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Phosphatidic acid binding proteins display differential binding as a function of membrane curvature stress and chemical properties

Priya Putta [a], Johanna Rankenberg [a,1], Ruud A. Korver [b,c], Ringo van Wijk [b,c], Teun Munnik [b,c], Christa Testerink [b,c], Edgar E. Kooijman [a,*]

[a] Biological Sciences, Kent State University, PO Box 5190, 44242 Kent, OH, USA
[b] University of Amsterdam, Swammerdam Institute for Life Sciences, Section of Plant Physiology, Postbus 94215, 1090, GE, Amsterdam, The Netherlands
[c] University of Amsterdam, Swammerdam Institute for Life Sciences, Section of Plant Cell Biology, Postbus 94215, 1090 GE Amsterdam, The Netherlands

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ABSTRACT

Phosphatidic acid (PA) is a crucial membrane phospholipid involved in de novo lipid synthesis and numerous intracellular signaling cascades. The signaling function of PA is mediated by peripheral membrane proteins that specifically recognize PA. While numerous PA-binding proteins are known, much less is known about what drives specificity of PA-protein binding. Previously, we have described the ionization properties of PA, summarized in the electrostatic-hydrogen bond switch, as one aspect that drives the specific binding of PA by PA-binding proteins. Here we focus on membrane curvature stress induced by phosphatidylethanolamine and show that many PA-binding proteins display enhanced binding as a function of negative curvature stress. This result is corroborated by the observation that positive curvature stress, induced by lyso phosphatidylcholine, abolishes PA binding of target proteins. We show, for the first time, that a novel plant PA-binding protein, Arabidopsis Epsin-like Clathrin Adaptor 1 (ECA1) displays curvature-dependence in its binding to PA. Other established PA targets examined in this study include, the plant proteins TGD2, and PDK1, the yeast proteins Opi1 and Spo20, and, the mammalian protein Raf-1 kinase and the C2 domain of the mammalian phosphatidylserine binding protein Lact as control. Based on our observations, we propose that liposome binding assays are the preferred method to investigate lipid binding compared to the popular lipid overlay assays where membrane environment is lost. The use of complex lipid mixtures is important to elucidate further aspects of PA binding proteins.

1. Introduction

Phosphatidic acid (PA) is a minor membrane phospholipid that is formed in response to several stress conditions in plants [1]. PA signaling has also been implicated in numerous cellular processes in mammals, as diverse as membrane trafficking and sperm hyperactivation [2,3]. While many proteins with PA-binding properties have been identified, it is still poorly understood what determines the specificity of proteins to bind PA [4,5]. Of particular note is the observation that while numerous PA-binding proteins have been identified and characterized, no PA-specific binding domain has emerged that is conserved in more than one protein. Previously, we identified the ionization properties and effective lipid shape of PA as two determinants that set PA apart from other, more abundant, anionic membrane lipids [6–9]. It is clear that certain amino acid residues such as tryptophan, lysine, arginine and histidine, are enriched in PA binding domains [1, 10]. We and others have subsequently shown that membrane lipid composition can strongly influence the degree of PA binding by PA binding proteins [9,11–13].

These observations suggest a need for careful evaluation and reevaluation of PA binding by PA binding proteins. Here we re-examine the binding of several PA specific binding proteins from plants, yeast, and mammals, to PA in model lipid membranes as a function of the membrane lipid phosphatidylethanolamine (PE). PE is zwitterionic, similar to phosphatidylcholine (PC), but carries a much smaller head group (in terms of actual size, but also in terms of hydration [*14*]). In fact, PE is a well-known non-bilayer forming lipid with negative spontaneous curvature (i.e. a Type II lipid, see below) [15–17].

The presence of non-bilayer lipids is essential in various cell processes, from cell fusion to cell fission, membrane trafficking to membrane remodeling, and are an indispensable part of the membrane bilayer composition [18–20]. Non-bilayer lipids can also be classified as Type I and Type II lipids based on the type of curvature stress they induce in the bilayer (See Fig. 1, and [*21*]). Type I lipids have a larger headgroup area compared to acyl-chain area. By definition, they have a positive spontaneous curvature, and thus cause positive curvature stress when

* Corresponding author.
E-mail address: ekooijma@kent.edu (E.E. Kooijman).
[1] Currently at: Department of Ophthalmology, University of Colorado School of Medicine, 12,800 East 19th Avenue, RC-1 North, 5402 E Mail stop 8311 Aurora CO 80045, USA.

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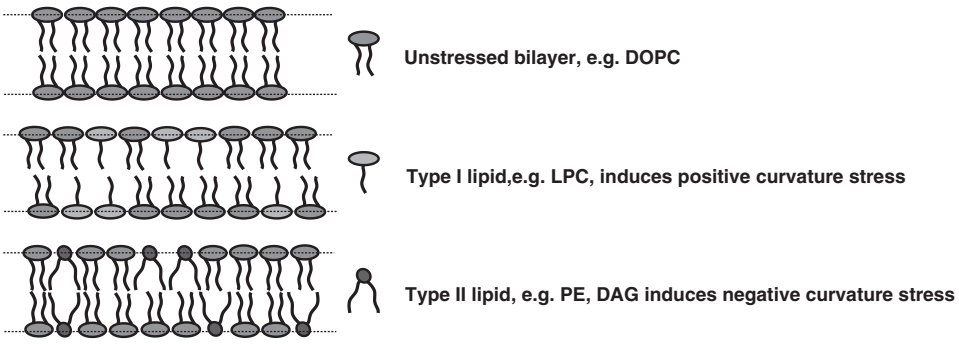

