Protein Lipid Overlay Assay

Simon Dowler,[1] Gursant Kular,[2] and Dario R. Alessi[1]*

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[1]MRC Protein Phosphorylation Unit, School of Life Sciences, MSI/WTB Complex University of Dundee, Dow Street, Dundee DD1 5EH, Scotland. [2]Inositol Lipid Laboratory, School of Life Sciences, MSI/WTB Complex University of Dundee, Dow Street, Dundee DD1 5EH, Scotland.

*Corresponding author. E-mail, d.r.alessi@dundee.ac.uk

Abstract

The protein lipid overlay (PLO) assay enables the identification of the lipid ligands with which lipid binding proteins interact. This assay also provides qualitative information on the relative affinity with which a protein binds to a lipid. In the PLO assay, serial dilutions of different lipids are spotted onto a nitrocellulose membrane to which they attach. These membranes are then incubated with a lipid binding protein possessing an epitope tag. The membranes are washed and the protein, still bound to the membrane by virtue of its interaction with lipid(s), is detected by immunoblotting with an antibody recognizing the epitope tag. This procedure requires only a few micrograms of protein and is quicker and cheaper to perform than other methods that have been developed to assess protein-lipid interactions. The reagents required for the PLO assay are readily available from commercial sources and the assay can be performed in any laboratory, even by those with no prior expertise in this area.

Introduction

A large number of proteins possess the ability to interact specifically with phospholipids. This interaction is usually mediated through a specific modular lipid binding domain contained within the protein. These domains include the pleckstrin homology (PH) domain (*1*); the Fab1p, YOTB, Vac1p, EEA1 (FYVE) domain (*2*); the PhoX homology (PX) domain (*3*, *4*); the Epsin NH_2-terminal homology (ENTH) domain (*5*, *6*); and the protein kinase C conserved regions 1 and 2, termed the C1 and C2 domains (*7*). Although the presence of one of the above lipid binding domains in a protein can readily be established through routine database searches, it is currently not possible to perform sequence alignments or structural modeling to reliably predict with which lipid a putative lipid binding domain of a protein will interact, nor the relative affinity of these interactions. This information can only be obtained experimentally by performing a lipid binding assay. Here, we describe a simple protocol called the protein lipid overlay (PLO) assay, which enables the lipid binding characteristics of proteins to be determined. The major steps of the PLO assay are summarized (Fig. 1). The lipid binding specificities of numerous PH, FYVE, and PX domain-containing proteins have been investigated using this assay. Yu and Lemmon used the PLO assay to study the lipid binding specificities of all of the 15 PX domains present in *Saccharomyces cerevisiae* (*8*). The specificities of many PH domains for their phosphoinositide ligands determined with the PLO assays has correlated well with the results obtained from more laborious and specialized methodologies used to assess protein-lipid interactions (*9*, *10*).

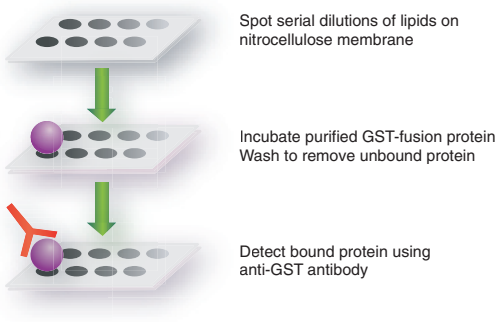

Fig. 1. The key steps of the protein lipid overlay (PLO) assay.

Materials

Costar Spin-X columns

0.44-μm syringe filters

Glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Bucks, UK)

Hybond C-extra nitrocellulose (Amersham Pharmacia Biotech)

Lipid-spotted membranes (Echelon Research Laboratories)

Note: Although these membranes are available, we have no experience using them and have always spotted the lipids onto a Hybond C-extra nitrocellulose membrane just before the assay.

