Chapter 15
Analysis of Phosphatidylinositol 3,4,5 Trisphosphate 5-Phosphatase Activity by *in vitro* and *in vivo* Assays

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Abstract Phosphatidylinositol 3,4,5 trisphosphate [PtdIns(3,4,5)P_3] is a potent membrane-bound signaling molecule transiently synthesized by phosphoinositide 3-kinase (PI3-kinase) in response to extracellular agonists. PtdIns(3,4,5)P_3 signals need to be strictly controlled. PtdIns(3,4,5)P_3 recruits and binds effectors that function in oncogenic signaling pathways. PtdIns(3,4,5)P_3 activates cell proliferation, growth, and migration as well as regulating insulin signaling. The inositol polyphosphate 5-phosphatase family of enzymes dephosphorylate and thereby modulate PtdIns(3,4,5)P_3 levels, attenuating PI3-kinase-dependent signaling. PtdIns(3,4,5)P_3 5-phosphatase enzyme activity can be assessed *in vitro* by analysis of the hydrolysis of radiolabeled or fluorescently labeled PtdIns(3,4,5)P_3 and *in vivo* by visualization of the recruitment and turnover of the PtdIns(3,4,5)P_3-specific biosensor GFP-PH/ARNO or other PtdIns(3,4,5)P_3 binding proteins at the plasma membrane.

Keywords Phosphatidylinositol 3,4,5 trisphosphate · inositol polyphosphate 5-phosphatase · thin layer chromatography · confocal immunofluorescence · plasma membrane · growth factor stimulation · phosphoinositide biosensor

Abbreviations 5-phosphatase: Inositol polyphosphate 5-phosphatase; DMSO: Dimethyl sulfoxide; PI3-kinase: Phosphoinositide 3-kinase; PH: Pleckstrin homology; PtdIns(4,5)P_2: Phosphatidylinositol 4,5 bisphosphate; PtdIns(3,4,5)P_3: Phosphatidylinositol 3,4,5 trisphosphate; PtdSer: Phosphatidylserine; TLC: Thin layer chromatography.

15.1 Introduction

Phosphoinositide 3-kinase (PI3-kinase) phosphorylates phosphatidylinositol 4,5 bisphosphate [PtdIns(4,5)P_2] at the plasma membrane following agonist stimulation to transiently generate the lipid signaling molecule

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phosphatidylinositol 3,4,5 trisphosphate [PtdIns(3,4,5)P_3] [1]. PtdIns(3,4,5)P_3 regulates many cellular processes, including cell proliferation, inhibition of apoptosis, insulin signaling, actin dynamics, and cell migration [2]. PtdIns(3,4,5)P_3 recruits proteins that contain modular domains such as pleckstrin homology (PH) domains found in the serine threonine kinase Akt and its activating kinase PDK1. PtdIns(3,4,5)P_3 localizes PH domain-containing proteins at the plasma membrane and thereby regulates their allosteric activation [1]. In addition to Akt, the PH domains of ARNO, Btk, and GRP1 also specifically bind to PtdIns(3,4,5)P_3 [3–7].

The signals generated by PtdIns(3,4,5)P_3 are regulated by PTEN and/or the inositol polyphosphate 5-phosphatases (5-phosphatases) [8]. The 5-phosphatases specifically remove the 5-position phosphate from the inositol ring of the membrane-bound phosphoinositides PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, and PtdIns(3,5)P_2, forming PtdIns(4)P, PtdIns(3,4)P_2, and PtdIns(3)P, respectively. Additionally, some 5-phosphatases can also hydrolyze the soluble inositol phosphates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 at the 5-position phosphate, forming Ins(1,4)P_2 and Ins(1,3,4)P_3, respectively. Currently, 10 mammalian and four yeast family members have been identified that all contain a conserved 300 amino acid 5-phosphatase domain [9]. Each 5-phosphatase displays varying substrate preferences [10], but most are capable of hydrolyzing both the phosphoinositides and inositol phosphates. To date, nine of the 10 mammalian 5-phosphatases exhibit PtdIns(3,4,5)P_3 5-phosphatase activity *in vitro*. PtdIns(3,4,5)P_3 5-phosphatases include Synaptojanin-1 and -2, SHIP-1 and -2, PIPP, 72-kDa 5-phosphatase, OCRL, SKIP, and the type II 5-phosphatase, which regulate numerous cellular processes including synaptic vesicle recycling, immune cell function, insulin signaling, neurite elongation, and endosomal trafficking [8, 11–13].

The gold standard for demonstrating PtdIns(3,4,5)P_3 5-phosphatase activity is via analysis of total cellular PtdIns(3,4,5)P_3 levels in intact cells. Cells are labeled with [^{3}H]-inositol followed by short-term growth factor stimulation, extraction of lipids, deacylation, and HPLC analysis of lipid reaction products in comparison to known standards [14]. However, this may be technically challenging depending on the cell type and cannot be undertaken for transiently transfected cells with less than 50% transfection efficiency or a mixed cell population. Also, such analysis cannot determine localized PtdIns(3,4,5)P_3 turnover on specific membrane microdomains such as membrane ruffles, or in specific subcellular compartments of highly specialized cells such as the neurite growth cone.