Fig. 1. Effect of Type I and Type II lipids on membrane structure.

incorporated in flat bilayer membranes. Type I lipids make up only a small fraction of membrane lipids and often have important signaling functions, or are merely present transiently during the remodeling of membrane lipids [22]. In contrast to Type I lipids, Type II lipids have a small headgroup area compared to acyl-chain area and have negative spontaneous curvature, and thus induce negative curvature stress. The presence of Type II lipids in membranes leads to reduced headgroup packing and thus tends to facilitate protein binding via the increased insertion of small hydrophobic and amphipathic domains into lipid membranes [20,23,24]. Membrane curvature stress is influenced by the presence of Type I and Type II lipids in the bilayer ([*18,21,25*], where the Type II lipid PE is the most common but diacylglycerol (DAG) is also an important Type II lipid [26].

While PE changes the curvature stress of membranes, as shown schematically in Fig. 1, PE also affects the charge of phosphomonoester-containing membrane lipids. Interaction of the primary amine in the headgroup of PE with phosphomonoesters in lipid headgroups leads to an increase in the negative charge of the phosphate (e.g. PA [6], ceramide-1-phosphate [27], and polyphosphoinositides [28]). PE thus has two distinct effects: first, it induces negative curvature stress; and second, it increases the negative charge of PA; both of which influence peripheral membrane protein binding.

Here we investigate the interaction of several peripheral membrane proteins from plants, yeast, and mammals with the membrane lipid phosphatidic acid (PA) in model lipid membranes. We show that PE generally facilitates PA binding, and replacing PE by the Type I lipid, lysophosphatidylcholine (LPC) inhibits protein binding. Inclusion of diacylglycerol (DAG), a Type II lipid, unexpectedly reduces membrane binding of PA-target proteins. These results can be understood in terms of the physicochemical properties of the lipids making up our model membranes. They also underscore the importance of studying binding interactions in well characterized membrane models that most closely mimic the real biomembrane environment.

2. Materials and methods

The lipids that were used for the liposome binding assays were as follows: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC), 1–2-dioleoyl-*sn*-glycerol (DOG), and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) (Avanti Polar Lipids, Alabaster, AL, USA). Lipids were used as received after purity was confirmed via (HP)TLC. Concentration of lipid stocks (in chloroform/methanol 2:1) was checked regularly (together with integrity via TLC) by phosphate assay according to Rouser [29]. All chemicals were from Sigma Aldrich and VWR unless specified.

2.1. Liposome binding assay

Liposome binding assay was performed according to Julkowska et al. [30]. The interaction of PA with its binding proteins in complex lipid mixtures was examined using the following assay. Briefly, 400 nmol total lipid (lipid composition as indicated in the results) in $CHCl_3$:MeOH (9:1) was used to prepare lipid films and dried under N_2 (g) followed by vacuum drying for a minimum of 2 h. 500 μl of freshly prepared extrusion buffer (250 mM Raffinose pentahydrate, 25 mM Tris–HCl pH 7.5, and 1 mM DTT) was added to each lipid film and allowed to hydrate for 40–50 min with occasional vortexing to stimulate MLV formation followed by brief 30 s sonication (bath sonicator). This suspension was extruded 13× over polycarbonate membranes with 0.1 μm pore size using the Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA). The liposome suspension was diluted in three volumes of freshly prepared 1× binding buffer (150 mM KCl, 25 mM Tris–HCl pH 7.5, 1 mM DTT, and 0.5 mM EDTA). The liposomes were pelleted by centrifugation at 21,000 × *g* for 45 min at room temperature. After careful removal of the supernatant the liposome pellet was resuspended in 25 μl 1× binding buffer. 25 μl containing 500 ng of GST- tagged protein of interest (2500 ng for ECA1) was added. Protein was pre-diluted in freshly prepared 6× binding buffer (750 mM KCl, 150 mM Tris–HCl pH 7.5, 6 mM DTT, 3 mM EDTA) so that each (25 μl) protein sample contains protein in 1× binding buffer. Total sample is thus 50 μl, and was incubated on an orbital shaker for 30–40 min at room temperature to facilitate binding. These samples were then centrifuged at 16,000 × *g* for 30 min at room temperature. The pellet was resuspended in 300 μl 1× binding buffer transferred to a new tube and centrifuged at 16,000 × *g* for 30 min at room temperature (pellet wash to reduce background binding). The resulting pellet was resuspended in 33 μl of 1× Laemmli sample buffer [30] and 20 μl of the pellet was loaded on the gel. Pellet samples were heated at 95 °C for 5 min before loading onto the SDS gel. Total protein sample contained 25 μl of 500 ng protein, 25 μl of 1× binding buffer, and 16.7 μl of 4× Laemmli sample buffer and 20 μl of this was loaded on the gel to give the maximum amount of protein binding to the pellet. After SDS–PAGE samples were subjected to Western analysis using *anti*-GST antibody (Santa Cruz). The western blot images were taken using Chemidoc-IT-TS2 machine or via film development and processed using ImageJ software. The resulting values were normalized to total protein sample and plotted using SigmaPlot.

2.2. Protein expression and purification of PA binding proteins

The following proteins were examined for the effect of curvature stress on PA binding; from *Arabidopsis thaliana*: Epsin-like Clathrin Adaptor1 (ECA1) [31,32], TGD2 [33], and PH2 domain of PDK1 [30,34]; from *Saccharomyces cerevisiae*: Opi1 (103–191), Opi1 (113–191) [12], and Spo20 [35]; and mammalian: PA binding domain of Raf-1 kinase [9,36], and the C2 domain of Lact (as a control) [37,38].

