Chapter 13

Identification of Lipid Binding Modulators Using the Protein-Lipid Overlay Assay

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Abstract

The protein-lipid overlay assay is an inexpensive, easy-to-implement, and high-throughput methodology that employs nitrocellulose membranes to immobilize lipids in order to rapid screen and identify protein-lipid interactions. In this chapter, we show how this methodology can identify potential modulators of protein-lipid interactions by screening water-soluble lipid competitors or even the introduction of pH changes during the binding assay to identify pH-dependent lipid binding events.

Key words Lipid-protein overlay assay, Phospholipids, Phosphoinositides, Inositol 1,3-bisphosphate, EEA1 FYVE, Dishevelled-2 DEP, Phafin2, Tollip, pH effect

1 Introduction

Lipids not only function in membrane organization but are also engaged in the spatiotemporal regulation of protein-mediated signaling transduction pathways and intracellular membrane trafficking. The function of a lipid is highlighted by the structure and net charge of its head group as well as the length and saturation of its fatty acid chains. Abnormal changes in lipid metabolism are associated with genetic defects that trigger human diseases such as cancer, diabetes, and neurodegenerative diseases [1]. Thus, targeting lipid-protein interactions is an emerging area of biomedical research for drug development. Indeed, there is accumulated literature that emphasizes lipid binding proteins as prospective drug targets [2–4]. Protein-lipid binding can be simply assessed by various biochemical techniques, including liposome-based assays, such as the liposome flotation and the liposome microarray-based assays [5, 6]. Although these robust methodologies provide the ability for screening protein-lipid binding by using liposomes of different compositions, their preparation and stability, and the elevated cost

Iulia M. Lazar et al. (eds.), *Proteomics for Drug Discovery: Methods and Protocols*, Methods in Molecular Biology, vol. 1647, DOI 10.1007/978-1-4939-7201-2_13,

of the instrumentation required for screening lipid binding limits their use. Biophysical techniques, including surface plasmon resonance, isothermal calorimetry, solution NMR titrations, and microscale thermophoresis, allow the quantification of protein-lipid interactions [7], but they also require specialized instrumentation. One powerful and relatively quick methodology to identify potential protein lipid ligands is the protein-lipid overlay assay (PLOA). This assay, described by Alessi and colleagues in 2002, consists of immobilizing serial dilutions of a lipid of interest onto a nitrocellulose membrane. This membrane is then incubated with the protein of interest (typically a fusion protein), washed, and protein binding to the lipid is detected by the sequential addition of an antibody against the epitope tag and a chemiluminescent antibody [8]. This 2-day method requires 1–10 μg of protein and microgram amounts of lipid. PLOA can be used as a first high-throughput screening to identify novel protein-lipid interactions. Indeed, PLOA has been used to investigate the specificity of 33 *Saccharomyces cerevisiae* PH domains for phosphoinositide binding [9]. Also, the assay has been modified by designing miniaturized nitrocellulose membranes arrays, which contained duplicated sets of 51 lipids to survey *S. cerevisiae* novel protein-lipid interactions [10]. Echelon Inc. markets a series of lipid strips and arrays, in which lipids are prespotted on nitrocellulose membranes at amounts as high as 100 pmol of lipid per spot. Alternatively, and as described in detail below, lipid-spotted nitrocellulose membranes can be made in the laboratory by spotting the lipid of interest onto the membrane. These lipid-containing membranes can last for at least 6 months if they are stored at 4 °C and protected from the light.

By using PLOA, however, one should consider that the lipid is not organized in a lipid bilayer as occurs under physiological condition and that other lipids from the membrane structure always accompany the lipid under investigation. Thus, despite its simplicity and sensitivity, the PLOA method exhibits some limitations including nonspecific binding, or, lack of it. Thus, the use of an alternative method to support the lipid binding results is recommended [7, 11]. However, if the lipid ligand is consistently identified using PLOA and other orthogonal assays, then additional features of protein-lipid interactions can be investigated using PLOA. For example, lipid binding-deficient mutants of a protein can be investigated to identify potential lipid binding sites [12, 13]. The presence of cofactors for protein-lipid interactions can also be studied using PLOA. For example, the requirement of the lectin-like oxidized low-density lipoprotein receptor-1 for Ca^{2+}, but not Mg^{2+}, in order to associate with phosphatidylserine could be identified using this methodology [14]. Also, PLOA can be used to identify cooperativity in lipid binding. Syntenin binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) through its PDZ domains, but enhancement of the binding in the presence of a