Alternative techniques that have recently been developed include analysis of the plasma membrane recruitment of PtdIns(3,4,5)P_3-specific biosensors in transiently transfected cells. The advantage of this technique is that single-cell analysis of PtdIns(3,4,5)P_3 turnover can be determined. In addition, many of the 5-phosphatases are large proteins, which may be challenging to express as purified recombinant proteins. Furthermore, the 5-phosphatases often form multiprotein complexes with receptors and/or other signaling proteins, which may affect their enzyme activity. To enable analysis of PtdIns(3,4,5)P_3 5-phosphatase activity of specific 5-phosphatases in transfected cells or to

quantify PtdIns(3,4,5)P_3 5-phosphatase activity in multiprotein complexes, an immunoprecipitation-based assay has been developed. We describe the use of radiolabeled ^{32}P-PtdIns(3,4,5)P_3; however, a commercially available PtdIns(3,4,5)P_3 substrate together with malachite green may be equally effective.

15.2 Materials

15.2.1 In vitro PtdIns(3,4,5)P_3 5-Phosphatase Assays

15.2.1.1 Transfection of COS-1 Cells

1. Dulbecco's Modified Eagle's Medium (DMEM, Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 U/ml of penicillin/0.1% streptomycin (Sigma, St Louis, MO), and 2 mM L-glutamine (JRH Biosciences).
2. Trypsin (JRH Bioscicences): 0.12% Trypsin, 0.02% EDTA, 0.04% glucose.
3. 25x dextran/chloroquine: 400 µg/ml of DEAE dextran (Sigma), 100 µM chloroquine (Sigma). Sterilize by filtration through a 0.2-µm filter. Store at −20°C indefinitely. A working aliquot can be stored at 4°C for two weeks.
4. Phosphate buffered saline (PBS): Prepare a 10x stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4 (adjust to pH 7.4). Store at room temperature and dilute one volume with nine volumes of water to prepare a working solution. Sterilize by autoclaving.
5. 10% DMSO in PBS: Mix one volume of DMSO (Sigma) with nine volumes of sterile PBS.
6. Lysis buffer: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 (v/v), store at 4°C, and use ice-cold. One Complete Mini Protease Inhibitor Cocktail tablet (Roche Diagnostics, Mannheim, Germany) should be dissolved in 10 ml of the lysis buffer just prior to use for harvesting the cells following transfection (this solution cannot be stored).
7. Cell scrapers (Falcon, Bedford, MA).

15.2.1.2 Immunoprecipitation of Recombinant Proteins

1. Tris-saline: Prepare a 10x stock with 200 mM Tris-HCl, pH 7.4, 1.5 M NaCl, and store at room temperature. Dilute one volume with nine volumes of water to prepare a working solution.
2. Protein A Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden).

15.2.1.3 Labeling of PtdIns(3,4,5)P_3 Substrate

1. PtdIns(4,5)P_2 (Sigma) is resuspended at 2 mg/ml in a mixture of chloroform:-methanol:10 mM HCl (20:9:1). Lipid stocks should be stored in 100-µl

aliquots in dark glass vials at −20°C. The lids should be sealed with Parafilm or an equivalent to prevent evaporation of the solvent.
2. L-α Phosphatidyl-L-serine (PtdSer) stock (Sigma) is resuspended at 5 mg/ml in a mixture of chloroform:methanol:10 mM HCl (20:9:1) and stored as above for PtdIns(4,5)P_2.
3. Nitrogen cylinder with regulator.
4. Lipid resuspension buffer: 20 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA.
5. PtdSer carrier lipid: Place 5 μg of PtdSer per reaction into a microcentrifuge tube and dry under a stream of nitrogen. Resuspend the lipid in 10 μl of lipid resuspension buffer and sonicate for 5 min in a water-bath sonicator. PtdSer carrier lipid should be prepared fresh on the day required but can be stored for several hours at room temperature.
6. Recombinant PI3-kinase: obtained from Dr Meredith Layton, Ludwig Institute, Australia [15]. Store at −20°C.
7. 20x kinase buffer: 400 mM HEPES, pH 7.8, 100 mM $MgCl_2$, 20 mM EGTA.
8. Unlabeled 2.5 mM ATP (Sigma). Store at −20°C.
9. Adenosine 5'-[γ-^{32}P]triphosphate ~3,000 Ci/mmol, 10 mCi/ml. Store at −20°C (Perkin Elmer Life Sciences, Boston, MA; we use frozen, not the 4°C stabilized label).
10. 1 M HCl. Measure 45.8 ml of water into a 50-ml tube and add 4.2 ml of concentrated HCl. Be careful when handling concentrated HCl; only use it in a fume hood.
11. Chloroform-saturated 2 M KCl: Dissolve 3.73 g of KCl in 25 ml of water and add 5 ml of chloroform. Mix the solution by shaking vigorously and then allow the phases to separate. Only use the top aqueous layer for lipid extractions. The solution can be stored at room temperature for months as long as the chloroform layer is replaced as it evaporates.
12. Chloroform:methanol (1:1, v/v): Mix equal volumes of chloroform and methanol. This solution should be freshly prepared before use, ensuring that the solution does not turn cloudy.