The ECA1-GST fusion protein was cloned using primers containing Gateway attB1 and attB2 recombination sites, (forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGAACGCTACAGTCATG, reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAAATGAGGCCAGTGCTAC) to amplify AtECA1 (At2G01600) from Arabidopsis Col-0 cDNA. The resulting PCR product was then used to perform a Gateway BP reaction

with pDONR221 to create pDONR221-AtECA1. The pDONR221-AtECA1 vector and a Gateway compatible pGEX vector were used for the Gateway LR reaction, which resulted in the pGEX-AtECA1 vector.

The GST:TGD2 fusion protein was cloned by using primers containing *BglII* and *MfeI* restriction sites (forward: gaagatctGGTTTTCAAATG CGGTCGAAG, reverse: gccaattgTCATAGTAGCCTGCTTAGGGA). The GST:Opi1 (103–191) and GST:Opi1 (113–191) fusion proteins were cloned by using primers containing *BglII* and *EcoRI* restriction sites (forward (103): gaagatctGATGAGTTCTTCACCAAC, forward (113): gaagatctCTGTCGCGGGCGATTG, reverse: ggaattcTTACGATGTCTCGTCC TCGCCAG). The GST:Spo20 fusion protein was cloned by using primers containing *BamHI* and *EcoRI* restriction sites (forward: cgggatccATGG ACAATTGTTCAGGAAGCA, reverse: ggaattcTTAACTAGTCTTAGTGGCGT CA). The GST:Lact-C2 fusion protein was cloned by using primers containing *BglII* and *EcoRI* restriction sites (forward: gaagatctTGCACTGA ACCCCTAGGCC, reverse: ggaattcCTAACAGCCCAGCAGCTCC). The resulting PCR products of Lact-C2, Opi1 (103–191), Opi1 (113–191), Spo20 and TGD2 were subcloned between *BamHI* and *EcoRI* restriction sites of the pGEX-KG vector.

The proteins examined in this work were expressed and purified from *E.coli* cells containing a GST-tag. Briefly, *E.coli* BL21 cells were grown in 5 ml LB media overnight with 5ul ampicillin, and transferred to 100 ml 2× YT media with 100ul ampicillin and incubated at 37 °C until midlog phase. Protein expression was induced with IPTG for 4 h/18 °C. Cells were pelleted at 5000 x g for 15 min and flash frozen until further use. The bacteria were lysed via probe sonication (TBSTxTW buffer- 50 mM Tris, 50 mM NaCl, 0.05% Tween, 0.015% Tirton, 0.5 mM EDTA, 1 mM EGTA, 0.15 mM PMSF, 25× protease inhibitor) and proteins were purified using glutathione beads and eluted with GST elution buffer (50 mM Tris, 10 mM glutathione, pH 8.0) [39]. Protein concentration was determined spectroscopically via a Bradford assay and/or using a nanodrop (Thermo scientific Nanodrop 2000).

2.3. Cryo- transmission electron microscopy

A FEI Vitrobot (Mark IV) plunge freezer was used to prepare vitrified cryo-TEM specimens from the liposome solutions. Details of the sample prep can be found in Frederik et al. [40]. Cryo-TEM observation was