Sodium dodecyl sulfate (SDS)-poylacrylamide gel electrophoresis (SDS-PAGE) supplies

Chemicals and Reagents

2-Mercaptoethanol (2-ME)

Acetic acid

Acetone

Ampicillin

Bradford protein assay reagent

Calcium chloride

Chloroform Complete Protease Inhibitor tablets (Roche Molecular Biochemicals)

Coomassie Blue stain

Dipalmitoyl phosphoinositide derivatives (Echelon Research Laboratories, Salt Lake City, UT, or Cell Signals, Lexington, KY)

Dithiothreitol (DTT)

Dulbecco's Modified Eagle's Medium (DMEM)

Enhanced Chemoluminescence (ECL) reagents (Amersham Pharmacia Biotech)

Ethanol

Fatty acid free bovine serum albumin (BSA) (Sigma-Aldrich)

Fetal calf serum (FCS)

Glutathione

Glycerol

Isopropanol

Isopropyl thio-β-D-galactoside (IPTG)

Luria Bertani (LB) medium

Methanol

N, *N*-bis [2-hydroxyethyl]-2-aminoethanesulphonic acid-HCl (Hepes)

N-bis [2-hydroxyethyl]-2-aminoethanesulphonic acid Tris (hydroxymethyl) methylamine (Tris)

Sodium dodecyl sulfate (SDS)

Sodium chloride

Sodium ethylenebis(oxyethylenenitrilo)-tetraacetate (EGTA)

Sodium ethylenediaminetetraacetate (EDTA)

Sodium fluoride

Sodium hydrogen phosphate (Na_2HPO_4)

Sodium orthovanadate

Sodium β-glycerophosphate

Sucrose

Triton X-100

Polyoxyethylene sorbitan monolaurate (Tween 20)

Antibodies, Cells, and Plasmids

BL21 *Escherichia coli*

Human embryonic kidney (HEK) 293 cells

pEBG2T or pEBG3T vectors, available from authors on request (not available commercially)

pGEX plasmids, Amersham Pharmacia Biotech

Mouse monoclonal antibody against glutathione-*S*-transferase (GST), Sigma-Aldrich

Mouse secondary antibodies coupled to horseradish peroxidase (HRP), Pierce

Equipment

VibraCell Sonicator, Sonics & Materials, Danbury, CT

Automated Biorad Western Processor, optional

Cold room

Recipes

Recipe 1: Lysis Buffer

Tris-HCl	50 mM, pH 7.5
Sucrose	270 mM
Sodium orthovanadate	1 mM
EDTA	1 mM
EGTA	1 mM
Sodium β-glycerophosphate	10 mM
Sodium fluoride	50 mM
Triton X-100	1% w/v
2-M	10 mM

Prepare in distilled H_2O and add one Complete Protease Inhibitor tablet per 50 ml of lysis buffer.

Recipe 2: Lysis Buffer + Salt

Add 0.5 M NaCl to Lysis Buffer (Recipe 1).

Recipe 3: Buffer A

Tris-HCl	50 mM, pH 7.5
EGTA	0.1 mM
Sucrose	0.27 M
2-ME	10 mM

Prepare 1000 ml in distilled H_2O and store at 4°C.

Recipe 4: Buffer A + 20 mM Glutathione

Add 0.15 g glutathione to 25 ml of Buffer A 5 (Recipe 3) and readjust pH to 7. Store at –20 °C.

Recipe 5: SDS-Sample Buffer

Tris-HCl	50 mM, pH 6.8
SDS	2% (by mass)
Glycerol	10% (by vol)
DTT	10 mM

Prepare 50 ml in distilled water and store at –20°C.

Recipe 6: DMEM + FCS

Add 50 ml of FCS to 1 liter of DMEM.

Recipe 7: 2.5 M $CaCl_2$

Add 3.68g of $CaCl_2 \bullet 2H_2O$ to 10 ml of distilled H_20. Filter sterilize and store at –20°C.