15.2.1.4 PtdIns(3,4,5)P_3 5-Phosphatase Assays

1. TLC plates: Partisil K6 Silica Gel 60 Å (250-μm layer) channeled concentration zone TLC plates (Whatman, Maidstone, UK). TLC plates need to be oxalate-treated prior to use by incubating plates horizontally in oxalate solution [1% potassium oxalate (w/v), 50% ethanol (v/v), 2 mM EDTA] for 30 s. Drain off the excess liquid and then dry the plates vertically on an absorbent paper towel overnight at room temperature (*see* Note 2). Oxalate-treated TLC plates can be stored at room temperature for months before use.
2. Hairdryer.
3. TLC tank: Cover the two widest sides of the tank with pieces of gel blotting paper (Schleicher and Scheull Bioscience, Dassel, Germany). Add freshly

prepared tank solvent (130 ml of propan-1-ol, 8 ml of glacial acetic acid, 680 µl of orthophosphoric acid, and 61.32 ml of water; ensure that the solution is clear) to the TLC tank, seal the lid in place with vacuum grease, and allow the tank to equilibrate at room temperature overnight (*see* Note 3).
4. Super Rx medical X-ray film (Fuji, Tokyo, Japan).

15.2.1.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Running gel buffer: 0.75 M Tris-HCl, pH 8.8. Store at room temperature.
2. Stacking gel buffer: 0.25 M Tris-HCl, pH 6.8. Store at room temperature.
3. 10% SDS (Amresco, Solon, OH). Dissolve 100 g of SDS in 1 L of water. Store at room temperature. SDS powder is a respiratory irritant and should only be handled in the fume hood.
4. 30% acrylamide/bis solution (because unpolymerized solution is neurotoxic, exposure should be avoided; Bio-Rad Laboratories, Hercules, CA).
5. Ammonium persulfate (APS): 14 mg/ml of APS (Bio-Rad Laboratories) in water.
6. N,N,N',N'-tetramethylethylene-diamine (TEMED, Sigma).
7. Running buffer: 25 mM Tris-HCl, 190 mM glycine, 0.1% (w/v) SDS; store at room temperature.
8. Prestained molecular weight markers: Page Ruler (Fermentas, Burlington, Ontario, Canada).
9. 2x Laemmli buffer: 40 mM Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 0.002% bromophenol blue (w/v), 10% 2-mercaptoethanol (v/v).
10. Transfer buffer: 25 mM Tris-HCl, 190 mM glycine, 20% (v/v) methanol; store at room temperature.
11. PVDF membrane (Pall Life Sciences, Pensacola, FL).
12. Gel blotting paper (Schleicher and Scheull Bioscience).
13. Blocking buffer: 5% skim milk (w/v) in Tris-saline, prepared fresh.
14. Primary antibody: appropriate antibody to detect the protein of interest diluted in 10 ml of Tris-saline.
15. Secondary antibody: appropriate HRP-conjugated secondary antibody diluted in 10 ml of Tris-saline.
16. Western Lightning enhanced chemiluminescence solution (Perkin Elmer Life Sciences, Boston, MA) and Super Rx medical X-ray film (Fuji).

15.2.2 In vivo PtdIns(3,4,5)P_3 5-Phosphatase Assays

15.2.2.1 Transfection of COS-1 Cells with GFP-PH/ARNO

1. GFP-PH/ARNO plasmid was obtained from Dr. Tamas Balla (National Institutes of Health, Bethesda, MD).
2. 13-mm coverslips (Menzel, Braunschweig, Germany). Coverslips should be placed into a glass jar and sterilized by autoclaving.

3. Epidermal growth factor (EGF; BD Biosciences, Bedford, MA) or other appropriate growth factor. Store at −20°C in single-use aliquots to avoid freeze-thawing. Prepare stimulation media by adding EGF to serum-free DMEM to a final concentration of 100 ng/ml.

15.2.2.2 Immunofluorescence

1. 3% paraformaldehyde (BDH, Victoria, Australia). Paraformaldehyde is toxic and should be prepared in a fume hood. Heat approximately 300 ml of water to 80°C in a glass beaker, stirring continuously on a heated stirrer. Add 15 g of paraformaldehyde, stirring continuously for 30 min at 80°C. At this stage, most of the paraformaldehyde will have gone into solution; however, the solution will still appear cloudy. Add 50 ml of 10x PBS, stirring continuously at 80°C (for a final concentration of 1x PBS). After the addition of the 10x PBS, the solution should appear clear. Adjust the pH to 7.4 using HCl and then add water to a final volume of 500 ml. Freeze in aliquots at −20°C. Paraformaldehyde can be stored indefinitely at −20°C, but avoid freeze-thawing.
2. 0.1% Triton X-100 (Sigma) in PBS: Mix 50 µl of Triton X-100 with 50 ml of PBS and vortex. Store at room temperature.
3. Staining dish. To prepare the dish, two pieces of gel blotting paper are placed in the bottom of an agar plate and dampened with water. The blotting paper is then covered with a piece of Parafilm (or equivalent) and the coverslips placed on top of the Parafilm, cell side facing up. This setup can be reused, but the gel blotting paper needs to be dampened before each use.
4. 1% BSA fatty acid free (Bovogen, Victoria, Australia) in PBS: Mix 0.5 g of BSA with 50 ml of PBS; vortex. Store in aliquots at −20°C.
5. Primary antibody to detect protein of interest diluted in 1% BSA.
6. Secondary antibody conjugated to AlexaFluor®594 (or equivalent) diluted in 1% BSA. Protect the solution from the light.
7. Glass slides (Menzel).
8. Slow Fade solution (Molecular Probes, Eugene, OR).