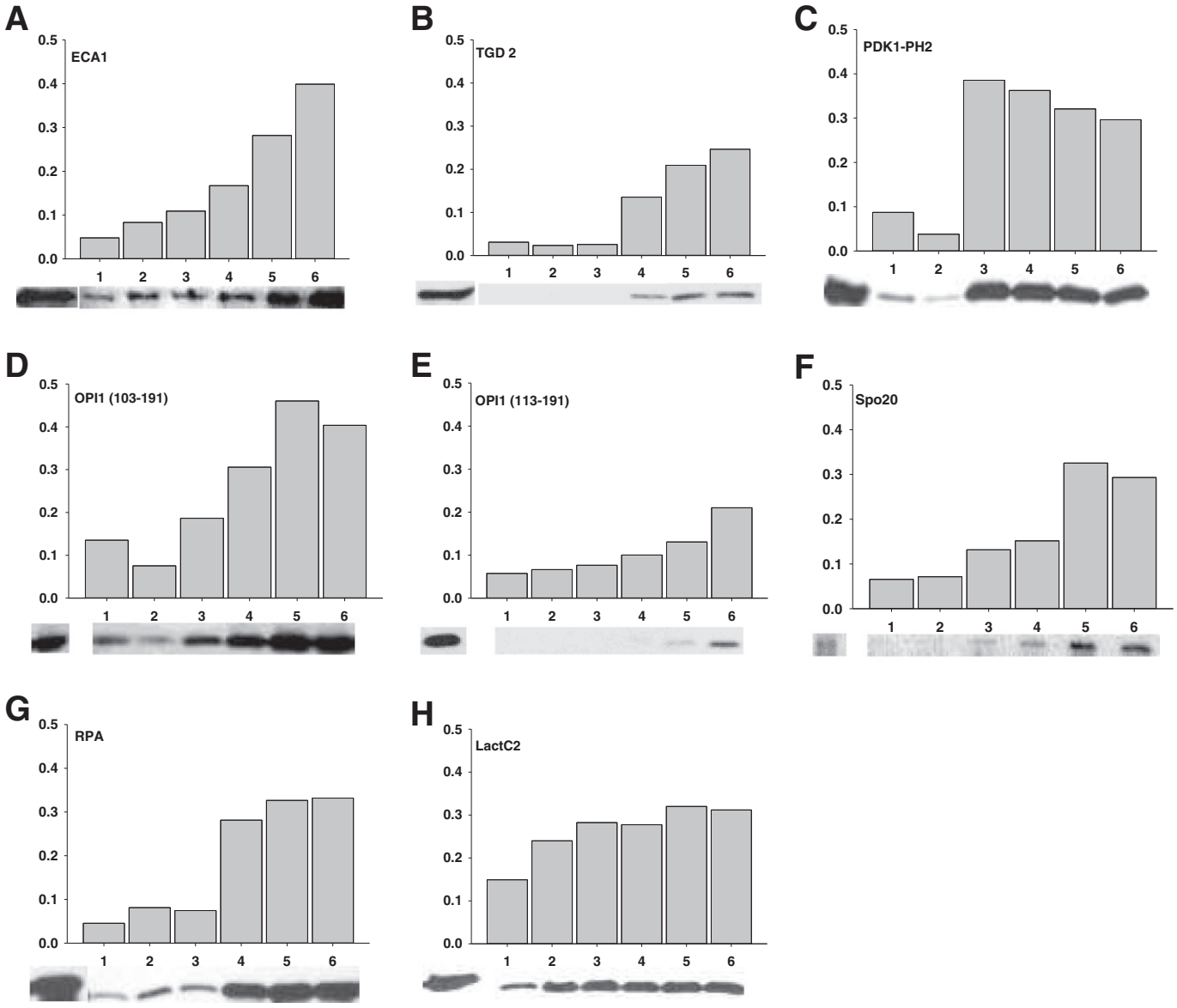

Fig. 2. Effect of PE on PA binding by PA target proteins. A) ECA1, B) TGD2, C) PH2 domain of PDK1, D) Opi1 (103–191), E) Opi1 (113–191), F) Spo20, G) PA-binding domain of Raf1-kinase, and H) C2 domain from Lact. Shown are representative data from a minimum of 3 independent vesicle binding experiments. Binding values are represented as ratios of total protein. Vesicle lipid composition is as follows: Lanes 1–6 DOPC/DOPE/DOPA, 1–100/0/0, 2–50/50/0, 3–90/0/10, 4–75/15/10, 5–60/30/10, 6–45/45/10. Total protein is shown on the left.

performed on a FEI Tecnai F20 transmission electron microscope. The basic experimental setup and procedure can be found in Gao et al. 2014 [41].

3. Results

3.1. PE increases PA-binding by PA binding proteins

PE is a non-bilayer lipid that can make-up over 70% of the membrane lipid composition and is found primarily on the inner leaflet of the plasma membrane in mammalian and plant cells [42]. The presence of PE lipids was shown to affect the activity of various peripheral membrane proteins and some integral membrane proteins [18,20,43,44]. To investigate the effect of PE on PA binding by PA-targets we added PE (DOPE) to our matrix lipid phosphatidylcholine (DOPC). We replace some of the DOPC by the same molar quantity of DOPE and keep the concentration of DOPA constant at 10 mol%. In this way we prepared liposomes with 15, 30 and 45 mol% PE and compare the amount of bound protein to controls consisting of pure DOPC, DOPC-DOPE 50:50, and DOPC-DOPA 90:10.

In order not to bias our study we picked PA-targets from different organisms that have different PA binding domains, and are well characterized for PA specificity. An exception is the ECA1 protein, which was identified in a screen for PA-binding proteins involved in plant salt stress signaling [31]. Here we characterize the lipid binding specificity of ECA1 in model membrane systems for the first time. Fig. 2 summarizes our results for the effect of PE on membrane binding for the plant proteins ECA1, TGD2 and PDK1; the yeast proteins, Opi1, and Spo20; and the mammalian proteins Raf-1 kinase (RPA) and Lact C2. The latter is used as a control.

Fig. 2A-C shows the results for the three plant proteins, ECA1, TGD2 and PDK1. Both ECA1 and TGD2 show that increasing PE concentration increases protein binding. The PH2 domain of the plant protein PDK1 deviates from this trend as it shows more or less saturated binding for membranes containing 10 mol% PA and 90 mol% PC, addition of PE does not lead to more protein binding.

Similar trends are observed for the two Opi1 constructs and Spo20 (Fig. 2D-F); i.e., PE facilitates binding of these PA-targets to PA (as shown previously [11,12]). We have previously shown that RPA binding to PA is sensitive to the presence of PE in model membranes [9]. Here we observe a similar trend. Inclusion of PE together with PA (Fig. 2G) leads to an increase in binding of RPA. Binding data for our control Lact C2, shown in Fig. 2H, confirms that the protein binds to lipid membranes, and has moderate affinity even for PC and PC/PE membranes. Incorporation of PA appears to increase binding but binding of Lact C2 is not affected by increasing PE concentration as expected since it is a phosphatidylserine (PS) specific binding protein.

The data from Fig. 2 illustrates that PE increases PA binding in most of the PA binding proteins we investigated. However, the effect of PE on the binding to PA by PA-effector proteins is two-fold. Not only does DOPE increase the negative curvature stress of the membrane (it's spontaneous curvature is −30 Å (17)), it also increases the negative charge of PA via the electrostatic hydrogen bond switch [9,45].

