Fluorescence spectroscopy measures yeast \( PAH1 \)-encoded phosphatidate phosphatase interaction with liposome membranes

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Abstract Phosphatidate (PA) phosphatase, the enzyme that catalyzes the penultimate step in triacylglycerol synthesis, is a cytosolic enzyme that must associate with the membrane where its substrate PA resides. Fluorescence spectroscopy was used to measure the interaction of yeast \( PAH1 \)-encoded PA phosphatase with model liposome membranes. PA phosphatase contains five tryptophan residues and exhibited inherit fluorescence that increased upon interaction with phosphatidylcholine liposomes. The interaction was enhanced by inclusion of other phospholipids and especially the substrate PA. Interaction was dependent on both the concentration of phosphatidylcholine-PA liposomes as well as the surface concentration of PA in liposomes. Mg\(^{2+}\) ions, which were required for catalysis, did not affect PA phosphatase interaction with phosphatidylcholine-PA liposomes. PA phosphatase was a substrate for protein kinase A, protein kinase C, and casein kinase II, and these phosphorylations decreased PA phosphatase interaction with phosphatidylcholine-PA liposome membranes.—Xu, Z., W-M. Su, and G. M. Carman. Fluorescence spectroscopy measures yeast \( PAH1 \)-encoded phosphatidate phosphatase interaction with liposome membranes. J. Lipid Res. 2012. 53: 522–528.

Supplementary key words phospholipid • triacylglycerol • liposomes • yeast

The yeast \( Saccharomyces cerevisiae \) serves as a eukaryotic model to study the biochemistry and physiological roles of phosphatidate (PA) phosphatase (1), the enzyme that catalyzes the dephosphorylation of PA yielding diacylglycerol (DAG) and \( P_i \) (2). The gene encoding the enzyme in yeast is \( PAH1 \) (3), and in fact, its discovery led to the revelation that the lipodystrophic defect in the fatty liver dystrophy (\( fdl \)) mouse (4, 5) was a PA phosphatase deficiency because of mutations in the \( lip1 \) gene (3, 6). The PA phosphatase reaction requires Mg\(^{2+}\) ions and is based on a \( DXDXT(V/T) \) catalytic motif within a haloacid dehalogenase-like domain in the enzyme (3, 7). The DAG produced by the enzyme is utilized for triacylglycerol (TAG) synthesis, and for the synthesis of the major phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (1, 8). PA phosphatase also plays a major role in controlling the cellular content of its substrate PA (3), the precursor of phospholipids that are synthesized via the liponucleotide intermediate CDP-DAG (1, 8). In addition, PA plays a signaling role in the regulation of phospholipid synthesis gene expression (9).

The importance of PA phosphatase in yeast is exemplified by an assortment of \( pah1 \Delta \) mutant phenotypes. For example, cells bearing the \( pah1 \Delta \) mutation exhibit elevated levels of PA and reduced levels of DAG and TAG (3, 7). The elevated PA content causes the induction of phospholipid synthesis gene expression and the aberrant expansion of the nuclear/ER membrane (3, 7, 10), whereas the reduced capacity to synthesize DAG and TAG causes a defect in lipid droplet formation (11) and an acute sensitivity to fatty acid-induced toxicity (12), respectively. In addition, loss of PA phosphatase causes a respiratory deficiency phenotype and sensitivity to growth at elevated temperature (3, 10).

Mutations in mammalian lipins (counterpart of yeast \( PAH1 \)-encoded PA phosphatase) also cause defects in lipid metabolism and cell physiology. Mice lacking lipin 1 exhibit defects in adipose tissue development, lipodystrophy, insulin resistance, and peripheral neuropathy (4, 5, 13–16). In humans, lipin 1 mutations are associated with metabolic syndrome, type 2 diabetes, and recurrent acute myoglobinuria in children, whereas mutations in lipin 2

Abbreviations: DAG, diacylglycerol; ER, endoplasmic reticulum; PA, phosphatidate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol.