15.3 Methods

15.3.1 In vitro PtdIns(3,4,5)P_3 5-Phosphatase Assays

The method outlined below describes *in vitro* PtdIns(3,4,5)P_3 5-phosphatase assays performed using radiolabeled PtdIns(3,4,5)P_3 and immunoprecipitated proteins from intact cells. These assays can also be performed with purified recombinant 5-phosphatases as well as immunoprecipitated endogenous 5-phosphatases or multiprotein complexes that may contain 5-phosphatases immunoprecipitated from mammalian cells.

15.3.1.1 Transfection of COS-1 Cells

COS-1 cells may be transiently transfected with a construct encoding the 5-phosphatase of interest, or a protein that complexes with a 5-phosphatase, prior to immunoprecipitation of the recombinant protein from cell lysates. While the method describes the transfection of one 5-phosphatase construct, it can easily be adapted to assess the effects of other proteins such as 5-phosphatase-binding partners on PtdIns(3,4,5)P_3 5-phosphatase activity by transiently co-transfecting the cells with multiple constructs. The 5-phosphatase construct can also be transfected into different cell types, although the transfection method may need to be optimized. The cells can also be treated with specific growth factors or inhibitors as required.

1. COS-1 cells are maintained in DMEM supplemented with 10% fetal bovine serum in 100-mm tissue culture dishes.
2. For transfection, 100-mm tissue culture dishes of COS-1 cells at ~80% confluence are split 1:2 and incubated for 4 h at 37°C in a humidified 5% CO_2 incubator to allow the cells to adhere. One 100-mm tissue culture dish is required for the transfection of each construct.
3. Replace the media with 5 ml of DMEM without fetal bovine serum.
4. Add 200 µl of 25x dextran/chloroquine and 5 µg of a DNA construct encoding either the 5-phosphatase of interest or the empty vector alone as a negative control. The amount of DNA added to the transfection may need to be increased depending on the activity of the promoter.
5. Mix gently and incubate at 37°C for 2 h in a humidified 5% CO_2 incubator (*see* Note 4).
6. Discard the media/dextran/chloroquine/DNA mix.
7. Add 5 ml of 10% DMSO in PBS and incubate for 2 min at room temperature. The time of this incubation is critical.
8. Aspirate the DMSO/PBS mix.
9. Add 8 ml of DMEM containing serum and incubate the cells at 37°C overnight in a humidified 5% CO_2 incubator.
10. Split the cells 1:2 to generate two 100-mm tissue culture dishes for each construct and incubate at 37°C in a humidified 5% CO_2 incubator overnight.
11. Wash the cells twice with ice-cold Tris-saline.
12. Add 1 ml of ice-cold lysis buffer to each dish (with protease inhibitors) and scrape the cells from the dish with a cell scraper.
13. Incubate at 4°C for 1 h, with gentle agitation.
14. Centrifuge 13,000 × *g* for 10 min.
15. Transfer the Triton-soluble supernatant to a fresh tube for immunoprecipitation of the recombinant protein of interest. This protocol will generate 2x 1 ml of Triton-soluble supernatant for each construct.

15.3.1.2 Immunoprecipitation of Recombinant Proteins

The transiently expressed recombinant 5-phosphatase or binding partner or receptor is immunoprecipitated from cell lysates using specific antibodies. It is

also possible to immunoprecipitate endogenous 5-phosphatase proteins from cells or multiprotein complexes that contain a 5-phosphatase.

1. For each construct, transfer 100-µl aliquots of protein A Sepharose [50% slurry (v/v)] to two microcentrifuge tubes. Transfer an additional 100 µl of protein A Sepharose to a separate tube as a blank control reaction for the PtdIns(3,4,5)P_3 5-phosphatase assay.
2. Centrifuge the protein A Sepharose for 15 s at 13,000 × *g* and then discard the supernatant.
3. Wash the protein A Sepharose pellets three times with 1 ml of ice-cold lysis buffer (without protease inhibitors) and discard the final wash.
4. Set the blank tube aside at 4°C for use in the PtdIns(3,4,5)P_3 5-phosphatase assays described in Subheading 15.3.1.4.
5. Add 1 ml of the Triton-soluble supernatant prepared as described in Subheading 15.3.1.1.
6. Incubate at 4°C overnight with gentle agitation (a rotating wheel is ideal; *see* Note 5).
7. Centrifuge the immunoprecipitations at 1,000 × *g* for 10 s and remove the supernatant.
8. Wash the pellets three times with 1 ml of ice-cold lysis buffer (without inhibitors).
9. Wash the pellets three times with 1 ml of ice-cold 1x kinase buffer and discard the final wash.
10. Use one immunoprecipitation for PtdIns(3,4,5)P_3 5-phosphatase assays as described in Subheading 15.3.1.4. Add 50 µl of 2x Laemmli buffer to the other immunoprecipitation and analyze by Western blotting (Subheading 15.3.1.5) to confirm the expression/immunoprecipitation of the recombinant protein of interest.

