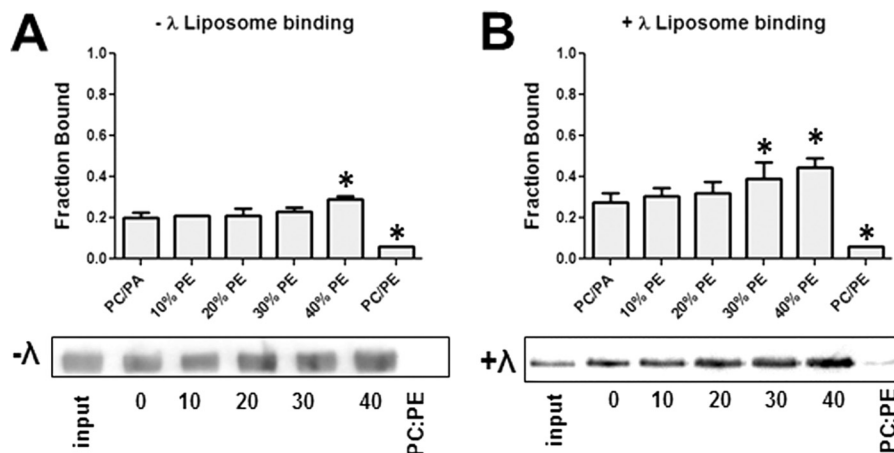
Kinetic data for lipin 1b  $\pm$ - $\lambda$  phosphatase using liposomes composed of 10 mol% PA and 90 mol% (PC + PE) with PE mol% indicated

	$K_m^{\text{app}}$ $\mu\text{M}$	$k_{\text{cat}}$ $s^{-1}$	$k_{\text{cat}}/K_m^{\text{app}}$ $s^{-1}\mu\text{M}^{-1}$
<b>Lipin 1 +<math>\lambda</math></b>			
0% PE	46 $\pm$ 19	1.07 $\pm$ 0.12	0.02
15% PE	29 $\pm$ 5	1.70 $\pm$ 0.10	0.06
30% PE	32 $\pm$ 3	5.50 $\pm$ 0.10	0.17
45% PE	23 $\pm$ 11	8.84 $\pm$ 0.90	0.38
60% PE	19 $\pm$ 11	10.33 $\pm$ 1.10	0.54
<b>Lipin 1 -<math>\lambda</math></b>			
0% PE	39 $\pm$ 20	0.97 $\pm$ 0.12	0.03
15% PE	29 $\pm$ 11	1.68 $\pm$ 0.14	0.06
30% PE	33 $\pm$ 5	2.51 $\pm$ 0.10	0.08
45% PE	46 $\pm$ 12	3.28 $\pm$ 0.25	0.07
60% PE	32 $\pm$ 7	5.00 $\pm$ 0.30	0.16

*in vivo* studies have demonstrated that phosphorylation can affect the association of lipin 1 with intracellular membranes, suggesting that phosphorylation is more likely to affect lipin 1 membrane interaction. To examine this question *in vitro*, we investigated how the presence of PE affects lipin 1 binding to PA-containing liposomes. A well characterized fluorescence

spectrometric method has been developed for examining yeast Pah1p association with liposomes (11). Despite repeated attempts, we were unsuccessful in adapting this method for mammalian lipin 1, most likely due to the high number of tryptophan residues compared with yeast Pah1p (12 *versus* only 5 in Pah1p). Therefore, we employed a liposome binding assay that uses liposome flotation through a density gradient to examine lipin 1 association with membranes. PC liposomes of 100 nm in diameter were prepared by extrusion and contained either no PA or 20 mol% PA. The concentration of PA was increased to 20 mol% to enhance the level of lipin 1 association with the liposomes. PC:PE:PA liposomes were similarly generated by using increasing concentrations of PE at the expense of PC, while maintaining PA at 20 mol%. 200 ng of purified lipin 1 was first incubated with the liposomes to allow binding, and the bound and free lipin 1 was separated by flotation centrifugation through a step gradient. In the absence of PA only a modest amount of lipin 1 binding to liposomes was observed (Fig. 3, A and B). In PC:PA liposomes, the amount of lipin 1 recovered increased steadily through replacement of the PC with PE.

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**FIGURE 3. Effect of PC or PE on lipin 1 physical association with liposomes.** *A*, phosphorylated ( $-\lambda$ ) lipin 1 was mixed in buffer B with PC liposomes containing PA at 20 mol% and the indicated concentrations of PE replacing PC or PE:PC liposomes without PA. After 20 min of incubation, the lipin 1-liposome mixture was subjected to liposome flotation, and the amount of lipin 1 recovered after flotation was measured. The graph illustrates the amount of lipin 1 bound to liposomes as a percent of the total amount of lipin 1 in the binding reaction  $\pm$  S.D. Each binding assay was performed at least three times, and the asterisk indicates  $p < 0.05$  when comparing PC:PA liposomes with 40% PC:PE:PA liposomes and PC:PE liposomes. *Right*, shown is a representative immunoblot of lipin 1 (anti-FLAG) recovered after flotation. *Input* is 20% of the amount incubated with the liposomes. *B*, dephosphorylated ( $+\lambda$ ) lipin 1 binding to PC:PE:PA liposomes was measured as described in *A*. Each binding assay was performed at least three times, and the asterisk indicates  $p < 0.05$  when comparing PC:PA liposomes with 30 and 40% PC:PE:PA liposomes and PC:PE liposomes