Recipe 8: Transfection Buffer

Hepes	50 mM, pH 6.96
NaCl	0.28 M NaCl
Na_2HPO_4	1.5 mM

Recipe 9: Blocking Buffer

Tris-HCl	50 mM, pH 7.5
NaCl	150 mM
Tween 20	0.1% (by vol)
Fatty acid-free BSA	2 mg/ml

Prepare 250 ml in distilled H_2O and store at 4°C.

Recipe 10: TBST

Tris-HCl	50 mM, pH 7.5
NaCl	150 mM
Tween 20	0.1% (by vol)

Prepare 1000 ml in distilled H_2O and store at 4°C.

Instructions

The PLO assay has been performed with the PH domains of dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP) 1 [which binds both phosphatidylinositol 3,4,5-trisphosphate, PI(3,4,5)P_3, and phosphatidylinositol 3,4-bisphosphate, PI(3,4)P_2 (*9*)]; tandem pleckstrin homology domain-containing protein (TAPP) 1 and TAPP2 [PI(3,4)P_2 (*11*, *12*)]; phosphatidylinositol 4-phosphate [PI(4)P] adaptor protein (FAPP) 1 and FAPP2 [PI(4)P (*10*)]; *Arabidopsis thaliana* PH domain protein-1 (AtPH1) [phosphatidylinositol 3-phosphate, PI(3)P (*10*)]; phospholipase C (PLC) δ1 [PI(4,5)P_2 (*13*)]; Grp1 [PI(3,4,5)P_3 (*14*, *15*)], as well as the FYVE domain of EEA1 [PI(3)P (*2*)]; and the PX domain of serum- and glucocorticoid-induced protein kinase-3 (SGK3), which binds PI(3)P as well as other phosphoinositides (*16*, *17*) (Fig. 2). The PLO assay was also used to study the effect of a single-amino acid mutation on the PH domain of the adaptor protein DAPP1 (*9*); this change converts the PH domain from a protein that binds both PI(3,4,5)P_3 and PI(3,4)P_2 to a domain that interacts only with PI(3,4)P_2 (*11*) (Fig. 3).

To perform the PLO assay, it is necessary first to express the epitope-tagged protein or protein domain in a nonproteolyzed form. The final preparation of the protein must also be free from detergents, because they will interfere with the lipid binding assay. Below, we describe the two procedures that we use to express and purify GST fusion proteins in *E. coli* or HEK 293 cells that yield purified protein compatible with use in the PLO assay. These protocols could readily be adapted to purify proteins with other epitope tags, including hexahistidine or the Flag epitope.