15.3.1.3 Labeling of PtdIns(3,4,5)P_3 Substrate

The radiolabeled PtdIns(3,4,5)P_3 substrate is generated by incubating unlabeled PtdIns(4,5)P_2 with recombinant PI3-kinase and [γ^{32}P]ATP to produce PtdIns([^{32}P]3,4,5)P_3. The protocol outlined below describes the use of recombinant PI3-kinase from a noncommercial source. However, active recombinant PI3-kinase is also available from a number of commercial sources such as Upstate (Lake Placid, NY), Biomol (Plymouth Meeting, PA), and Jena Bioscience (Jena, Germany).

1. Add 6.7 µl of PtdIns(4,5)P_2 stock and 2.4 µl of PtdSer stock to a microcentrifuge tube.
2. Dry the lipids under a stream of nitrogen gas using a glass Pasteur pipette attached via plastic tubing to the regulator on the nitrogen cylinder (*see* Note 6).
3. Resuspend the lipids in 200 µl of lipid resuspension buffer (*see* Note 7).
4. Sonicate for 5 min in a water-bath sonicator.

5. Mix 18 µl of water, 2 µl of 2.5 mM ATP, 1 µg of recombinant PI3-kinase (in 5 µl), 5 µl of 20x kinase buffer, 50 µl of PtdIns(4,5)P_2/PtdSer mix, and 20 µl [γ^{32}P]ATP (3,000 Ci/mmol) (ensure that appropriate shielding is used when working with [γ^{32}P]ATP; *see* Note 8).
6. Incubate the reaction at room temperature overnight.
7. Stop the reaction by adding 100 µl of 1 M HCl.
8. Add 10 µl pf PtdSer carrier lipid, 200 µl of chloroform:methanol (1:1), and 500 µl of chloroform-saturated 2 M KCl to the substrate reaction.
9. Vortex briefly, and then centrifuge at 10,000 × *g* for 5 min to separate the organic and aqueous phases.
10. Transfer the bottom organic layer to a clean microcentrifuge tube using a Gilson pipette (or equivalent). It is very important not to transfer any of the top layer during this step. Depress the plunger of the pipette almost to the first stop, and then insert the pipette tip into the bottom organic layer. Fully depress the plunger to the first stop to expel a small amount of air and any of the top aqueous layer within the pipette tip. Carefully draw up almost all of the bottom organic layer and withdraw the pipette.
11. Dry the PtdIns([^{32}P]3,4,5)P_3 under a stream of nitrogen as above. Care must be taken during this step to prevent spillage of the radioactive substrate (*see* Note 8).
12. Resuspend the lipid in 500 µl of lipid resuspension buffer.
13. Sonicate for 5 min in a water-bath sonicator.

15.3.1.4 PtdIns(3,4,5)P_3 5-Phosphatase Assays

To measure PtdIns(3,4,5)P_3 5-phosphatase activity *in vitro*, the radiolabeled PtdIns([^{32}P]3,4,5)P_3 substrate generated in Subheading 15.2.1.3 is incubated with the recombinant immunoprecipitated 5-phosphatase (Subheadings 15.3.1.1 and 15.3.1.2) and then the lipid reaction products are extracted and separated by thin layer chromatography.

1. Add 5 µl of 20x kinase buffer and 50 µl of PtdIns([^{32}P]3,4,5)P_3 to the immunoprecipitate pellets prepared in Subheading 15.3.1.2.
2. Incubate for 30 min at 37°C. This time can be shortened to 10 min if submaximal hydrolysis of PtdIns(3,4,5)P_3 is required to compare subtle differences in 5-phosphatase activity.
3. Stop the reactions by adding 100 µl of 1 M HCl (*see* Note 9).
4. Add 10 µl of PtdSer carrier lipid, 200 µl of chloroform:methanol (1:1), and 500 µl of chloroform-saturated 2 M KCl to each reaction.
5. Vortex briefly and then centrifuge at 10,000 × *g* for 5 min to separate the organic and aqueous phases.
6. Transfer the bottom organic layer to a clean microcentrifuge tube, ensuring that none of the top layer is transferred (see Subheading 15.3.1.3, step 10).
7. Pipette the whole organic layer from a reaction onto the loading zone of a lane on an oxalate-treated TLC plate. Heat the plate with a hairdryer on the lowest warm setting during loading to facilitate solvent evaporation (*see* Note 10).

8. Place the plate into the prepared TLC tank with the samples at the bottom and incubate at room temperature until the solvent front almost reaches the top of the TLC plate (approximately 4–5 h).
9. Remove the plate from the TLC tank and place on an absorbent paper towel behind suitable shielding to dry.
10. Cover the plate with plastic wrap and place into an X-ray film cassette with an intensifying screen. Tape the edges of the plate to the cassette to prevent the plate from moving during exposure.
11. Expose the plate to X-ray film at room temperature (start with a 2-h exposure, but an overnight or longer exposure may be required if the signal is low).
12. Determine the percentage of PtdIns(3,4,5)P_3 hydrolysis by comparing the intensity of the PtdIns(3,4)P_2 spot to the PtdIns(3,4,5)P_3 spot for each sample by densitometry. An example of the results obtained is shown in Fig. 15.1a.