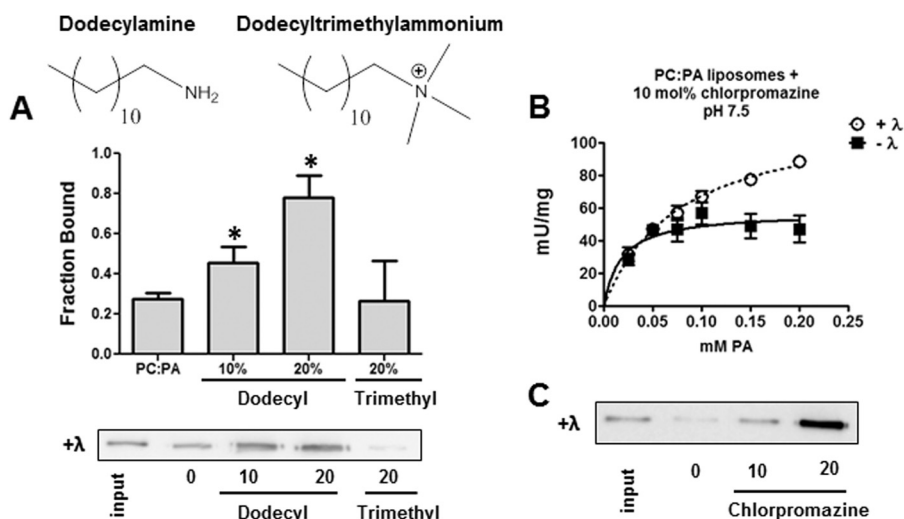
Under all conditions phosphorylated lipin 1 showed greatly reduced binding levels but a similar dependence on PE. Because liposomes were prepared in the presence of EDTA and no  $Mg^{2+}$  was added to the binding reaction, it does not appear that lipin 1 enzymatic activity is required for liposome association. In addition, the PAP activity of dephosphorylated lipin 1 shows a substantial decline in the apparent  $K_m$  with increasing mol% of PE when measured with PC:PE:PA liposomes (Table 1). Finally, we should point out that although some of the concentrations of PA and PE used in the measurements of lipin 1 PAP activity and binding to membranes are higher than normally seen in cells, we do this to maximize activity and binding in an *in vitro* assay system. Thus, PE increases lipin 1 membrane association in the presence of PA and, therefore, lipin 1 PAP activity, with a relative preference for dephosphorylated lipin 1.

**Di-anionic PA; pH and Amphiphilic Amines**—We next investigated why PE both enhanced overall PAP activity and illuminated differences in phosphorylated *versus* dephosphorylated lipin 1. Phosphatidic acid is unique among the glycerolipids in that it has the smallest headgroup, giving it a cone shape. Furthermore, as a phosphomonoester, the second  $pK_a$  of PA falls within the intracellular physiologic pH range. Biophysical studies of PA in model membranes showed that PE is capable of forming a hydrogen bond with an oxygen of the phosphate group and causing deprotonation of PA (20). This effectively reduces the second  $pK_a$  from 7.9 to 6.9, thus deprotonating PA to a  $-2$  charge more readily at physiological pH. Some PA binding proteins prefer to associate with di-anionic PA, suggesting that the enhanced activity and membrane association we observed could be due to the effects of PE on the charge of PA (28). Like PA, PE is a cone-shaped phospholipid. However, our finding that the inclusion of PE in liposomes increases lipin 1 PAP activity could be due to either steric effects on access to PA, effects of PE on PA charge, or both. To identify the determinant(s) for PE on lipin 1 enzymatic activity, we examined lipin 1 binding and activity in the presence of a membrane-

incorporated primary amine other than PE. Dodecylamine and dodecyltrimethylammonium mimic the amines of PE and PC respectively, but they are amphiphilic amines. It has been previously demonstrated that dodecylamine, but not dodecyltrimethylammonium, can deprotonate PA at pH 7.0 (28). Therefore, we generated PC:PA liposomes and PC:PA liposomes containing an increasing amount of dodecylamine or dodecyltrimethylammonium substituted for PC and determined the degree of lipin 1 binding by liposome flotation. As shown in Fig. 4A, increasing concentrations of dodecylamine increased the association of lipin 1 with liposomes. Dodecyltrimethylammonium, as a quaternary amine incapable of hydrogen bonding with PA, was without effect. For reasons that are not completely clear, both amines completely inhibited lipin 1 enzymatic activity (not shown).

Chlorpromazine is another amphiphilic amine that has been previously used in PAP enzymatic assays (29). We generated liposomes composed of a 1:1 ratio of PA to chlorpromazine at 10 mol% in PC and measured lipin 1 PAP activity using an increasing concentration of substrate. As demonstrated in Fig. 4B, the addition of chlorpromazine to PAP assays at pH 7.5 uncovers the difference between phosphorylated and dephosphorylated lipin 1, similar to the addition of PE to the micelles. There was a reduction in PAP activity of about 5-fold when compared with PA:PC liposomes alone, possibly reflecting a competition for binding to PA (29). We next measured whether chlorpromazine enhanced the binding of lipin 1 to liposomes (Fig. 4C). Similar to PE and dodecylamine, the addition of chlorpromazine to PC:PA liposomes substantially increased lipin 1 binding.

To more firmly substantiate the hypothesis that lipin 1 prefers di-anionic PA, we measured the enzymatic activity of lipin 1 in TX/PA micelles that maintain a constant molar ratio of TX to PA at 9.1 mol% at pH 7.0, 7.5, and 8. Compared with pH 7.0 (Fig. 5A), at pH 7.5 (Fig. 5B) there was an increase in overall activity with no difference between phosphorylated and



**FIGURE 4. Effect of amphiphilic amines on lipin 1 association with membranes and PAP activity.** *A*, shown is dephosphorylated lipin 1 (+ $\lambda$ ) binding to liposomes containing the indicated mol% concentration of dodecylamine and dodecyltrimethylammonium. Binding assay was performed by flotation as described in Fig. 3*A*. Each binding assay was performed at least three times, and the asterisk indicates  $p < 0.05$  when comparing PC:PA liposomes with PC:PA liposomes containing 10 and 20 mol% dodecylamine. *Bottom*, shown is a representative immunoblot of lipin 1 (anti-FLAG) recovered after flotation at the indicated concentrations of chlorpromazine. *B*, shown is phosphorylated ( $-\lambda$ ) and dephosphorylated (+ $\lambda$ ) lipin 1 PAP activity using 100 nm PC:PA liposomes containing PA at 10 mol% and chlorpromazine at 10 mol%. PAP assays were performed as a function of PA molar concentration at pH 7.5. *C*, dephosphorylated lipin 1 (+ $\lambda$ ) binding to liposomes was performed as described in Fig. 3*A* but with the indicated mol% of chlorpromazine.

dephosphorylated lipin 1 in either case. This is consistent with previous findings that human lipin 1 has a pH optimum at pH 7.5 (8). Increasing the pH to 8, which is above the second  $pK_a$  of PA, would cause  $>80\%$  of the PA to be deprotonated. Fig. 5*C* shows that increasing the pH to 8 resulted in phosphorylated lipin 1 having a significant decrease in activity when compared with dephosphorylated lipin 1. The kinetic data is summarized in Table 2. Thus, three different model systems that manipulate the relative  $pK_a$  of PA in membranes or micelles gave similar results to enzymatic and binding activity by uncovering a difference in phosphorylated and dephosphorylated lipin 1.