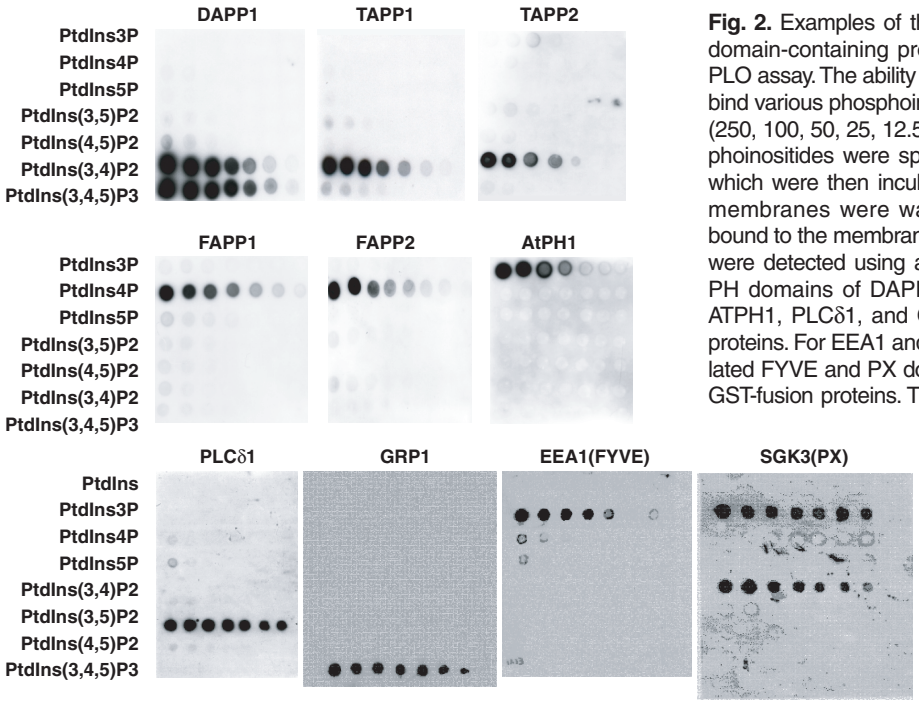

Fig. 2. Examples of the interactions of PH, FYVE, and PX domain-containing proteins with lipids observed using the PLO assay. The ability of the indicated GST fusion proteins to bind various phosphoinositides was analyzed. Serial dilutions (250, 100, 50, 25, 12.5, 6, and 3 pmol) of the indicated phosphoinositides were spotted onto nitrocellulose membranes, which were then incubated with the indicated proteins. The membranes were washed and the GST-fusion proteins bound to the membrane by virtue of their interaction with lipid were detected using an antibody that recognizes GST. The PH domains of DAPP1, TAPP1, TAPP2, FAPP1, FAPP2, ATPH1, PLCδ1, and Grp1 were expressed as GST fusion proteins. For EEA1 and SGK3 (also known as CISK), the isolated FYVE and PX domains, respectively, are expressed as GST-fusion proteins. The results shown in this figure, except the PX domain of SGK3, are taken from data we have published previously (*10*, *27*).

Purification of Recombinant GST-Fusion Proteins from *E. coli*

Preparation of the bacterial lysate

1. Ligate cDNA encoding the protein of interest into the pGEX bacterial expression vectors such that the protein of interest will be NH_2-terminal to GST.
2. Transform into BL21 *E. coli*.
3. Grow a 500-ml culture in LB medium containing 0.1 mg/ml ampicillin at 37°C until the absorbance at 600 nm is between 0.3 OD and 0.6 OD.
4. Induce protein expression by adding IPTG to a final concentration of 250 μM.
5. Culture the cells for either 3 hours at 37°C or for 16 to 18 hours at 26°C.

   Note: Choose the expression condition that minimizes degradation of the expressed GST-fusion protein.
6. Pellet the cells by centrifugation at 1000*g* for 5 min at room temperature.
7. Resuspend the cell pellet in 25 ml of ice-cold Lysis Buffer (Recipe 1).
8. Place the cell suspension on ice.
9. Lyse the cells by one round of freeze-thawing in liquid nitrogen.
10. To fragment the DNA, sonicate the lysed cells on ice with 30-s bursts in a VibraCell Sonicator with the amplitude set at 60 and tuned to a minimum output wattage of less than 18 W.
11. Leave the lysate on ice for 30 s between each burst to prevent overheating.
12. Repeat the sonication and cooling until the viscosity of the lysate is reduced, indicating that the bacterial genomic DNA has been fragmented.
13. Clarify the lysates by centrifugation at 20,000*g* for 30 min at 4°C.
14. Filter the supernatant through a 0.44-μm pore syringe filter.

Affinity chromatography to purify the GST-fusion proteins

GST-fusion proteins are purified by affinity chromatography using immobilized glutathione-Sepharose 4B, referred to here as "beads."

1. Equilibrate 1 ml of beads with Lysis Buffer (Recipe 1) by resuspending and pelleting the beads three times in 15 ml of Lysis Buffer (Recipe 1) at 3000*g* for 30 s. Suspend the beads in 2 ml Lysis Buffer.

   Note: Equilibration is best performed just before use.

2. Incubate 25 ml of lysate for 1 hour at 4°C on a rolling platform with 1 ml of equilibrated beads.

3. Wash the beads four times with 10 volumes of Lysis Buffer + Salt (Recipe 2), pelleting the beads by centrifugation at 15,000*g* for 30 s after each wash and aspirating the wash buffer.