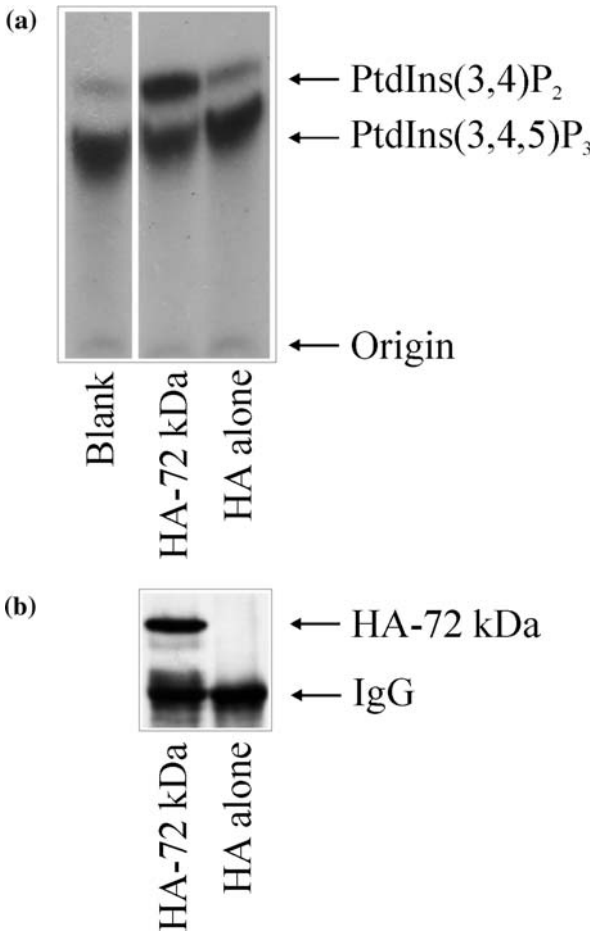

Fig. 15.1 *In vitro* PtdIns(3,4,5)P_3 5-phosphatase assays. (a) COS-1 cells were transiently transfected with plasmids encoding HA empty vector alone (HA alone) or HA-72-kDa 5-phosphatase (HA-72-kDa). Forty-eight hours after transfection, cells were harvested and the 1% Triton X-100-soluble fractions were immunoprecipitated with HA antibodies. HA alone and HA-72-kDa immunoprecipitates were subjected to PtdIns([^{32}P]3,4,5)P_3 5-phosphatase assays and the lipid products were resolved by TLC. The origin and migration of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are shown on the right. (b) COS-1 cells were transiently transfected and immunoprecipitated as in (a). HA alone and HA-72-kDa immunoprecipitates were subjected to SDS-PAGE and immunoblotted with HA antibodies. The migration of HA-72-kDa 5-phosphatase (HA-72 kDa) and IgG is indicated on the right

15.3.1.5 Western Blotting of Immunoprecipitated Recombinant Proteins

Following PtdIns(3,4,5)P_3 5-phosphatase assays with radiolabeled substrate, duplicate immunoprecipitates prepared in Subheading 15.3.1.2 are Western blotted with appropriate antibodies to confirm the expression and immunoprecipitation of the recombinant 5-phosphatase.

1. The following instructions are for a Bio-Rad mini-gel electrophoresis and transfer system but can be adapted to suit other systems. The glass plates should be wiped clean with methanol prior to use.
2. Prepare a 0.75-mm-thick 10% gel by mixing 2.5 ml of running gel buffer, 1.6 ml of 30% acrylamide/bis solution, 50 µl of 10% SDS, 0.57 ml of water, 250 µl of 14 mg/ml APS, and 10 µl of TEMED. Pour into the prepared casting apparatus, leaving space (~2 cm) for the stacking gel, and overlay with distilled water. Allow the gel to polymerize (~15 min).
3. Pour off the water from the running gel.
4. Prepare the stacking gel by mixing 2.5 ml of the stacking gel buffer, 0.65 ml of 30% acrylamide/bis solution, 50 µl of 10% SDS, 1.55 ml of water, 250 µl of 14 mg/ml APS, and 10 µl of TEMED. Fill up the casting apparatus with the stacking gel mix and then insert the comb. Allow the gel to polymerize (~15–30 min).
5. Remove the comb and rinse the wells with running buffer using a disposable plastic transfer pipette. Insert thin strips of gel blotting paper into the wells to remove the buffer from the wells. Assemble the gel unit into the tank.
6. Boil the samples at 100°C for 10 min and then centrifuge briefly to pellet the protein A Sepharose. Load 30 µl of a sample (avoiding the protein A Sepharose) into a well. Load prestained molecular weight markers into one well and then fill all of the wells with running buffer.
7. Fill the tank with running buffer, connect the gel unit to the power supply, and run at 200 V until the blue dye front has just run off the gel.
8. Disconnect the electrophoresis unit from the power supply, disassemble, and discard the stacking gel.
9. Cut a sheet of PVDF membrane just larger than the gel. Mark one corner of the membrane with a soft pencil to allow identification and orientation of the membrane in subsequent steps. Briefly wet the membrane in methanol and then rinse in water followed by transfer buffer.
10. Lay the transfer cassette open on the bench. Wet a sponge in transfer buffer and place on the transfer cassette followed by a piece of gel blotting paper. Wet another piece of gel blotting paper, transfer the gel to the blotting paper, and place into the transfer cassette. Carefully overlay the PVDF membrane onto the gel, remove any air bubbles, and then cover with another two pieces of gel blotting paper and a sponge soaked in transfer buffer.
11. Place the transfer cassette into the transfer tank, ensuring that the gel is facing the cathode, and then place an ice block and magnetic stirrer into the tank.