The ability of a soluble enzyme to act on a lipid substrate is constrained by two factors; movement of the enzyme through three dimensions to bind to a membranous surface and movement across the surface to bind to the substrate and catalyze the reaction. A kinetic model that accounts for these types of reactions has been described (for review, see Ref. 30). This means that the action of soluble enzymes acting on an insoluble lipid at a membrane interface can be broken down in to two steps. The first step, defined as the “bulk step,” is the enzyme binding to a membranous surface either non-specifically (surface binding model) or specifically (phospholipid binding model). The second step, or “surface step,” measures the combination of the enzyme binding the substrate and subsequent catalysis. Thus, to examine how the enzyme binds to a membrane surface, in the bulk step the total concentration of substrate is varied. On the other hand, to examine the kinetics of the surface step or how the membrane-bound enzyme catalyzes the conversion of successive substrate molecules to product, the concentration of substrate within the membranous surface is varied while keeping the molar concentration constant. Examining the activity of the enzyme under these two conditions allows determination of the kinetic rates of each enzymatic step simply as a function of molar or surface concentration. We examined lipin 1 activity as a function of the surface concentration of PA (mol%) while

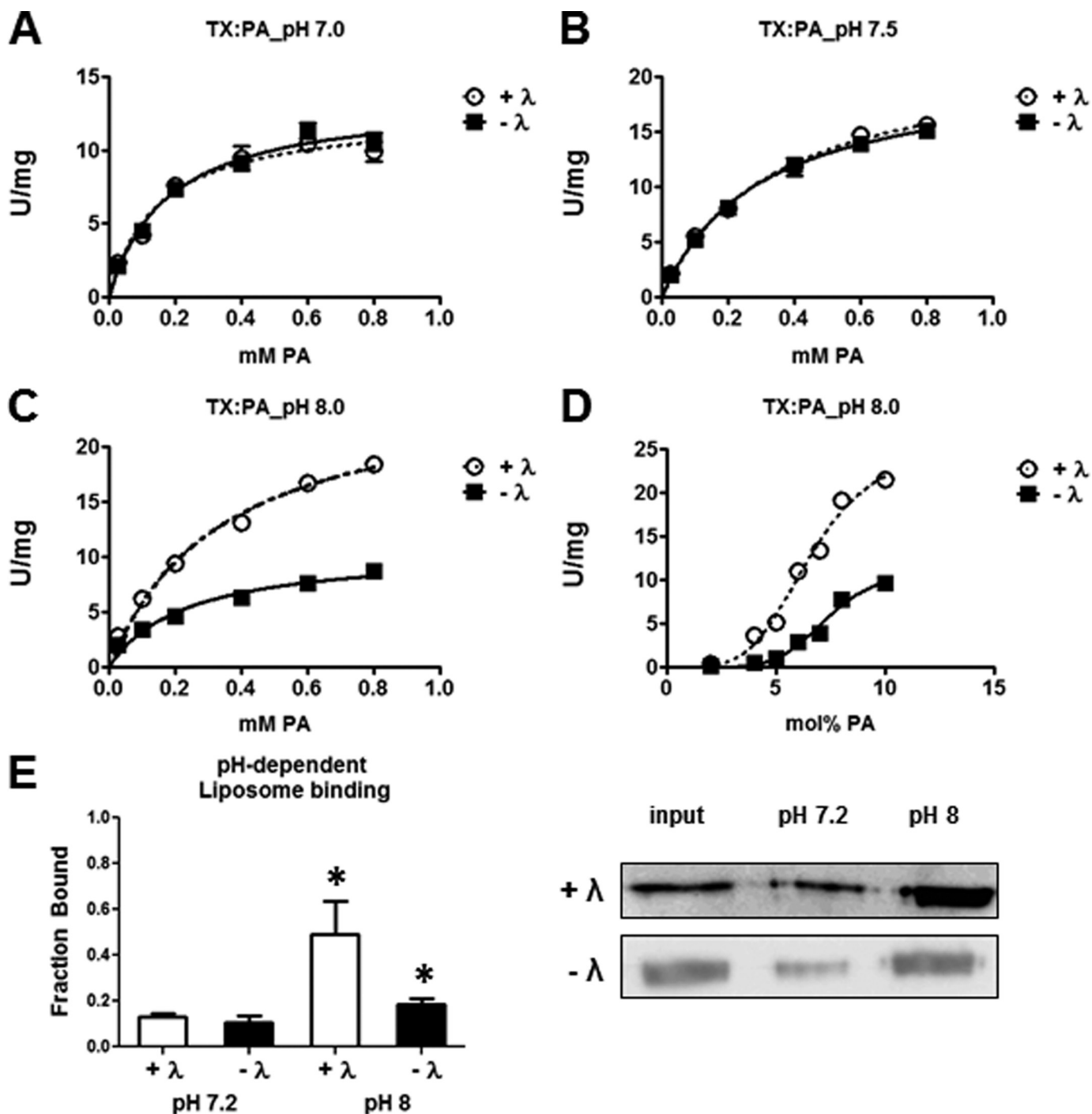
keeping the total PA concentration constant at saturating levels (1 mM PA). Under these conditions, rather than exhibiting Michaelis-Menten kinetics, lipin 1 functions according to an allosteric sigmoidal model with positive cooperative kinetics. Fig. 5*D* demonstrates that phosphorylated lipin 1 has a significant decrease in  $V_{max}$  compared with dephosphorylated lipin 1. Calculation of the apparent  $K_m$  values according to the formula  $K_{prime} = K_m^h$  ( $h$ , Hill number) gives values of 6.4 mol% for dephosphorylated lipin 1 and 7.4 mol% for phosphorylated lipin 1. This gives  $k_{cat}$  values of  $37.7 s^{-1}$  for phosphorylated lipin 1 and  $84.9 s^{-1}$  for dephosphorylated lipin 1. Phosphorylated lipin 1 also shows a 50% increase in the Hill number compared with dephosphorylated lipin 1 (Table 3).