3.2. LPC inhibits binding of PA-binding proteins

In order to investigate just the effect of curvature stress further we choose to study membrane binding by PA binding proteins as a function of a lipid with opposite curvature to PE, namely the Type I lipid LPC. Not only is LPC a typical Type I glycerophospholipid, it also does not affect the charge of PA (LPC has the same headgroup as PC).

Type I lipids induce positive curvature stress and are known to affect protein binding to lipid bilayers [46]. In order to evaluate the effect of positive membrane curvature stress on the capacity of PA-targets to bind to PA we introduced increasing concentrations of LPC by replacing the matrix lipid DOPC to obtain mixtures containing 5, 10, 15, 20, and 25 mol% LPC, and a constant 10 mol% DOPA. Comparing the binding of PA-binding proteins between PC/PA and PC/LPC/PA liposomes thus addresses the role of (positive) curvature stress while keeping the charge constant.

In Fig. 3A we show that the newly characterized PA binding protein ECA1 is sensitive to positive curvature stress as its binding to PA is severely inhibited. Aside from ECA1 we also investigated the effect of LPC on the binding of an entirely different PA binding domain, namely the PH2 domain of PDK1. Fig. 3B shows that increasing concentrations of LPC also inhibit binding for PDK1-PH2. Finally, Fig. 3C shows that as the concentration of LPC is increased, from 5 to 15 mol% a gradual decrease in Opi1 binding to PA is observed. At higher concentrations the attenuation of PA binding appears to saturate; i.e., LPC concentrations of 20–25 mol% appear to fully inhibit Opi1 binding similar to our observations for ECA1 and PDK1-PH2. These data thus show that membrane curvature stress plays an important factor in PA binding for PA-binding proteins.

3.3. DAG alters liposome morphology and inhibits PA binding in the liposome binding assay

In order to further explore the effect of membrane curvature on PA binding by PA-binding proteins we selected another Type II lipid that is not expected to affect the charge of PA. Unlike PE, diacylglycerol (DAG) does not have a primary amine headgroup, and therefore is not expected to significantly affect the charge of PA. DAG is known to induce negative curvature stress to membranes [16,17]. Under physiological conditions the concentration of DAG is around 1–2 mol% although this can increase up to 10 mol% in oncogenic cells [47,48]. Low concentrations of DAG

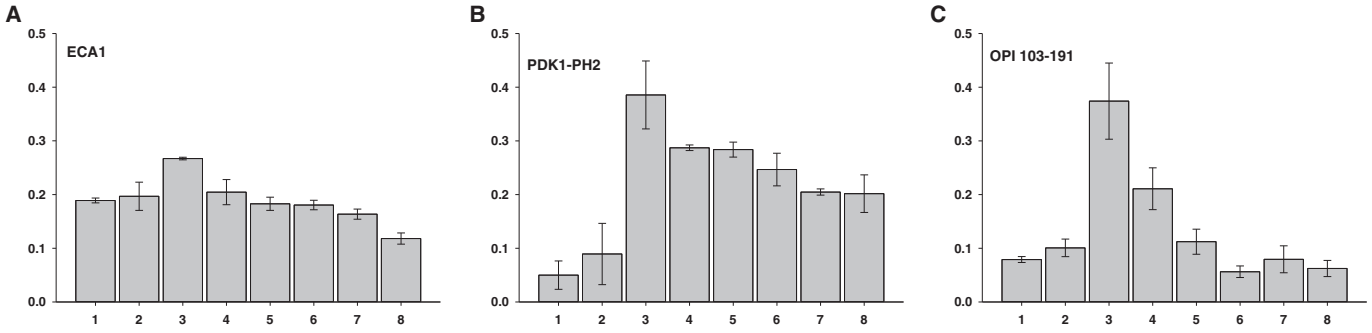

Fig. 3. LPC decreases binding of PA-targets to PA. A) ECA1, B) PH2 domain of PDK1, and C) Opi1 (103–191). Shown are the average and SD of a minimum of 3 independent vesicle binding experiments. Binding values are represented as ratios of total protein. Representative data together with the Western blot are shown in Supplementary Fig. S1. Vesicle lipid composition is as follows: Lane 1–8: DOPC/LPC/DOPA, 1–100/0/0, 2–85/15/0, 3–90/0/10, 4–85/5/10, 5–80/10/10, 6–75/15/10, 7–70/20/10, 8–65/25/10.

cause significant changes in membrane protein activity [49]. Diacylglycerol has the highest negative spontaneous curvature (~ − 10 Å) of any membrane glycerol-lipid measured to date due to its small hydroxyl headgroup [16,17].

Incorporation of DAG (dioleoylglycerol (DOG)- 5–25 mol%, identical to our concentrations tested for LPC) surprisingly shows no clear trend in PA-target binding. However, at higher concentrations of DAG (15 and 20 mol% of DOG) we consistently observe a significant decrease in protein binding. Fig. 4 shows the results for 15 and 20 mol% of DOG in PC/PA model membranes for the PA effector Opi1 (lanes 3 and 5). These results are compared to the effect of PE on Opi1 binding (lanes 4 and 6) to clearly contrast the opposing effects of both Type II lipids. Similar results were observed for PDK1-PH2 (data not shown). The reduction in binding observed for the DOG containing liposomes is in stark contrast to our results for PE, which show that the Type II lipid PE increases (Fig. 2) and the Type I lipid LPC decreases (Fig. 3) protein binding to PA by PA-targets.