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are responsible for anemia and inflammatory disorders associated with Majeed syndrome (17–20).

In yeast [as well as in mammalian cells (21, 22)], PA phosphatase is primarily associated with the cytosolic fraction of the cell (3, 23, 24). Yet, the association of the enzyme with the membrane where its substrate PA resides is essential to its function in vivo (3, 23, 24). The association of PA phosphatase with the membrane is primarily regulated by the phosphorylation of the enzyme (23, 24); phosphorylation favors a cytosolic location, whereas dephosphorylation allows for a membrane association (24). The phosphorylated enzyme is recruited to the nuclear/endoplasmic reticulum (ER) membrane where it is dephosphorylated by the protein phosphatase complex Nε1p-Spo7p (10, 23, 24). This in turn leads to anchoring of PA phosphatase to the membrane via an amphipathic helix located at the N-terminus of the protein (23).

As an initial step in gaining mechanistic insight into the interaction of PAH1-encoded PA phosphatase with membranes, we utilized a convenient fluorescence assay with purified yeast PA phosphatase and model liposome membranes. We found that the interaction of PA phosphatase with liposomes caused the intensity of its inherent fluorescence to increase, and the interaction was enhanced by the presence of PA in the liposomes. The fluorescence assay proved useful in monitoring the effects of phosphorylation on the interaction of PA phosphatase with liposome membranes.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade or better. Lipids were from Avanti Polar Lipids. Reagents for electrophoresis, immunoblotting, and protein assay were from Bio-Rad. Polyvinylidene difluoride paper and the enhanced chemiluminescence Western blotting detection kit were from GE Healthcare. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies were from Thermo Scientific. Lambda protein phosphatase and casein kinase II were from New England Biolabs. Protein kinase A from Thermo Scientific. Lambda protein phosphatase and carbonate filter (100 nm diameter).

Preparation of liposomes

Liposomes (unilamellar phospholipid vesicles) were prepared by the lipid extrusion method of MacDonald et al. (27). In brief, chloroform was evaporated from the indicated phospholipids under nitrogen to form a thin film. The phospholipids were then redissolved in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA. After five cycles of freeze-thawing, the phospholipid suspensions were extruded 11 times through a polycarbonate filter (100 nm diameter).

Dephosphorylation reactions

Phosphorylation reactions were measured at 30°C for the indicated time intervals and amounts of protein kinase enzymes in a total volume of 20 μl. The reaction mixture for protein kinase A contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 μM [γ-32P]ATP (10,000 cpm/pmol), and 0.5 μg recombinant PA phosphatase. The reaction mixture for protein kinase C contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.375 mM EDTA, 0.375 mM EGTA, 1.7 mM CaCl₂, 20 μM DAG, 50 μM PS, 20 μM [γ-32P]ATP (10,000 cpm/pmol), and 0.5 μg recombinant PA phosphatase. The reaction mixture for casein kinase II contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 50 μM [γ-32P]ATP (10,000 cpm/pmol), and 0.5 μg recombinant PA phosphatase. At the end of the phosphorylation reactions, samples were treated with 2× Laemmli’s sample buffer, followed by SDS-PAGE (26). SDS-polyacrylamide gels were dried and subjected to phosphorimaging analysis.

The PA phosphatase isolated from yeast was dephosphorylated with 50 units of lambda protein phosphatase in 50 mM Tris-HCl (pH 7.5), 2 mM MnCl₂, 2 mM dithiothreitol, 0.01% Brij 35, 0.1 mM EDTA, 100 mM NaCl.

Preparation and analysis of PA phosphatase-liposome interaction

Recombinant PA phosphatase (105 nM) was incubated for 10 min with the indicated concentrations of PC-PA liposomes in 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl, 105 nM PA phosphatase, and the indicated concentrations of liposomes in a total volume of 0.2 ml. The excitation wavelength was 280 nm, and the emission spectra were collected from 300–450 nm after a 10 min incubation period. The slit width was set at 5 nm for both excitation and emission beams. The spectra were corrected for light scattering effects by subtracting blanks that contained liposomes but lacked PA phosphatase.