Finally, to ensure that the observed changes in lipin 1 PAP activity were caused by changes in PA association, we measured the ability of lipin 1 to bind to PC:PA liposomes at pH 7.2 and 8.0. At pH 7.2, the majority ( $>80\%$ ) of the PA will be mono-anionic, whereas at pH 8.0 most of the PA will be di-anionic. As seen in Fig. 5*E*, the binding to PC:PA liposomes is greatly increased for dephosphorylated lipin 1 at pH 8.0 when compared with pH 7.2. Phosphorylated lipin 1 binding is also increased at pH 8.0 but not nearly to the extent of dephosphorylated lipin 1.

**Inhibition of *mTOR* Increases Lipin 1 PAP Activity**—Because phosphorylation of lipin 1 inhibits physiological activity by decreasing membrane association, we were interested in examining which phosphorylated residues were responsible. We identified that mouse lipin 1 has at least 19 and as many as 23 phosphorylation sites (4). Instead of primarily a cytosolic localization, as observed with wild type lipin 1, mutation of 21 of these Ser/Thr residues to Ala promoted localization to the ER/nucleus suggesting an increase in membrane association (5). To examine whether the identified Ser/Thr residues are the relevant phosphorylated sites that control membrane association, we purified wild type lipin 1 (WT) and the 21xA lipin 1



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**FIGURE 5. Effect of pH on lipin 1 PAP activity.** A–C, phosphorylated (–λ) and dephosphorylated (+λ) lipin 1 PAP activity using Triton X-100 mixed micelles containing 9.1 mol% PA was measured as a function of the molar concentration of PA in at the indicated pH. D, phosphorylated (–λ) and dephosphorylated (+λ) lipin 1 PAP activity using Triton X-100 mixed micelles containing 1 mM final concentration of PA was measured as a function of the surface concentration of PA (mol%) at pH 8.0. The surface concentration of PA was 2, 4, 5, 6, 7, 8, and 10 mol%. E, phosphorylated (–λ) and dephosphorylated lipin 1 (+λ) binding to 100 nm PC:PA liposomes at pH 7.2 and 8.0 were performed as described in Fig. 3A. Input is 20% of the amount incubated with the liposomes. Each binding assay was performed at least three times, and the asterisk indicates  $p < 0.05$  when comparing binding between pH 7.2 and pH 8.0 for both +λ and –λ. Right, shown is a representative immunoblot of lipin 1 (anti-FLAG) recovered after floatation at the indicated pH.

mutant (21xA) and examined enzymatic activity as a function of PA concentration (Fig. 6A). Only the wild type lipin 1 was subjected to dephosphorylation with λ-phosphatase. Fig. 6A demonstrates that mutating the 21 Ser/Thr residues to Ala generates a lipin 1 protein that has similar activity as a lipin 1 protein that has had all phosphates removed by λ-phosphatase *in*

*vitro*. This demonstrates that all of the Ser/Thr sites that can be phosphorylated and significantly affect activity are contained within the 21 sites mutated in the 21xA mutant.

Of the identified phosphorylated sites in lipin 1, only two have been demonstrated to be downstream of mTOR activation. However, inhibition of mTOR causes a dramatic change in



**TABLE 2**

 Kinetic data for lipin 1b +/-  $\lambda$  phosphatase using TX/PA micelles at varying pH

Kinetic constants with respect to molar PA concentration.

		$K_m^{app}$	$k_{cat}$	$k_{cat}/K_m^{app}$
		$\mu M$	$s^{-1}$	$s^{-1} \mu M^{-1}$
pH 7	+ $\lambda$	150 $\pm$ 40	41.6 $\pm$ 6.80	0.280
	- $\lambda$	180 $\pm$ 40	45.2 $\pm$ 3.20	0.250
pH 7.5	+ $\lambda$	330 $\pm$ 50	74.9 $\pm$ 4.30	0.230
	- $\lambda$	300 $\pm$ 20	69.2 $\pm$ 2.10	0.231
pH 8	+ $\lambda$	340 $\pm$ 60	86.3 $\pm$ 6.80	0.254
	- $\lambda$	230 $\pm$ 70	35.5 $\pm$ 4.0	0.154

**TABLE 3**

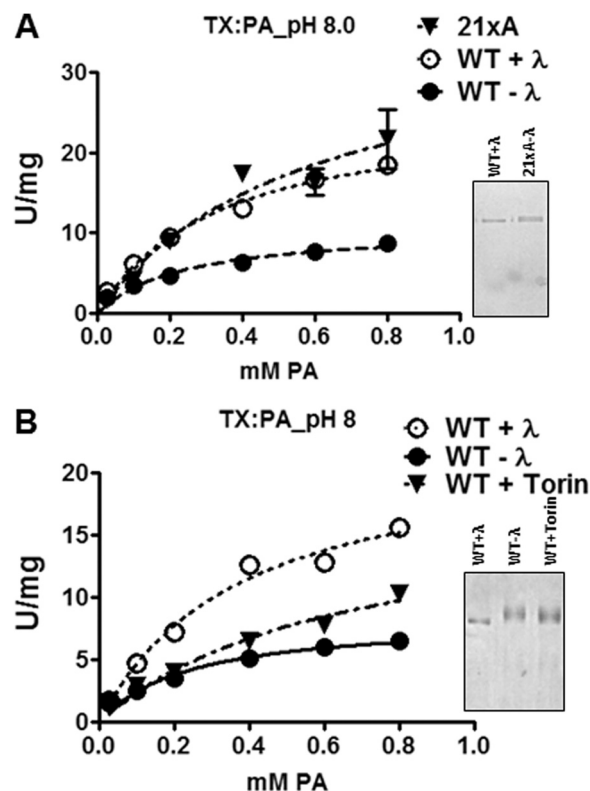
 Kinetic data for lipin 1b +/-  $\lambda$  phosphatase using TX:PA micelles at varying pH

Kinetic constants with respect to surface PA concentration.