What is the reason we observe this apparent discrepancy for DOG even though its negative spontaneous curvature is significantly higher than that of DOPE ($C_{DOG} = -1/10$ Å^{-1}, and $C_{DOPE} = -1/30$ Å^{-1} [16]? Dynamic light scattering showed that the DOG lipid mixtures formed structures of around 125 nm identical to our other lipid mixtures. Additionally, thin layer chromatography showed that DOG was incorporated into vesicles. Next we turned to cryo-TEM to visualize the integrity of our liposomes after extrusion. The images revealed that while there were indeed vesicles present in the liposomal dispersion containing DOG, a fraction (approximately ~25%) of these contained massive amounts of internal membrane structures. The cryo-TEM images show irregular corrugated "rosette" like structures for lipid dispersions containing 25 mol% DOG (Fig. 5A). In contrast, lipid dispersions containing 25 mol% of LPC (Fig. 5B) or very high concentration of PE (45 mol%, Fig. 5C) formed regular bilayer vesicles. The "rosette" like structures appear to explain the irregular protein binding to DAG containing liposomes. A strong reduction in protein binding at the higher DAG concentrations is likely due to the inaccessibility of a significant fraction of the membrane.

4. Discussion

4.1. Negative curvature stress increases PA-binding by PA binding proteins

Phosphatidic acid (with unsaturated acyl-chains) was shown to induce negative curvature stress in model membranes [7,8]. Coupled with the location of the phosphomonoester headgroup, close to the acyl-chain / headgroup interface, the negative spontaneous curvature of PA suggested to us that negative curvature stress may play an important role in membrane binding of PA-target proteins. We subsequently showed for mammalian Raf-1 kinase that binding to PA is sensitive to negative curvature stress in the membrane [9]. Here, we replicate this observation and show that Raf-1 kinase binding is indeed sensitive to PE. Furthermore, we reproduce this behavior for the yeast proteins Opi1 [12], and Spo20 [11,35].

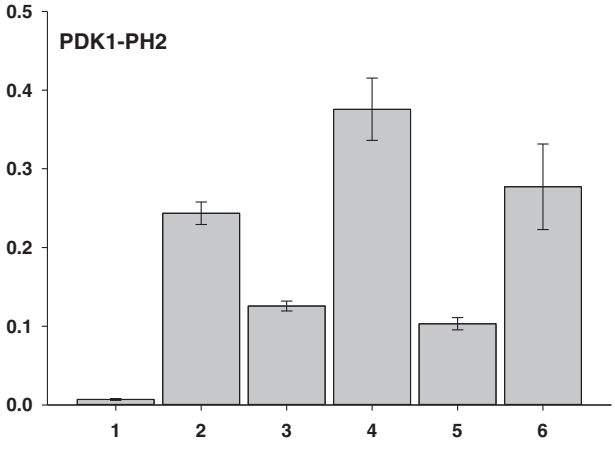

Fig. 4. High concentrations of diacylglycerol reduce binding of Opi1 to PA containing model membranes. Binding values are represented as ratios of total protein. Shown are the average and SD of a minimum of 3 independent vesicle binding experiments. Binding values are represented as ratios of total protein. Representative data together with the Western blot are shown in Supplementary Fig. S2. Vesicle composition is as follows; Lane 1: DOPC- 100, Lane 2: DOPC/DOPA- 90/10, Lane 3: DOPC/DOG/DOPA- 75/15/10, Lane 4: DOPC/DOPE/DOPA- 75/15/10, Lane 5: DOPC/DOG/DOPA- 70/20/10, Lane 6: DOPC/DOPE/DOPA 70/20/10.

In these studies we use a liposome binding assay [30] to study the protein interaction with PA for two reasons. First, large unilamellar vesicles (LUVs) mimic the biological system (i.e. bilayer structure) in that the curvature of LUVs is similar to that found in biomembranes. The actual curvature of most cellular membranes is negligible compared to the size of individual membrane components. Only when the vesicle radius decreases below ~80 nm or so do curvature effects on protein binding become observable [50]. Secondly, curvature stress, as induced by non-bilayer lipids, can be accurately mimicked in model membranes. The actual curvature stress in biomembranes is likely to be different from that in our model membranes due to the spatial and temporal variations in biomembrane composition. Our model membranes do allow for exquisite control of membrane composition and allow us to model both negative and positive curvature stress.

Although the liposome binding assay is not a quantitative assay to study specific binding constants, it accurately reveals qualitative information on lipid-binding specificity and specific membrane environment, i.e. membrane curvature stress. In our studies of PA binding by PA-targets we find significant variability between experiments (i.e. in the absolute amount of membrane binding for each condition investigated). However, the assay faithfully recapitulates the trends between different membrane lipid compositions (i.e. lipid specificity and membrane environment), and is thus a reliable method to assess lipid specificity.

We show here for the first time, that ECA1 binds to PA containing vesicles in vitro. ECA1 was identified as an A/ENTH domain containing protein. Silkov et al. (2011) first described its membrane binding characteristics and structural features [51]. The N-terminal A/ENTH domain showed a high affinity for PI [3–5]P_3 and a lower affinity for PI [4,5]P_2, but PA was not tested in their study. Subsequently, AtECA1 was found to bind clathrin and localize to the growing cell plate of dividing cells [32]. Most recently, AtECA1 was identified as a PA-binding protein in a quantitative proteomics approach, and was found to be recruited to the membrane of root cells upon salt stress [31]. Considering these results, AtECA1 is hypothesized to function as a clathrin adaptor in clathrin mediated endocytosis. Aside from showing that ECA1 binds to PA containing membranes in vitro we also show that this binding is sensitive to membrane lipid composition and curvature stress (Figs. 2 and 3).