12. Fill the tank with transfer buffer, connect to the power supply, and switch on the magnetic stirrer. Transfer at 250 mA for 1 h.
13. Disassemble the transfer apparatus and confirm that the prestained molecular weight markers have transferred to the membrane. Discard the gel and blotting paper.
14. Incubate the membrane in 50 ml of blocking buffer for 1 h at room temperature with gentle agitation.
15. Briefly rinse the membrane three times with Tris-saline and then place it into a heat-sealable plastic bag. Add the primary antibody to the bag and remove the air bubbles. Heat-seal the top of the bag and incubate at 4°C overnight with gentle agitation (*see* Note 12).
16. Remove the primary antibody (*see* Note 13) and then wash the membrane three times with Tris-saline for 5 min each wash (*see* Note 14).
17. Place the membrane into a heat-sealable bag and add the secondary antibody. Again remove the air bubbles and heat-seal the bag. Incubate at room temperature for 1 h with gentle agitation.
18. Wash the membrane as in step 16.
19. Mix 2.5 ml of each of the ECL reagents in a plastic container. Incubate the membrane in the ECL mix for 1 min, making sure that the membrane is evenly coated.
20. Remove the blot from the ECL reagent and gently blot with paper towel to remove excess liquid. Cover the membrane with plastic wrap, place into an X-ray cassette, and secure with tape. Expose the membrane to film for a suitable time (start with a 2-min exposure and adjust the time accordingly). An example of the results is shown in Fig. 15.1b.

15.3.2 In vivo PtdIns(3,4,5)P_3 5-Phosphatase Assays

The protocol described below outlines the measurement of plasma membrane PtdIns(3,4,5)P_3 levels in COS-1 cells. However, this method can be easily adapted to any cell line that can be transfected with the GFP-PH/ARNO construct. In addition, other PtdIns(3,4,5)P_3-specific GFP-PH fusion proteins can be used including GFP-PH/Btk. This method can also be utilized to analyze the turnover of PtdIns(3,4,5)P_3 in specialized cellular compartments such as neurite growth cones. To analyze the generation of PtdIns(3,4)P_2, the product of PtdIns(3,4,5)P_3 hydrolysis by 5-phosphatases, the same protocol can be repeated using the PH domain of TAPP1, which binds only PtdIns(3,4)P_2 [16].

15.3.2.1 Transfection of COS-1 Cells with GFP-PH/ARNO

COS-1 cells are co-transfected with the GFP-PH/ARNO biosensor and a construct encoding the 5-phosphatase of interest or a protein that may regulate 5-phosphatase activity. While this method describes PtdIns(3,4,5)P_3 analysis in

cells overexpressing a 5-phosphatase, knockdown of the protein of interest by RNAi methods can also be used.

1. Transfect a 100-mm dish of COS-1 cells using the dextran/chloroquine method described in Subheading 15.3.1.1. Follow steps 1–9 and add 5 μg of GFP-PH/ARNO DNA in step 4 in addition to 5 μg of a construct encoding the 5-phosphatase of interest or empty vector alone as a control. Incubate the cells at 37°C overnight.
2. Trypsinize the cells with 2 ml of trypsin and then add 6 ml of DMEM containing serum. Sterilize a pair of forceps with 70% ethanol and transfer four coverslips per transfection to separate wells of a 24-well dish. Add 400 μl of media containing serum and 100 μl of cells (*see* Note 15). Incubate at 37°C for ~6 h.
3. Replace the media with 500 μl of DMEM without serum and incubate overnight (16 h) at 37°C.
4. Thaw the paraformaldehyde fix and prepare the stimulation media containing 100 ng/ml of EGF (*see* Note 16). Label the wells of the 24-well dish 0, 1, 5, and 10 min for each transfection.
5. Remove the media from the 10-min wells and add 500 μl of stimulation media. Incubate at 37°C for 5 min, remove the media from the 5-min wells, and add 500 μl of stimulation media. Incubate at 37°C for 4 min and then repeat for the 1-min wells. Incubate the dish for a further 1 min at 37°C.
6. Immediately aspirate the culture media from all of the wells and add ~500 μl of paraformaldehyde fix (*see* Note 17). Incubate for 20 min at room temperature.
7. Remove the paraformaldehyde and add ~500 μl of 0.1% Triton X-100 in PBS. Incubate for 2 min at room temperature.
8. Remove the 0.1% Triton X-100 in PBS and wash the coverslips three times with PBS (*see* Note 18).

15.3.2.2 Immunofluorescence

Following transient transfection, the cells are stimulated with a growth factor to promote plasma membrane PtdIns(3,4,5)P_3 production and are then fixed, permeabilized, and stained with specific antibodies to detect the recombinant 5-phosphatase.