		$K_m^{app}$	$k_{cat}$	$k_{cat}/K_m^{app}$	Hill no.
		mol%	$s^{-1}$	$s^{-1} mol\%^{-1}$	
pH 8	+ $\lambda$	6.4 $\pm$ 0.16	84.9 $\pm$ 10.2	13.3	4.31 $\pm$ 0.9
	- $\lambda$	7.4 $\pm$ 0.11	37.7 $\pm$ 5.7	5.10	6.10 $\pm$ 1.5

the intracellular localization of lipin 1, suggesting that mTOR activity is sufficient to regulate lipin 1 association with membranes. Therefore, not all of the phosphorylated sites identified play a significant role in the control of lipin 1 association with membranes. To more specifically address the dependence of mTOR-mediated phosphorylation of lipin 1 on the control of membrane association, we treated cells for 16 h with 250 nM torin1 and purified lipin 1 from control and treated cells (Fig. 6B). The electrophoretic mobility of lipin 1B from cells treated with torin1 was only modestly increased when compared with control cells, suggesting that the phosphorylation sites reliant on mTOR activity are only partially responsible for the phosphorylation-induced mobility changes. This is consistent with previous observations on lipin 1 mobility using rapamycin to inhibit mTORC1 activity in 3T3-L1 adipocytes (4). Despite the small change in electrophoretic mobility (Fig. 6B, inset), there was a significant increase in the PAP activity of lipin 1 (WT+Torin) from torin1-treated cells when compared with highly phosphorylated lipin 1 obtained from cells with active mTOR (WT - $\lambda$ ) (Fig. 6B). Lipin 1 that was devoid of all phosphorylated sites (WT + $\lambda$ ) had a similar  $k_{cat}$  (74.9  $s^{-1}$ ) to the lipin 1 lacking the mTOR-directed phosphorylation sites (58.8  $s^{-1}$ ). Both were more than 2-fold higher than phosphorylated lipin 1 (27.8  $s^{-1}$ ). Thus, the majority of the effects of phosphorylation on lipin 1 PAP activity require mTOR kinase activity, suggesting that other kinase(s) have a less significant contribution in this respect.

*The Polybasic Domain Is Required for Lipin 1 Phosphorylation to Affect Activity*—In 2007, Kooijman *et al.* (28) described an electrostatic/hydrogen-bond switch as a mechanism for PA-binding proteins. This theory posits that basic residues within PA-binding proteins, particularly lysine residues, are capable of forming hydrogen bonds causing deprotonation of PA and enhanced membrane association. Lipin 1 has a highly basic stretch with 9 lysine and arginine residues in a row at amino acids 153–161. This domain is required for interaction with PA, but curiously, had no effect on lipin 1 enzymatic activity (31). To determine whether this region is involved in sensing di-anionic PA in the membrane, we mutated all nine basic residues