Immunoblot analysis of PA phosphatase-liposome interaction

Recombinant PA phosphatase isolated from yeast was dephosphorylated with 50 units of lambda protein phosphatase in 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl in a total volume of 0.2 ml. Following incubation, the reaction mixture was subjected to centrifugation at 100,000 g for 1 h, and the liposome pellet was resuspended in the same volume as the supernatant. Samples (25 μl) from each fraction were separated by SDS-PAGE (26) and then subjected to immunoblot analysis (28) using anti-PA phosphatase antibodies (24). The anti-PA phosphatase antibodies were used at a concentration of 2 μg/ml, and alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies were used at a dilution of 1:5,000. Immune complexes were detected using the enhanced chemiluminescence Western blotting detection kit. Fluorimaging was used to acquire images from immunoblots, and the relative densities of the images were analyzed using ImageQuant software. The signals were in the linear range of detectability.

PA phosphatase assay

PA phosphatase activity was measured at 30°C for 20 min by following the release of water-soluble P, from chloroform-soluble PA (29). The P, produced in the reaction was measured with malachite green-molybdate reagent as described by Han and Carman (30). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.5 mM MgCl₂, 10 mM 2-mercaptoethanol, enzyme protein, and either PC-PA liposomes or Triton X-100-PA mixed micelles as the substrate. The molar ratios of PC to PA and Triton X-100 were determined.
compared with the constant determined for PC liposomes (Figs. 2B and 3B). We questioned whether MgCl$_2$ (0.5 mM) affected the interaction of PA phosphatase with PC-PA liposomes given that Mg$^{2+}$ is required to elicit a maximum turnover number for PA phosphatase activity in vitro (3). For these experiments, EDTA was omitted from the buffer used to prepare liposomes. Mg$^{2+}$ did not have a major effect on the interaction of the enzyme with PC-PA liposomes as the $K_d$ values with and without MgCl$_2$ were not significantly different (Fig. 4). This indicated the

Dissociation constants ($K_d$) for the interaction of PA phosphatase with liposomes were calculated using equation 1 (31)

$$\frac{F_0 - F}{F} = \left(\frac{F_{max} - F}{F_{max}}\right) - \frac{(K_d/n)}{1}$$  

where $K_d$ is the dissociation constant of the lipid-protein complex, $n$ is the number of binding sites per lipid, $F$ is the fluorescence intensity at each titration point, $F_{max}$ is the fluorescence intensity of membrane bound protein, which equals to the limiting fluorescence intensity when all protein was bound to the vesicles in the solution, $F_0$ is the minimal fluorescence when no lipid vesicle was added, $C$ is the lipid concentration. The apparent binding constant $K_d = K_d/n$ represents the dissociation constant of protein-lipid interaction and reflects the affinity of a protein to the membrane (31). Statistical analyses were performed with SigmaPlot software. The p values < 0.05 were taken as a significant difference.

RESULTS

Tryptophan fluorescence of PA phosphatase increases with liposome interaction

We sought a convenient assay to measure the interaction of yeast PAH1-encoded PA phosphatase with model liposome membranes. Because PA phosphatase contains five tryptophan residues (Trp$^{15}$, Trp$^{388}$, Trp$^{390}$, Trp$^{420}$, Trp$^{637}$), an amino acid with intrinsic fluorescence (32, 33), we examined fluorescence spectroscopy as a method to assay the interaction of the enzyme with liposomes. For this assay, we utilized PA phosphatase enzyme that was expressed and purified from E. coli (3). The fluorescence spectrum of the recombinant PA phosphatase in aqueous solution showed a maximum at 350 nm (Fig. 1), indicating a polar environment surrounding the tryptophan residues. The interaction of PA phosphatase with PC liposomes resulted in an increase in fluorescence emission intensity of the tryptophan residues as well as a shift from 350 to 343 nm in the wavelength of the maximum emission (Fig. 1).