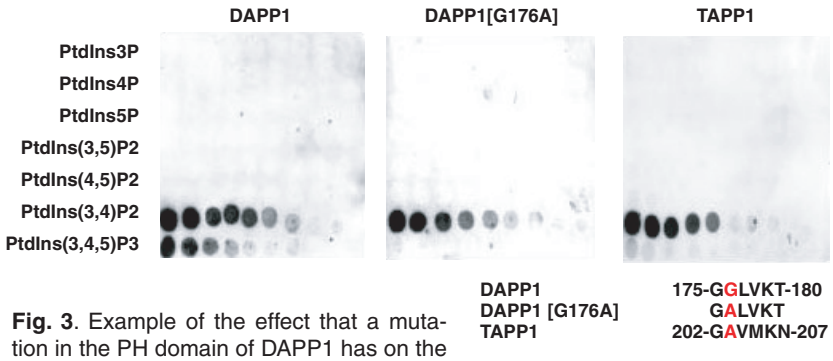

Fig. 3. Example of the effect that a mutation in the PH domain of DAPP1 has on the lipid binding specificity of this protein. A single amino acid substitution converts DAPP1 into a TAPP1-like PH domain. The ability of the indicated wild-type and mutant DAPP1 and TAPP1 GST fusion proteins to bind PI(3,4,5)P_3 and PI(3,4)P_2 was analyzed using the PLO assay. Serial dilutions (1000, 500, 250, 125, 63, 31, 16, 8, 4, 2, 1, and 0.5 pmol) of the indicated phosphoinositides were spotted onto nitrocellulose membranes, which were then incubated with the indicated proteins. The membranes were washed, and the GST-fusion proteins bound to the membrane by virtue of their interaction with lipid were detected using an antibody that recognized GST. These results are taken from data published previously (*11*).

4. Wash the beads ten times with 10 volumes of Buffer A (Recipe 3), pelleting the beads by centrifugation at 3000*g* for 0.5 min after each wash and aspirating the wash buffer.

   Note: This step removes residual Triton X-100.

5. Elute the bound GST-fusion protein at room temperature with 2 ml of Buffer A + Glutathione (Recipe 4)
6. Remove the beads by filtration through a 0.44-μm filter and save the eluate.
7. Measure the concentration of protein using Bradford reagent.
8. Aliquot the samples in 0.1-ml portions and snap freeze in liquid nitrogen. Store at −80°C.
9. Solubilize between 2 and 10 μg of purified protein in SDS Sample Buffer (Recipe 5).
10. Resolve the protein by SDS-PAGE.
11. Visualize the bands by Coomassie-staining the gel.

Purification of GST-Fusion Proteins from HEK 293 Cells

In our experience, certain lipid binding domains cannot be expressed in a soluble or unproteolyzed form in *E. coli*. In this situation, we express the domain as a GST-fusion protein in HEK 293 cells by subcloning the cDNA to be expressed into either the pEBG2T or pEBG3T vectors (*10*, *18*) that encode the expression of proteins with NH_2-terminal GST tags in mammalian cells. The DNA is then introduced into the cells using a modified calcium phosphate method (*19*).

1. Subclone the cDNA for the protein domain into either pEBG2T or pEBG3T and, after confirmation of the inserted sequence, prepare DNA suitable for transfection.
2. Culture 20 10-cm plates of HEK 293 cells in DMEM + FCS (Recipe 6) until the cells are 20% confluent.
3. For each of the 10-cm diameter plates to be transfected, mix 10 μg of plasmid DNA with sufficient sterile water to yield 0.45 ml.
4. Add 50 μl of sterile 2.5 M $CaCl_2$ (Recipe 7) and vortex the solution for 15 s.

5. Add 0.5 ml of sterile Transfection Buffer (Recipe 8) and vortex for 1 min.

   Note: It is essential that the pH of the Transfection Buffer be exactly 6.96, or the transfection efficiency is vastly reduced.