The other plant proteins that we characterized here for the first time for PE dependency are TGD2 [33], and the PH2 domain of PDK1 [30]. Each of these plant proteins is involved in a different function [4]. TGD2 was identified as a component of a lipid transport complex in the chloroplast envelope [52]. Its subcellular localization showed association with the chloroplast envelope membrane. Here, the protein is able to allow transfer of ER-derived PA [33,53] to plastids using its lipid binding C-terminus. TGD2 is part of a large complex, also including TGD1, TGD3 and TGD4 [53,54].

The 3-phosphoinositide-dependent protein kinase 1 (PDK1) is a master regulator of ACG-kinases and contains a pleckstrin homology domain (PH-domain) which interacts with phosphatidic acid (PA) and phosphoinositides [34]. PDK1 is selectively activated in vivo by interaction with phospholipids PA and PIP_2 [55] and, amongst other functions, is believed to play a role in nutrient sensing [55], response to pathogens, polar auxin flow (through phosphorylation of PINOID (PID) [56]) and osmotic stress [57]. Thus the PA binding characteristics of PDK1 are important in many plant cell processes. Unlike ECA1 and TGD2 the

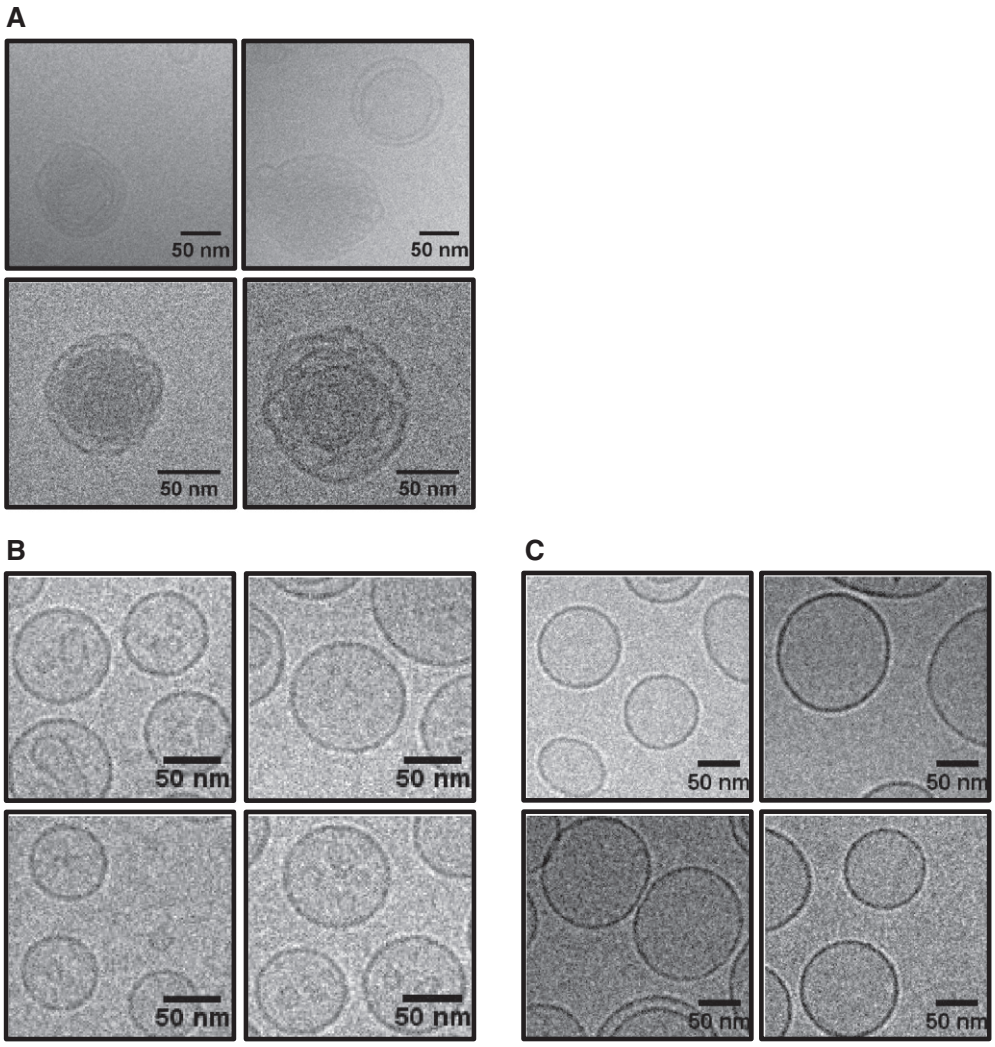

Fig. 5. Cryo-TEM images of three liposome compositions used in this study. A) DOPC/DOPA/DOG 65:10:25 B) DOPC/DOPA/LPC 65:10:25 and C) DOPC/DOPA/DOPE 45:10:45 M ratio. Liposomes were prepared as discussed in the materials and methods. Cryo-TEM measurements were replicated at least twice on individually prepared liposome dispersions.

PDK1 PH2 domain is not sensitive to increases in PE concentration. One possibility is that the 10 mol% of DOPA used in our studies supplies sufficient negative curvature stress (and/or sufficient charge, see below) to essentially maximize membrane binding. It should also be noted that PDK1-PH2 has more intrinsic membrane affinity than our other PA-binding proteins. This is also supported by our data on the effect of LPC where membrane binding of PDK1-PH2 is not completely inhibited.