The effect of liposome composition on PA phosphatase tryptophan fluorescence was examined. For PC liposomes and liposomes containing phosphatidylserine (PS), PE, phosphatidylinositol (PI), or PA, there was a dose-dependent increase in PA phosphatase fluorescence (Fig. 2A), indicating an increase in PA phosphatase-liposome interaction. Of the liposomes examined, the one composed of PC-PA had the greatest effect on the interaction. Moreover, the interaction of PA phosphatase with the PC-PA liposomes was dependent on the surface concentration of PA (Fig. 3), which further substantiated dependence for this phospholipid. The dissociation constant ($K_d$) for PC-PA liposomes (at 10 mol % PA) was 4.4-fold lower when compared with the constant determined for PC liposomes (Figs. 2B and 3B). We questioned whether MgCl$_2$ (0.5 mM) affected the interaction of PA phosphatase with PC-PA liposomes given that Mg$^{2+}$ is required to elicit a maximum turnover number for PA phosphatase activity in vitro (3). For these experiments, EDTA was omitted from the buffer used to prepare liposomes. Mg$^{2+}$ did not have a major effect on the interaction of the enzyme with PC-PA liposomes as the $K_d$ values with and without MgCl$_2$ were not significantly different (Fig. 4). This indicated the
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by centrifugation. The supernatant and liposome fractions were then subjected to immunoblot analysis using anti-PA phosphatase antibodies. This analysis showed a concentration-dependent increase in the PA phosphatase enzyme that physically interacted with liposome membranes (Fig. 5). At the highest concentration (0.4 mM) of phospholipid used in this experiment, about 50% of the PA phosphatase was associated with liposome membranes.

PA phosphatase is catalytically active with PC-PA liposome membranes

The yeast PA phosphatase enzyme is routinely measured when its substrate is presented in a Triton X-100-PA mixed micelle (3, 34, 35). To confirm that the PA phosphatase was catalytically active when the substrate was presented in PC-PA liposomes, the activity was measured with 0.5 mM MgCl₂ under the reaction conditions used to measure enzyme-liposome interaction. The PA phosphatase activity measured with PC-PA liposomes was 3.5 μmol/min/mg. As expected (3), no activity was observed when MgCl₂ was omitted from the reaction. The PA phosphatase activity measured with PC-PA liposomes was 40% less than the activity (5.9 μmol/min/mg) measured with Triton X-100-PA mixed micelles. This lower level of activity might be attributed to the fact that the enzyme would not have access to PA on the inner leaflet of the liposome membrane.

independent nature of the binding and catalytic steps in the PA phosphatase reaction.

PA phosphatase physically interacts with PC-PA liposome membranes

We next confirmed that PA phosphatase physically interacted with PC-PA liposomes. PA phosphatase was incubated with PC-PA liposomes, followed by the separation of the liposome-bound and the free form of the enzyme.

Fig. 3. Effect of PA concentration on the interaction of PA phosphatase with liposomes. A: recombinant PA phosphatase (105 nM) was incubated with the indicated concentrations of liposomes composed of the indicated surface concentrations of PA. Following 10 min incubation, the increase in PA phosphatase fluorescence was measured. The data are plotted with respect to total phospholipid concentration in the liposomes. The values reported were the average of three experiments ± SE. B: dissociation constants (K_d) were determined from the data shown in A.

Fig. 4. Effect of magnesium on the interaction of PA phosphatase with PC-PA liposomes. Recombinant PA phosphatase (105 nM) was incubated with the indicated concentrations of PC-PA liposomes in the absence and presence of 0.5 mM MgCl₂. Following 10 min incubation, the increase in PA phosphatase fluorescence was measured. The data are plotted with respect to total phospholipid concentration in the liposomes. The values reported were the average of three experiments ± SE. The dissociation constants (K_d) were determined from the data are shown in the inset.