syndecan-2 peptide can be observed using PLOA [15]. Using the PLOA method, the C-terminal DDHD domain of the COPII component protein, p125A, shows low specificity for lipids, but becomes more specific when it is expressed together with the central SAM domain of the same protein [16]. PLOA can also be used to identify specificity for fatty acid chains. Thus, it was demonstrated that during the light period, *Arabidopsis* florigen FT preferentially binds to phosphatidylcholine fatty acids that are less saturated [17]. Protein binding to phospholipids can be coupled to proton pumps as suggested for the peripheral protein EEA1, whose FYVE domain binds PtdIns(3)P in a pH-dependent manner [18]. Thus, PLOA was proven to be efficient at identifying the pH dependency for the binding of Dishevelled-2 DEP domain to phosphatidic acid [19]. Several reports also used PLOA to identify negative modulators or competitors. For example, the adaptor protein Disabled-2 (Dab2) binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and sulfatides at overlapping sites within the N-terminal region of the protein [20]. Thus, sulfatide binding of Dab2 can be inhibited by an excess of soluble PtdIns (4,5)P_2 [21]. Likewise, binding of the adaptor protein Tom1 to Tollip specifically inhibits Tollip's PtdIns(3)P binding [22]. In the followings, we describe a protocol to use PLOA as a first step for screening molecules that can modulate protein-lipid interactions.

2 Materials

1. Nitrocellulose membrane (GE Healthcare Portrand Supported 0.45 μm NC).
2. Solution I: Prepare a solution of chloroform/methanol/water in a proportion of 65:35:8 (v/v) in a 1 mL glass vial (*see* **Note 1**).
3. Scissors, tweezers, and a soft pencil.
4. Microcentrifuge tubes, 0.5 mL.
5. Benchtop cooler rack.
6. Phospholipids (Echelon, Matreya, Cayman Chemicals, and Avanti Lipids products immobilize well on nitrocellulose membranes).
7. Refrigerated microcentrifuge and clinical centrifuge.
8. Water bath sonicator.
9. Bovine serum albumin (BSA) that is fatty acid-free (*see* **Note 2**).
10. Fusion proteins (*see* **Note 3**).
11. Washing buffer: 10 mM Tris–HCl (pH 8), 150 mM NaCl, 0.1% Tween-20 (*see* **Note 4**).
12. Blocking buffer: 3% fatty acid-free BSA in washing buffer (*see* **Note 5**).

13. Pre-developing buffer: 10 mM Tris–HCl (pH 8), 150 mM NaCl.
14. Aluminum foil.
15. Petri dishes of 100 and 40 mm diameters.
16. Primary rabbit anti-glutathione-*S*-transferase (GST) antibody: A commercial product can be obtained as a 0.51 μg/μL solution from Proteintech, and is recommended for usage in a dilution of 1:2000.
17. Donkey anti-rabbit Horseradish peroxidase (HRP)-coupled secondary antibody: A commercial product can be obtained from GE Healthcare, and is recommended for usage in a dilution of 1:10,000.
18. Modulator: Small drug or any macromolecule that alters the binding affinity of the lipid to the protein.
19. Chemiluminescent reagent kit: In this work, the Thermo Fisher SuperSignal West Pico Chemiluminescent Substrate product, which contains the stable peroxide solution and the luminol/enhancer solution, was used.
20. Platform shaker.
21. Cold room or cold box.
22. ChemiDoc XRS+ System and Image Lab software (Bio-Rad).

3 Methods

3.1 Spotting Lipids on Nitrocellulose Membranes

1. Cut pieces of nitrocellulose membrane that will fit five individual lipid spots (~4 cm × ~2.5 cm). Make sure to cut a corner to ascertain the orientation of the membrane. Keep the protective backing in place during the preparation of the lipid membranes. Take the pieces of nitrocellulose membrane and mark them with a soft pencil at the sites where the lipids will be placed.
2. Prepare microgram aliquot stocks of the phospholipid of interest. Centrifuge the original lipid vial inside a 50 mL centrifuge tube. Place tissue paper at the bottom of the centrifuge tube to protect the vial during centrifugation. Spin at 500 rpm for 3 min at 4 °C. This step prevents the loss of lipid during the vial opening. Resuspend the lipid (1 mg/mL) in Solution I, sonicate in a room temperature water bath for a few seconds, and aliquot micrograms of lipid solutions into 1 mL brown glass vials. Dry the solutions under N_2 gas in a fume hood. Store the dried lipids in glass vials sealed with Parafilm at −20 °C until use.
3. Resuspend the lipid stock at the desired concentration in Solution I. Mix gently using finger tapping. Prepare a serial dilution