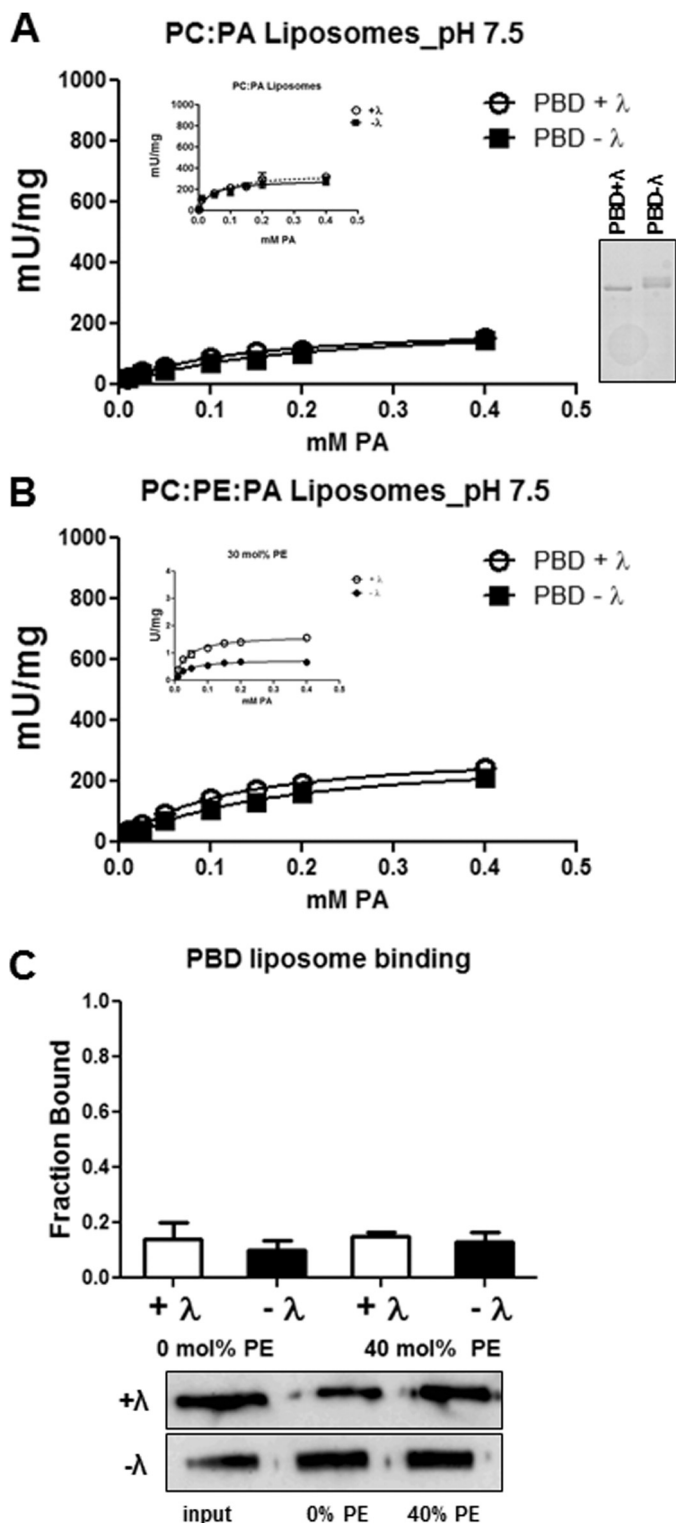


**FIGURE 6. Effect of mTOR inhibition and Ser/Thr mutation to A on lipin 1 PAP activity.** A, HEK293T cells were transfected with FLAG-tagged wild type lipin 1 or the 21xA mutant. Two days after transfection the cells were homogenized in buffer A, and lipin 1 was purified as described in Fig. 1A. While the protein was still attached to the FLAG beads, the wild type was treated with  $\lambda$ -phosphatase (WT + $\lambda$ ), whereas the 21xA mutant was not (21xA - $\lambda$ ). PAP activity was measured in TX/PA micelles (9.1 mol% PA) as a function of PA molar concentration at pH 8.0. The inset to the right is an SDS-PAGE gel of purified lipin 1 treated with phosphatase (WT + $\lambda$ ) and the lipin 1 21xA mutant untreated (21xA - $\lambda$ ) stained with Coomassie. U, units. B, HEK293T cells were transfected for 48 h with an expression vector for FLAG-tagged lipin 1b. 250 nM torin1 was added (WT+Torin) or not 16 h before the cells were homogenized in buffer A, and the lipin 1 contained in the extracts was bound to FLAG beads, purified, and treated with or without  $\lambda$ -phosphatase (WT + $\lambda$ , WT - $\lambda$ ) as described in Fig. 1A. PAP activity was measured in TX/PA micelles (9.1 mol% PA) as a function of PA molar concentration at pH 8.0. The inset to the right is a SDS-PAGE gel of purified lipin 1 +/-  $\lambda$ -phosphatase and lipin 1 treated with torin1 stained with Coomassie.

to alanine and examined the effect on lipin 1 activity. PAP activity of the lipin 1-PBD mutant was assayed using liposomes at 10 mol% PA and 90 mol% (PC + PE), with PE at 0 and 30 mol%. Fig. 7, A and B, have the respective wild type lipin 1 curves from Figs. 1D and 2B inserted as insets for comparison. Mutation of the PBD site greatly increases the apparent  $K_m$  when compared with wild type lipin 1 and largely prevents the decrease in  $K_m$  and increase in turnover that occurs upon replacing 30 mol% of the PC in the liposomes with PE (Table 4). More importantly, mutation of the PBD sites completely eliminates the effect of phosphorylation on lipin 1 PAP activity, suggesting that the function of phosphorylation is to prevent the PBD from interacting with PA. Because PA would be mostly di-anionic in the PC:PE:PA liposomes, this suggests that the PBD is required for recognition of the electrostatic charge of PA.

Mutation of the PBD in lipin 1 eliminates the decrease in the apparent  $K_m$  with PE, consistent with the involvement of the PBD in substrate binding. Dephosphorylated wild type lipin 1

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**FIGURE 7. Role of the PBD in lipin 1 PAP activity.** A, HEK293T cells were transfected with FLAG-tagged PBD mutant of lipin 1. Two days after transfection the cells were homogenized in buffer A and the PBD mutant of lipin 1 (PBD) was purified and treated with  $\lambda$ -phosphatase or not as described in Fig. 1A. PAP activity for dephosphorylated (PBD +  $\lambda$ ) or phosphorylated (PBD -  $\lambda$ ) PBD mutant was measured in PC:PA liposomes (90:10) mol% as a function of PA molar concentration at pH 7.5, as described in Fig. 1D. The inset within the graph are the data from wild type lipin 1 taken from Fig. 1D and shown for convenience. The inset to the right is a SDS-PAGE gel of purified lipin 1-PBD +/ -  $\lambda$ -phosphatase stained with Coomassie. mU, milliunits. B, PAP activity for dephosphorylated (PBD +  $\lambda$ ) or phosphorylated (PBD -  $\lambda$ ) PBD mutant was measured in PC:PE:PA liposomes (60:30:10) mol% as a function of PA molar

**TABLE 4**

Kinetic data for the PBD mutant of lipin 1b +/-  $\lambda$  phosphatase using liposomes composed of 10 mol% PA and 90 mol% (PC + PE) with PE mol% indicated

	$K_m^{APP}$ $\mu M$	$k_{cat}$ $s^{-1}$	$k_{cat}/K_m^{APP}$ $s^{-1}\mu M^{-1}$
PBD + $\lambda$			
0% PE	120 $\pm$ 20	0.64 $\pm$ 0.04	0.005
30% PE	110 $\pm$ 10	1.00 $\pm$ 0.05	0.009
PBD - $\lambda$			
0% PE	190 $\pm$ 50	0.69 $\pm$ 0.09	0.004
30% PE	170 $\pm$ 30	0.98 $\pm$ 0.09	0.006