Fig. 5. Immunoblot analysis of PA phosphatase interaction with PC-PA liposomes. A: Recombinant PA phosphatase (PAP) (105 nM) was incubated with the indicated concentrations (shown in B) of PC-PA liposomes. Following 10 min incubation, the liposome-bound and free forms of PA phosphatase were separated by centrifugation at 100,000 g for 1 h. The pellet (P), which contained liposomes was resuspended in the same volume as the supernatant (S), and equal volumes (25 μl) of the fractions were subjected to immunoblot analysis using anti-PA phosphatase antibodies. A representative immunoblot is shown where the position of PA phosphatase is indicated by the arrow. B: The relative amounts of liposome-bound and free PA phosphatase were determined by ImageQuant analysis of four immunoblots ± SD.
Phosphorylation decreases the interaction of PA phosphatase with PC-PA liposomes

PA phosphatase isolated from yeast is a highly phosphorylated protein (25), and studies with yeast that express a phosphorylation-deficient form of the enzyme indicate that phosphorylation favors a cytosolic location whereas dephosphorylation favors a membrane association (23, 24). Using the fluorescence assay, we examined whether phosphorylation of PA phosphatase affected its interaction with PC-PA liposomes. We utilized the recombinant PA phosphatase as a pristine protein kinase substrate free of the phosphorylations that occur when the enzyme is expressed in yeast (25). PA phosphatase has putative target sites for protein kinase A, protein kinase C, and casein kinase II, and as shown in Fig. 6A, all three protein kinases phosphorylated PA phosphatase in dose- and time-dependent manners. These phosphorylated forms of PA phosphatase were then examined for their interactions with PC-PA liposomes using the fluorescence assay. The phosphorylations with protein kinase A, protein kinase C, and casein kinase II caused a decrease in the interaction of PA phosphatase with the PC-PA liposomes with increased Kd values of 3.8-fold, 2.3-fold, and 1.5-fold, respectively, when compared with the control unphosphorylated enzyme (Fig. 6B). Moreover, the Kd value of PA phosphatase that is endogenously phosphorylated in yeast was 3.2-fold greater than the Kd value of the unphosphorylated enzyme isolated from E. coli (Fig. 6B). As described previously (23), the dephosphorylation of the yeast-derived PA phosphatase resulted in an increase in lipidosome interaction as indicated by a 3.15-fold decrease in the Kd value for the enzyme (Fig. 6B).

DISCUSSION

PA phosphatase is an important regulatory enzyme of lipid metabolism (1), whose physiological function is dependent on its interaction with the nuclear/ER membrane (23, 24). In this work, we examined the interaction of yeast PA phosphatase with model lipidosome membranes. Whereas a number of approaches [e.g., circular dichroism (36), dual polarization interferometry (37), quartz crystal microbalance with dissipation monitoring (38), and surface plasmon resonance (39)] have been used to examine protein-membrane interactions, we chose fluorescence spectroscopy (40, 41) because PA phosphatase exhibited an inherent tryptophan fluorescence whose wavelength of maximum emission shifted and increased upon interaction with lipidosome membranes. Additional probes such as brominated lipid derivatives that are commonly used to follow tryptophan fluorescence quenching (36, 40–42) were not required for this assay. The change in fluorescence of PA phosphatase upon interaction with lipidosome membranes was consistent with tryptophan residues being present in a more hydrophobic environment (shift from 350 to 343 nm) and less association with charged amino acid residues (increase in fluorescence) (43).

PA phosphatase interacted with PC liposomes alone, and this interaction was enhanced when the liposomes contained another major membrane phospholipid. Thus, interaction of the enzyme with the membrane surface was not dependent on the presence of its substrate PA. Yet, the greatest interaction occurred with PC-PA liposomes. This interaction was dependent on both the concentration of PC-PA liposomes as well as the surface concentration of PA in the liposomes. That the interaction was greatest with PC-PA liposomes was consistent with in vivo studies showing that the translocation of PA phosphatase from the cytosol to the nuclear/ER membrane is dependent on the abundance of PA in the membrane (23). Moreover, the enzyme was catalytically active when its substrate was presented in the assay as PC-PA liposomes.