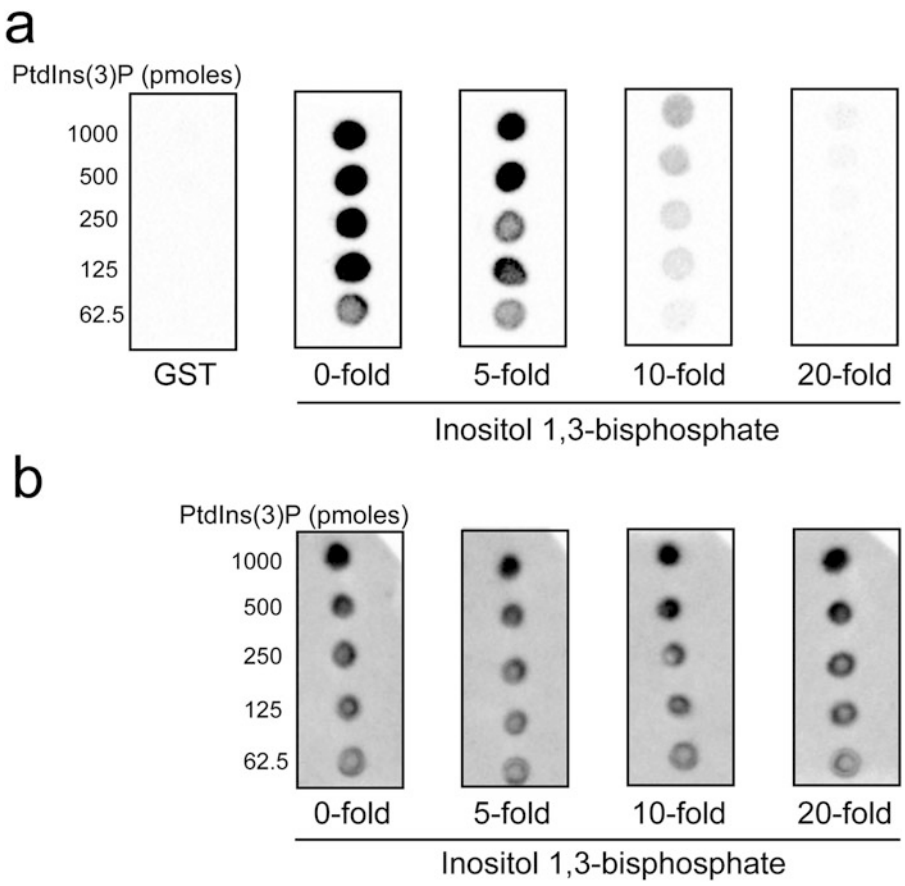

Fig. 1 Identification of modulators of protein-lipid binding using PLOA. In this experiment, GST-Tollip (**a**) and GST-Phafin2 (**b**) were probed for inhibition of PtdIns(3)P binding using the head group of the lipid, inositol 1,3-bisphosphate. As opposed to that observed for Phafin2, the results suggest that the head group of PtdIns (3)P competes with the lipid for Tollip's binding

of the lipid in the nano-picomolar range in solution I. Use 0.5 mL microcentrifuge tubes prechilled in the cooled benchtop rack. Keep all the solutions in the cooled benchtop rack.

4. Pipette 1 μL of each lipid onto the pre-labeled nitrocellulose membranes with the highest concentration of the lipid on top (*see* Figs. 1 and 2; *see* **Note 6**).
5. Dry the spotted lipid membranes for 1 h at room temperature, protecting them from light using aluminum foil. Membranes can be stored in petri dishes, wrapped with aluminum foil, at 4 °C.

3.2 Screening of Modulators of Lipid Binding Using LPOA

1. Remove the protecting backing from each piece of nitrocellulose membrane and, using tweezers, individually transfer the membranes (with the lipid spots facing down) into a 10 mm petri dish.