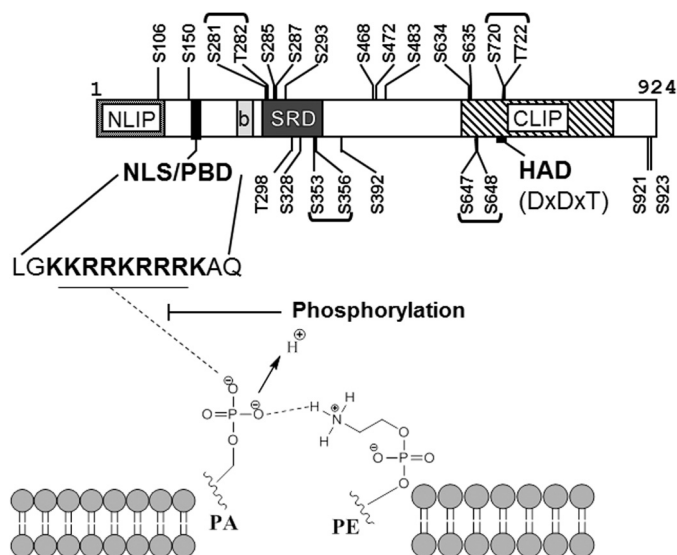
shows a decrease in  $K_m$  with increasing PE concentrations (Table 1). However, phosphorylation of the PBD mutant had little effect on activity (Table 4), suggesting that phosphorylation affects binding to substrate via the PBD. To confirm that the PBD mutation specifically eliminates the effects of phosphorylation on lipin 1 binding, we tested the ability of phosphorylated and dephosphorylated PBD mutant to bind to membranes by liposome floatation. Because the PBD mutant is largely defective in PA binding, it was necessary to use 1  $\mu g$  of protein for each binding reaction. Fig. 7C shows that without the PA binding PBD, the electrostatic charge of PA no longer influences the association of lipin 1, and phosphorylated lipin 1 can associate as readily as dephosphorylated lipin 1. This shows that in addition to PA binding through the PBD, a binding event that is regulated by phosphorylation, lipin 1 can associate with membranes independently of PA in a manner that is not regulated through phosphorylation.

## DISCUSSION

Lipin 1 phosphorylation has been proposed to play an important role in its function. When measured by biochemical fractionation of cells, hyperphosphorylated lipin 1 is found in the cytosol of 3T3-L1 adipocytes, whereas hypophosphorylated lipin 1 is localized to microsomal membranes. Overexpression of wild type lipin 1 shows cytosolic localization by immunofluorescent microscopy, whereas the 21xA mutant localizes to the ER/nucleus. Microscopic analysis of endogenous lipin 1 in 3T3-L1 adipocytes shows lipin 1 spread across the cytosol and localized to discrete punctuate spots, whereas treatment with torin1 causes a dramatic relocalization to the ER/nucleus. These changes in localization were found to play a crucial role in the regulation of the transcriptional activity of SREB1c (5). In addition, lipin 1 phosphorylation is thought to play a critical role in its enzymatic function, although evidence for this has been lacking until now. The studies presented here demonstrate that phosphorylation regulates the ability of the polybasic domain of lipin 1 to recognize di-anionic PA. We show that mTOR activity is required for phosphorylation of lipin 1 to negatively regulate lipin 1 PAP activity. In addition, while uncovering how phosphorylation regulates lipin 1 PAP activity,

concentration at pH 7.5 as described in Fig. 2B. The inset within the graph are the data from WT lipin 1 taken from Fig. 2B and shown for convenience. C, phosphorylated (- $\lambda$ ) and dephosphorylated (+ $\lambda$ ) PBD mutant binding to 100 nm PC:PE:PA liposomes (40:40:20 mol%) at pH 7.2 was performed as described in Fig. 3A. Input is 20% that of the amount incubated with the liposomes. Each binding assay was performed at least three times. Bottom, shown is a representative immunoblot of the PBD mutant (anti-FLAG) recovered after floatation.

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**FIGURE 8. Model representation of lipin 1 binding to PA.** Phosphorylated Ser/Thr residues are identified by number, where brackets indicate either or both residues may be phosphorylated, NLIP and CLIP are conserved NH<sub>2</sub>- and COOH-LIP in homology domains, HAD is haloacid dehalogenase-like domain identified by Dx DxT, NLS/PBD is the nuclear localization sequence/polybasic domain with the sequence shown below, SRD is the serine-rich domain previously identified to bind to 14-3-3, and *b* is the 33-amino acid alternatively spliced exon.

we made the very novel identification of effectors that act in conjunction with phosphorylation to control the PAP activity of lipin 1.

This study demonstrates that lipin 1 binding to its substrate is dependent on the electrostatic charge of PA. The electrostatic/hydrogen bond switch model proposes that PA binding proteins preferentially associate with di-anionic PA (Fig. 8). The ability of lipin 1 to associate with liposomes is dependent on both pH and the presence of hydrogen bond donor molecules in the liposomes such as PE or amphiphilic amines. This suggests that lipin 1 moves from three dimensions to the two-dimensional membrane surface via preferential interaction with di-anionic PA, presumably by electrostatic attraction. Furthermore, once bound, we propose that the PBD of lipin 1 functions as an anchor to bind lipin 1 to the two-dimensional membrane surface by the hydrogen bond between the primary amine of PE to PA “switching” to the primary amine of lysine within the PBD of lipin 1. The generation of a strong interaction between the PBD and PA would allow the catalytic site of lipin 1 to dephosphorylate multiple molecules of PA without lipin 1 releasing from the membrane surface. We suggest that this explains, at least in part, the functional cooperativity that is shown during surface step reaction kinetics. Lipin 1 can form homotetramers or heterotetramers with other lipin proteins. Although it remains to be determined whether the PBD-mediated association with PA must occur within the same lipin 1 molecule that contains the active site for functional cooperativity, previous reports have suggested that the catalytic sites within lipin 1 homo-tetramers function independently (32). Although mTOR has been demonstrated by immunofluorescence staining to control lipin 1 movement to membranes within cells, we have now shown that mTOR-directed phosphorylation regulates lipin 1 enzymatic function. Because mutation of the PBD elim-

inates the effects of phosphorylation on activity, we propose that mTOR-mediated phosphorylation prevents lipin 1 from recognizing PA containing two negative charges in membranes (Fig. 8). The requirement for the PBD for membrane interaction provides a rationale that explains why lipin 1 interacts with PA. Other negatively charged lipids such as unsaturated fatty acids and acyl-CoA esters have also been shown to promote PAP accumulation on membranes (33, 34). Conversely, neutralizing this negative charge with an amphiphilic amine displaces PAP activity from endoplasmic reticulum membranes in cell-free systems (34) and in hepatocytes (35). Future experiments will be necessary to determine whether these effects on PAP localization occur via interactions with the lipin 1 PBD.