The PA phosphatase protein contains an N-terminal amphipathic helix (amino acid residues 1 to 18) that is required for its interaction with the nuclear/ER membrane in vivo, and the fluorescence assay described in the present work has been used to substantiate this finding in vitro (23). In particular, alanine mutations of Leu8, Val11, and Trp16 (bulky residues within the hydrophobic phase of the amphipathic helix) cause a 3.6-fold decrease in enzyme interaction (as reflected in Kd values) with PC-PA liposomes.
liposomes (23). The binding function of the amphipathic helix, however, requires that the enzyme be in an unphosphorylated state (23). Presumably, the dephosphorylation causes a conformation change that allows for protein-membrane interaction (23). In vivo, the dephosphorylation of the enzyme at the nuclear/ER membrane is mediated by the protein phosphatase complex Nml1p-Spo7p (10). Yet, the requirement for this complex can be circumvented by a phosphorylation-deficient form of the enzyme (23, 24). It was fortuitous that our liposome binding studies were initially performed with purified recombinant PA phosphatase that is not subject to endogenous phosphorylation. Indeed, the liposome (lacking the Nml1p–Spo7p complex) interaction of the purified PA phosphatase isolated from yeast, which is endogenously phosphorylated (10, 25), was greatly reduced when compared with the recombinant enzyme. In previous studies using the fluorescence assay described here (23, 24), we showed that a phosphorylation-deficient PA phosphatase (alanine mutations of Ser\(^{110}\), Ser\(^{114}\), Ser\(^{168}\), Ser\(^{692}\), Thr\(^{725}\), Ser\(^{744}\), and Ser\(^{745}\) mutant isolated from yeast interacted with PC-PA liposomes better than that of wild-type enzyme isolated from yeast, and that the dephosphorylation of the wild-type enzyme with lambda protein phosphatase enhanced the interaction of the enzyme with PC-PA liposomes. These studies imply, but did not show, that phosphorylation inhibits the interaction of PA phosphatase with a membrane.

O’Hara et al. (25) have identified multiple sites of phosphorylation in \(PAH1\)-encoded PA phosphatase. Of these sites, the seven sites mentioned above are contained within the minimal Ser/Thr-Pro motif that is a target for protein kinases regulated during the cell cycle (25). Three of these sites (Ser\(^{692}\), Thr\(^{725}\), Ser\(^{744}\)) are targets of \(CDC28\)-encoded cyclin-dependent kinase (24). However, phosphorylation of these sites has little effect on PA phosphatase activity or its physiological function with respect to TAG synthesis (24). Thus, the phosphorylation of one or more of the remaining sites among the seven is needed to regulate enzyme function. The identity of the protein kinase(s) involved with the phosphorylation of these four sites is currently under investigation. Some of the identified sites that are not contained within a Ser/Thr-Pro motif are putative targets for protein kinase A, protein kinase C, and casein kinase II. In this work, we showed that the recombinant PA phosphatase was in fact a substrate for these three protein kinases. Moreover, the phosphorylations by these protein kinases caused a decrease in the interaction of PA phosphatase with PC-PA liposomes. That the effects of each protein kinase differed (as reflected in the respective \(K_p\) values), indicated that different sites of phosphorylation had varying effects on liposome membrane interaction. The data indicated that the site(s) phosphorylated by protein kinase A had the greatest effects. Studies are currently in progress to identify the target site(s) for each of these protein kinases.

Although the impetus for this work was to utilize a convenient method to examine the interaction of PA phosphatase with model liposome membranes, these studies also led to the discovery of new information about the enzyme. We learned that PA phosphatase can interact with membranes in the absence of PA but that the interaction was enhanced by the substrate. Moreover, interaction of the enzyme with the membrane was independent of its catalytic activity. We also learned that the activity of PA phosphatase could be measured with PC-PA liposomes, a model membrane system that more closely mimics a physiological membrane when compared with a detergent micelle. Finally, we discovered that PA phosphatase was a substrate for protein kinases A and C and casein kinase II, and that these covalent modifications affected enzyme-membrane interaction.

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