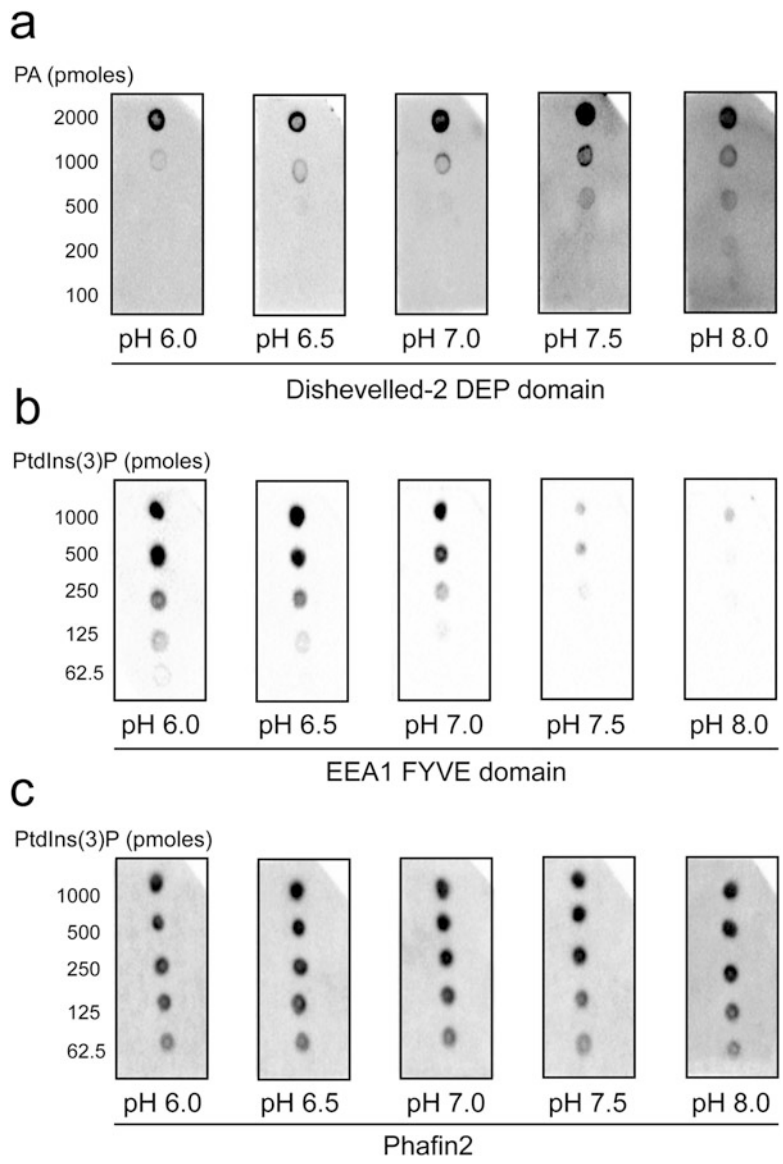

Fig. 2 PLOA showing an opposite pH-dependency of binding of EEA1 FYVE (**a**) and Dishevelled-2 DEP domain (**b**) for PtdIns(3)P and phosphatidic acid, respectively. On the other hand, PtdIns(3)P binding of Phafin2 is pH-independent (**c**)

2. Add 10 mL of blocking buffer to each of the petri dishes and gently block membranes for 1 h at room temperature using a platform shaker. Keep all petri dishes covered with aluminum foil throughout the experiment (*see* **Note 7**).
3. During the blocking incubation time, mix 1–10 μg of the fusion protein of interest without and with increasing molar ratios of the modulator in a final volume of 50–100 μL. Place the mixtures in a rotating mixer for 45 min at room temperature.

4. Discard blocking solution and wash membranes three times with washing buffer.
5. Add 10 mL of washing buffer to each of the Petri dishes (*see* **Note 8**). Add the fusion protein (as control) or the fusion protein-modulator mixtures to each of the Petri dishes and incubate with gentle agitation overnight at 4 °C (*see* **Note 9**). If the effect of pH in lipid binding is assayed (Fig. 2), the fusion protein should be incubated with the washing buffer at the specific range of pH values to be investigated.
6. Discard protein mixtures. Wash membranes three times with washing buffer for 10 min each time at room temperature by shaking on a platform shaker (*see* **Note 10**).
7. Prepare 40 mm petri dishes and add 4 mL of washing buffer containing 3% fatty acid-free BSA. Transfer the lipid membranes to each of these plates and add 2 μL of the rabbit anti-GST antibody commercial product. Incubate membranes gently on a platform shaker for 1 h at room temperature (*see* **Note 11**).
8. Transfer the membranes back to the 100 mm petri dishes. Wash membranes three times with washing buffer for 10 min each at room temperature by gently shaking on a platform shaker.
9. Transfer the membranes back to the 40 mm petri dishes and add 4 mL of washing buffer containing 3% fatty acid-free BSA. Add 0.4 μL of the secondary anti-rabbit-HRP conjugated antibody. Incubate the membranes gently on a platform shaker for 1 h at room temperature.
10. Transfer the membranes back to the 100 mm petri dishes. Wash membranes three times with washing buffer for 10 min each at room temperature by gently shaking on a platform shaker.
11. Wash membranes once with pre-developing buffer for 10 min at room temperature by gently shaking on a platform shaker (*see* **Note 12**).
12. Remove each of the membranes from the Petri dishes and transfer to a clean piece of transparent cellulose acetate sheet. Remove the excess of washing buffer using tissue papers. Do not let the membranes dry. Prepare the working solution of the SuperSignal West Pico Chemiluminescent Substrate mixture in the same cellulose acetate sheet, by mixing equal volumes of the stable peroxide solution and the luminol/enhancer solution. Place all the membranes (facing the lipid complexes down) in the mixture for 1 min. The membranes should be fully in contact with the working solution mixture, which will fluoresce due to the enzymatic activity of HRP.