As control we used the C2 domain of Lact which is known to bind to PS (i.e. another anionic lipid) containing membranes but is not a PA specific binding protein. Background binding to PC and PC/PE membranes by Lact C2 is higher than that of our PA binding proteins. Incorporation of PA appears to increase membrane binding but this is likely due to the addition of negative charge and not specific recognition of PA. Additionally, Lact C2 does not show enhanced binding upon addition of PE, in accordance with the observation that PS is not a Type II lipid [58].

The yeast SNARE protein Spo20 was originally identified as a PA binding protein by Nakanishi in 2004 [35] and its PA binding domain has been used as a PA-specific membrane sensor in cell studies, including plant cells [59]. Horchani et al. showed that Spo20 binding to POPA increased upon an increase in PE concentration using a flotation assay [11]. We confirm the same dependences for Spo20 using DOPA (unsaturated chains) with our liposome binding assay. Aside from showing that PE increased PA binding by Spo20 this recent work by the group of Bruno Antonny also showed that a Spo20 derived PA biosensor responds simply to an increase in negative charge [11]. Not only does an increase in PA lead to increased binding of Spo20, identical amounts of negative membrane charge induced by PS or PIP_2 ($PI4,5P_2$) resulted in similar levels of membrane binding. Similarly Ogawa et al. found for an artificial, tetravalent peptide developed to specifically recognize PA, that increasing concentrations of PA led to more membrane binding of the peptide probe [60]. However, this binding was not exclusive to PA as the peptide bound equally well to other anionic membranes in a similar manner as Spo20. These data suggest that careful and exhaustive evaluation of lipid affinity and specificity via liposome assays is needed for PA-targets.

4.2. Membrane curvature or negative charge?

The effect of PE on binding of PA targets is twofold; PE increases the negative curvature stress of the membrane, and the negative charge of PA via the electrostatic-hydrogen bond switch mechanism [6–9]. Recently, Eaton et al. showed that binding of Lipin 1 to PA is sensitive to the charge of PA as changes in pH and an amphiphile that increases the negative charge of PA increase Lipin 1-PA binding (an amphiphile that does not increase charge shows no increase in binding). Additionally, these authors showed that PE increased binding and concluded that an increase in charge was likely responsible. We show here that membranes containing LPC, a Type I lipid, significantly decreases binding of PA-targets to membranes containing PA. This decrease in binding

is found in comparison to PC/PA liposomes and charge is thus kept constant. The effect of PE on PA binding by PA-target proteins is thus likely dependent on not only charge but also on membrane curvature stress.

This dual effect can be rationalized as follows, an increase in charge increases the electrostatic attraction between cationic residues in PA-targets and thus stimulates binding. We previously showed computationally that the addition of the electrostatic-hydrogen bond switch mechanism significantly increases the affinity of PA-targets to PA [61]. Additionally, the increased negative curvature stress due to the presence of PE increases the availability of the PA headgroup that is normally buried deep in the lipid headgroup-acyl chain interface. It is expected that PA binding by PA targets will thus depend sensitively on the specific PA-binding domain with each domain having its own characteristics. A hypothesis supported by the observation that none of the domains identified to date show any amino acid similarity.

4.3. Diacylglycerol strongly affects membrane structure and results in a reduction in vesicle binding due to significant membrane deformation

We showed that, contrary to expectation, DOG at concentrations of 15 and 20 mol% inhibit PA-target binding to PA compared to PE (see Fig. 4). The cryo-TEM data clearly show unusual, rosette like vesicular structures, inside otherwise right sized vesicles (DLS did not show aberrant sizes). We do not observe these types of structures for our other complex lipid mixtures with LPC and PE (Fig. 5B and C respectively). The intra-liposomal structures do explain the reduction in PA-target binding to PA for the high DOG lipid membranes. The extra membrane structures inside these strange liposomes are not available for protein binding, and thus result in an apparent reduction of binding. What causes these intra-vesicular, corrugated, membranes? It is well known that DAG leads to membrane dehydration, and thus an increase in membrane fusion [62]. The structures we observe likely arise as a result of excessive fusion events that occurred during the vesicle extrusion process. These data show the need for careful liposome characterization for each complex lipid mixture evaluated. We suggest that cryo-TEM to visualize the membrane structures present after preparation of liposomes, is the best way to verify liposome integrity as DLS coupled with thin layer chromatography was not able to detect any issues with our vesicles containing high DAG content.

4.4. Elucidation of membrane binding via lipid overlay assays

Lipid overlay assays, where a single lipid is spotted from organic solution on a membrane, fail to represent lipids in a true bilayer structure thus making it unreliable for the study of protein–lipid interactions. This may be especially so for membrane lipids such as PA, for which no well-defined protein recognition motif has been identified, and which have a head group moiety that is buried within the hydrophilic region of the membrane. This is in contrast to the phosphoinositides where many well-defined protein domains are known and have been extensively characterized [63]. These PIPx binding domains form cage like structures where the PIPx headgroup fits specifically into the binding pocket and local membrane environment is not likely to affect protein binding significantly. Compared to the popular, but inaccurate, lipid overlay assay, the liposome binding assay mimics the membrane environment (bilayer structure) and the model liposomes used in these studies can have complex lipid composition and accurately represent the curvature stress induced by the presence of non-bilayer lipids.

5. Conclusion

This work highlights the need to study lipid protein interactions in the context of complex lipid mixtures in a lipid bilayer, especially when taking into account the detailed physical chemistry of individual membrane lipids. We show that curvature stress in the membrane has profound effects on the lipid-binding characteristics of established and novel PA-binding proteins.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2016.07.014.

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