6. Incubate the solution for 30 min at room temperature to induce the precipitation of DNA-$CaCl_2$ complexes.
7. Pipette the suspension dropwise onto the plate of cells.
8. Incubate the cells at 37°C in an atmosphere of 2.5% CO_2 for 16 to 20 hours.
9. Replace the media with fresh DMEM + FCS (Recipe 6) and incubate the cells at 37°C in an atmosphere of 5% CO_2 for an additional 24 hours.
10. Lyse each dish of cells in 1.0 ml of Lysis Buffer (Recipe 1).
11. Clarify the lysates by centrifugation at 13,000*g* at 4°C for 10 min.
12. Purify the GST fusion protein by using 1 ml of beads per 20 10-cm dishes according to the procedure described in "*Affinity chromatography to purify the GST-fusion proteins.*"

    Note: Typically, 0.1 to 1 mg of high purity nonproteolyzed GST-fusion can be obtained from 20 dishes of transfected HEK 293 cells.

Protein Lipid Overlay Assay

1. Reconstitute the lyophilized lipids to 1 mM stocks in a 1:1 solution of methanol and chloroform and store at −80°C before use.
2. Dilute the lipids in a 2:1:0.8 solution of methanol:chloroform:water to six to eight different concentrations ranging between 1 and 500 μM.
3. Spot 1 μl aliquots of the selected dilutions (which contain 1 to 500 pmol of lipid) onto Hybond-C extra membrane.
4. Allow to dry at room temperature for 1 hour.
5. Incubate the membrane with gentle rocking in Blocking Buffer (Recipe 9) for 1 hour at room temperature.
6. Incubate the membrane overnight at 4°C with gentle rocking in the fresh Blocking Buffer (Recipe 9) containing 1 to 10 nM of the GST-fusion protein (or other epitope-tagged protein).
7. Wash the membrane 10 times over 50 min in TBST (Recipe 10).
8. Incubate the membrane for 1 hour at room temperature with a 1:2000 dilution of anti-GST monoclonal antibody (or other antibody that recognizes the epitope) in Blocking Buffer (Recipe 9).
9. Wash the membrane 10 times over 50 min in TBST (Recipe 10).
10. Incubate the membrane for 1 hour with a 1:5000 dilution of the HRP-conjugated antimouse secondary antibody in TBST (Recipe 10).
11. Wash the membrane 12 times over 1 hour in TBST (Recipe 10).
12. Detect the lipid binding protein bound to the membrane by the ECL according to the manufacturer's instructions.

    Note: A strong signal is usually detected with 1 min of exposure of the film.

Troubleshooting

Reproducibility

We have noticed that aliquots of lipids that have been diluted in the 2:1:0.8 solution of methanol:chloroform:water when stored at −20°C yields reproducible results only for about 2 weeks. It is possible that lipids such as $PI(3,4,5)P_3$ may slowly hydrolyze when stored in this manner.

Optimizing the Signal

The blotting conditions described above were optimized for a low background-to-signal ratio using GST-fusion PH domains. For these domains, a concentration of 1 to 10 nM in the overnight incubation step is optimal; however, for other lipid binding domains or for proteins with epitope tags other than GST, concentrations outside of this range may yield better results. If the signal obtained is very strong or a high background is observed in the blots, then lower concentrations of GST-fusion protein should be used in the assay. Alternatively, the time of incubation of the lipid-spotted membrane with the lipid binding protein could be reduced from overnight to 1 to 2 hours. Conversely, if the signal is weak, then higher concentrations of lipid binding protein should be used. If the protein being investigated has a fast off rate dissociation constant from the lipid, the duration of the washing steps of the blot can be reduced from 12 washes over 1 hour to six washes over 20 to 30 min.