13. Remove the excess of solution and place another cellulose acetate sheet on the top of the membranes. Immediately expose the reaction at different time points using the chemiluminescence detector instrument.

4 Notes

1. The composition of the solution will depend on the nature of the lipid. For example, phosphoinositides are relatively polar and need water to be soluble. Other more hydrophobic lipids require chloroform only to be soluble. Solutions should be made fresh by the time that the lipid solutions are ready to be spotted. It is recommended to work with a benchtop cooler rack unit to prevent evaporation of the solution.
2. Fatty acid-free BSA (Sigma A7030) is the most suited reagent for blocking nonspecific binding of proteins to the nitrocellulose membrane. However, there are slight variations among lots of this product and, consequently, they affect the efficiency of blocking as well as the intensity of the binding signal. It is, therefore, recommended to compare results using more than one lot if possible. Both dried milk powder or albumin have also been suggested to be used as alternative blocking reagents for nonspecific binding, but results with these reagents are quite inconsistent in our hands.
3. Fusion proteins containing glutathione-*S*-transferase (GST) are the most suitable tag to be employed during PLOA. The major reason for the GST tag use is that it increases the yield of the protein of interest by increasing its solubility. However, other tags, including green fluorescent protein (GFP) and polyhistidine tags, also worked well in our hands. As occurs with most proteins, the location of a tag at either the N- or C-terminus does not have an effect in lipid binding. If this is not the case, it is recommended adding a linker of 4–7 amino acids (preferentially glycine residues), which can extend the distance between the lipid binding site and the tag. Proteins should be checked for optimal quality using SDS-PAGE analysis. Concentration of proteins should be carefully checked using two independent methods.
4. Tris-based buffers are relatively stable. However, large quantities of this buffer are required for this assay. It is, therefore, recommended to prepare a ten-fold concentrated stock of washing buffer (100 mM Tris–HCl, pH 8, 1.5 M NaCl) and store at room temperature. If the effect of the pH is tested, the same buffer is suitable for the assay at a pH range of 6–8.

5. Fatty acid-free BSA containing blocking solutions can be stored at −20 °C.
6. Efficient immobilization of lipids to nitrocellulose membranes occurs when their fatty acid chains are saturated and are at least 16 carbons in length.
7. Do not allow the nitrocellulose membranes to get dry throughout the experiment.
8. We do not recommend using the blocking solution during the overnight incubation of the protein mixtures with the lipid-bound membranes. The initial 1-h blocking step is sufficient to avoid nonspecific binding results. BSA may bind nonspecifically to the macromolecules to be assayed and, therefore, interfere with interpretation of the results.
9. If modulation of lipid binding is studied, it is recommended to have a negative control in the experiment to ensure that the modulator specifically targets the protein under investigation.
10. When using GST fusion proteins and the indicated antibodies, the effect of pH in protein-lipid binding is not critically affected after the incubation of the protein of interest. However, it is important to pay attention to the isoelectric point of the proteins to be studied. We have also carried out pH-dependent PLOA with buffers at the same pH at all times, which led to the same outcomes. This suggests that lowering the pH of the washing buffer does not significantly affect the strength of the antibody binding in later steps.
11. The use of 40 mm petri dishes in **steps 7** and **9** in Subheading 3.2 is suggested in order to minimize the volume of the antibodies used in the assay.
12. Tween-20 can interfere with the HRP-mediated fluorescence reaction.

Acknowledgments

We thank Janet Webster for critical reading and comments on the manuscript. We also thank Tiffany Radle and Morgan Vaughn for the optimization of the purification conditions of the GST-EEA1 FYVE domain. Work in the Capelluto laboratory is supported by the National Institutes of Health (NIAID).

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