There could be two separate membrane binding regions within the lipin family of proteins. The single *Saccharomyces cerevisiae* homolog of the lipin family contains an amino-terminal amphipathic helix that interacts with membranes and is inhibited by phosphorylation (13). Mutation of the amphipathic helix decreases membrane recruitment, renders the mutant Pah1p protein resistant to phosphorylation-mediated inhibition of membrane binding, and functionally eliminates the ability of Pah1p to participate in lipid synthesis. Mammalian lipin 1 and 2 contain predicted amphipathic helices at their amino terminus, but whether the helix is required for membrane association has not been determined. In contrast, although all mammalian lipins contain a nine-amino acid PBD, in *S. cerevisiae* there is no such conserved stretch, although there is a KKKEK region in a similar position (amino acids 171–176). However, the *Saccharomyces pombe* homolog contains no more than two consecutive basic amino acids. The *Arabidopsis thaliana* homologs also lack a PBD and yet functions similarly to yeast Pah1p (36). Evolutionarily, the nine-amino acid PBD found in mammals did not occur until urochordata (*Ciona intestinalis*) and is largely conserved in all descendant species. It should be pointed out that most PA-binding proteins do not have such a large stretch of basic residues and frequently require only one or two basic residues for PA binding. It is interesting that the mammalian lipins have evolved such a strategy. Lipin 1 can still associate with liposomes in the absence of PA (Fig. 3, A and B) and the absence of the PBD (Fig. 7C), albeit at greatly reduced efficiency. And increasing molar concentrations of PA:PC liposomes still increase Pah1p association even when the amphipathic helix was mutated such as to completely abolish membrane recruitment (13). Thus, both yeast and mammalian lipins may employ separable mechanisms for membrane binding. We suspect that the PBD is specific for PA association, whereas the amphipathic helix is more likely involved in general membrane binding and/or catalysis. However, future studies into the mechanism of lipin 1 membrane binding using combinations of the PBD/amphipathic helix will be necessary to fully identify the domains required for membrane binding, activity, and regulation by phosphorylation.

Lipin 1 is clearly required for phospholipid and neutral lipid synthesis. Insulin stimulation promotes triacylglycerol synthesis in adipose tissue (37). It may seem paradoxical that insulin stimulation increases lipin 1 phosphorylation and decreases its interaction with membranes and thus its enzymatic activity (4). However, a second major driving force for this reaction is



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increased fatty acid availability. This provides a feed-forward stimulus through the accumulation of fatty acids and PA in membranes that promotes the translocation of lipins to the endoplasmic reticulum and enables cells to match their rates of triacylglycerol synthesis to the fatty acid supply. This occurs during  $\beta$ -adrenergic stimulation of adipocytes, which promotes lipolysis, increases fatty acid accumulation, and increases lipin 1 membrane association, resulting in increased fatty acid esterification (4, 38, 39). This translocation would be favored by low insulin stimulated phosphorylation of lipin 1.

It is also possible that alterations in intracellular pH, combined with the effect of pH on the ability of lipin 1 to bind to PA, could contribute to the observations *in vivo*. To use adipocytes as an example, during insulin stimulation, lipin 1 is highly phosphorylated. Presumably this will decrease lipin 1 affinity for PA. However, adipocytes also increase fatty acid esterification to PA during the influx of fatty acids from lipoprotein lipase postprandially. Therefore, the concentration of PA at the ER will increase, overcoming the inhibitory effect of phosphorylation. On the other hand, stimulation of adipocytes with a catecholamine, such as epinephrine, causes a profound induction of triacylglycerol lipolysis. The high levels of fatty acids flooding the cell decrease intracellular pH (40). At the same time, insulin signaling pathways are inhibited, particularly the mTORC1 pathway (41). Thus, the reduction in pH decreases lipin 1 affinity for PA, yet at the same time lipin 1 phosphorylation is inhibited, thereby increasing its affinity for PA. We postulate that lipin 1 phosphorylation is a necessary counterbalance to maintain PAP activity during changes in intracellular pH, alterations in PC:PE ratios, and possibly intracellular fatty acid accumulation. Maintaining a relatively similar degree of lipin 1 affinity for PA could be important because allowing lipin 1 to have a high affinity for PA at any pH could promote lipin 1-mediated hydrolysis of PA generated via insulin stimulation of PLD at the plasma membrane or other membrane surfaces outside of the ER. In addition, lipin 1 might show increasing phosphorylation because of the importance of preventing lipin 1 from binding to the inner leaflet of the plasma membrane, an important site for the PA role in insulin-stimulated GLUT4 vesicle fusion (42, 43). Thus, as the intracellular pH changes, increasing or decreasing lipin 1 affinity for PA, phosphorylation is reciprocally regulated to maintain a relatively constant affinity for PA. We should point out that biochemical fractionation may not be an accurate measure of lipin 1 microsomal association, as changing the intracellular pH to the pH of the buffer during homogenization may artificially change lipin 1 association with membranes.

Determining that lipin 1 prefers to associate with membranes containing di-anionic PA has important implications in the control of intracellular PAP activity. Our studies show that intracellular pH will likely significantly affect lipin 1 enzymatic activity. Thus, lipin 1 may fall into a relatively novel class of proteins that function as pH sensors (44). In this case regulation of lipin 1 by pH could affect the production of phospholipids and triacylglycerols. Furthermore, changes in intracellular pH might also affect lipin 1 localization to the nucleus and thus regulate the ability of lipin 1 to function as a transcriptional co-activator or co-repressor. In addition, the relative levels of PE to other phospholipids may be important in the control of

lipin 1 localization; for example, conditions such as obesity can change the PC:PE ratio at the ER from 1.3 to 2.0 (45). Therefore, changes in the PC:PE ratio in conjunction with alterations in intracellular pH could change the activity of lipin 1 at the ER or cause aberrant lipin 1 localization to other intracellular membranes. We conclude that the translocation of the cytosolic reservoir of lipin 1 to membranes enables lipin 1 to perform its various physiological functions and that this translocation is modulated by the phosphorylation of lipin 1, especially through mTOR.

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