Controls

We strongly recommend that, as a positive control, one or more of the lipid-binding proteins (Figs. 2 and 3) be included to ensure that the assay is working reliably and the lipids have not degraded. The cDNAs required to express these lipid-binding domains can be requested from our group, as well as from a number of other laboratories. A mutant version of the protein that does not bind lipids should also be used as a negative control.

Related Techniques

Numerous approaches have been used to assess the interactions of proteins and lipids. Some of the other most frequently employed assays that we are aware of are listed below.

Lipid vesicle sedimentation assays have been widely used to investigate the phosphoinositide binding properties of proteins (*20*, *21*). Purified proteins are incubated with lipid vesicles containing a mixture of phospholipids that attempt to mimic the lipid composition of the plasma membrane and a small mole fraction of the test lipid. The vesicles also contain sucrose so that they can be sedimented by ultracentrifugation. After ultracentrifugation, if the protein binds to the lipid vesicles, then the protein redistributes from the supernatant to the pellet fraction. Microgram amounts of protein are required for this approach.

In isothermal titration calorimetry (*22*), the protein-lipid interactions are detected by measuring the small changes in temperature that are associated with the interaction of the proteins with the lipids. The data from these experiments can be used to calculate the thermodynamic properties of the interaction, such as Gibbs free energy, and kinetic parameters, such as the binding and dissociation constants. Milligram amounts of protein are required for this approach, and soluble forms of lipids are usually used.

The kinetics of protein binding to the test lipid can also be measured by surface plasmon resonance (SPR) (*23*, *24*). A biosensor chip is coated with a lipid monolayer containing a small mole fraction of the test lipid. The purified protein is washed over the chip, and protein bound to the chip by virtue of its interaction with the test lipid can be detected as a change in the defractive index across the chip. These experiments generate characteristic binding and dissociation curves that can be used to calculate the apparent binding and dissociation constants. Microgram amounts of protein are required for this approach.

The ability of a PH domain to bind water-soluble forms of a lipid, such as the inositol head groups of phosphoinositides, has also been determined by a gel filtration-based approach (*16*, *25*). The PH domain is incubated with a radioactively labeled inositol head group and then fractionated by gel filtration chromatography. If the labeled inositol interacts with the PH domain, a peak of radioactivity that co-elutes with the protein is observed on gel filtration. Microgram amounts of protein are required for this approach.

The ability of proteins to interact with phosphoinositide lipids has also been analyzed by incubating purified proteins, in the absence or presence of soluble phosphoinositides, with phosphoinositide lipids that have been covalently attached to agarose beads (*26*). The beads are then isolated by centrifugation and washed, and the associated proteins are separated by SDS-PAGE and detected by immunoblotting.

Notes and Remarks

The results obtained from a PLO assay are qualitative. If quantitative association constants are required, then SPR or isothermal titration calorimetry are the recommended methods. However, these approaches are substantially more laborious, require relatively expensive equipment, and necessitate significant expertise. The limitation of the gel filtration assay is that few lipids are available in radioactive and water-soluble forms that can be employed in this type of assay. Isothermal titration calorimetry also requires soluble forms of lipid that are not readily available. The advantage of the PLO assay over the other binding assays is that it enables tests for the lipid binding specificities of many protein samples for a range of naturally occurring lipids to be processed simultaneously. The results obtained from this type of assay provide a good qualitative indication of the relative affinity of different proteins or wild-type

and mutant forms of a protein for the lipid(s) with which they interact. Moreover, the primary results obtained from the PLO assay are in the form of a standard immunoblot, which is easy for the nonexpert to interpret in publications and visual presentations (Figs. 2 and 3). The PLO assay also has the potential to be adapted to a high-throughput format to enable the global comparison of the lipid binding specificities of all the proteins in the human genome that possess a certain type of lipid binding domain. The PLO assay could also be used as a basis for a screen to identify drugs that inhibit the interaction of a protein with its natural lipid ligand. Furthermore, if lipid binding proteins with fluorescent tags were employed, interaction of protein with the lipid could be detected directly using fluorescence technologies.

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