1. Transfer the coverslips from the tissue culture dish to a staining/Petri dish (*see* Note 19).
2. Block the coverslips with 40 μl of 1% BSA in PBS for 20 min at room temperature.
3. Incubate the coverslips with 40 μl of primary antibody (diluted in 1% BSA in PBS) for 1 h at room temperature.
4. Wash the coverslips three times with PBS (*see* Note 20).

5. Incubate the coverslips with 40 µl of AlexaFluor® 594-conjugated secondary antibody (or equivalent, diluted in 1% BSA in PBS) for 45 min at room temperature protected from the light.
6. Wash the coverslips three times with PBS.
7. Mount the coverslips on glass slides using 1.5 µl of antifade mounting solution per coverslip and seal the edges with nail polish (*see* Note 21).

15.3.2.3 Microscopy and Analysis

Images of fixed cells are acquired by confocal laser scanning microscopy. Single optical sections are taken through the middle of the cell such that the plasma membrane and the cytosol are visibly distinct (*see* Note 22). An example of this is shown in Fig. 15.2. Use ImageJ analysis software to quantify the recruitment of the GFP-PH/ARNO biosensor to the plasma membrane [17] (*see* Note 23). This is achieved by measuring and expressing the pixel fluorescence intensity at the plasma membrane as a ratio of the cytosolic fluorescence intensity, which is used to standardize for differential expression between individual cells. For each cell to be analyzed, the average pixel fluorescence intensity of three separate representative areas of defined size at the plasma membrane (e.g., 50 pixels) and three areas of defined size in the cytosol (e.g., 100 pixels) are determined (see Note 24). The ratio of plasma membrane to cytosolic pixel fluorescence intensity is then calculated [13]. At least 10 cells per time point should be analyzed from three separate experiments.

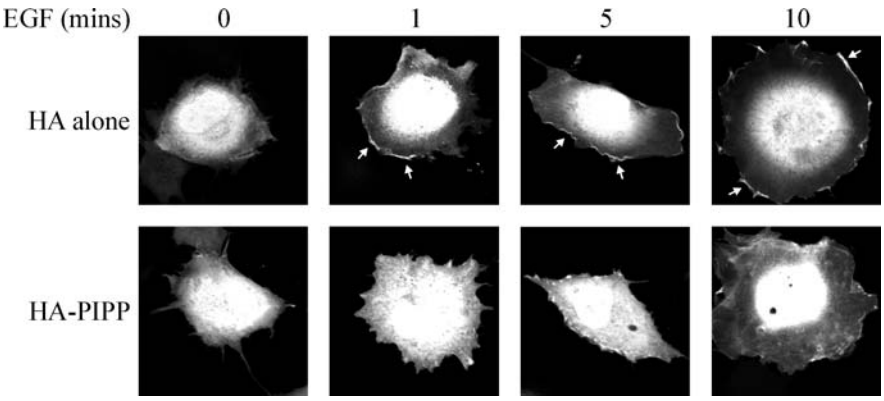

Fig. 15.2 *In vivo* PtdIns(3,4,5)P_3 5-phosphatase assays. COS-1 cells were transiently co-transfected with plasmids encoding GFP-PH/ARNO and either HA empty vector (HA alone) or HA-PIPP 5-phosphatase (HA-PIPP). Cells were serum-starved and, where indicated, stimulated with 100 ng/ml of EGF for 1, 5, or 10 min and stained with HA antibodies to identify co-transfected cells (not shown). Arrows indicate plasma membrane localization of GFP-PH/ARNO. Images were captured using confocal microscopy

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Notes

1. All solutions should be prepared in water with a resistivity of 18.2 MΩ-cm. All buffers and reagents used should be at room temperature unless otherwise stated.
2. While standard silica 60 TLC plates can be used, channeled concentration zone plates lanes allow quicker sample application and result in sharper bands with better resolution. Following oxalate treatment, TLC plates must be dried vertically and placed into the TLC tank in the same orientation in which they were dried. Concentration zone plates should be dried with the zone at the bottom. If concentration zone plates are not used, the bottom of the plate should be marked by ruling a line 1 cm from the bottom with a soft pencil, which serves as the origin on which the samples are loaded. If channeled plates are not used, the samples should be loaded 1 cm apart on the TLC plate.
3. The walls of the TLC tank need to be lined with gel blotting paper to saturate the chamber atmosphere with solvent vapor. Gel blotting paper can be stuck to the sides of the tank with tape, although most tapes are not able to withstand the solvents used in the tank. Alternatively, the paper can be placed against the sides of the tank and held in place with the TLC plate. Do not allow the gel blotting paper to come in contact with the silica of the TLC plate. The TLC tank should be poured at least the day before use to allow it to equilibrate properly. The tank can be used for two weeks providing the lid is tightly sealed with vacuum grease to prevent evaporation of the solvent.
4. Extending the dextran/chlroquine/DNA incubation to 3 h may improve the transfection efficiency. However, it is important to monitor the cells during this time as the longer incubation can promote cell death.
5. If protein degradation is an issue, the immunoprecipitations can be incubated for 2 h instead of overnight. Also, the samples can be precleared with protein A Sepharose prior to immunoprecipitation to decrease nonspecific binding if necessary.
6. Be careful when drying lipids under nitrogen (especially when labeled with ^{32}P) to prevent the lipids from being blown out of the tube by the gas stream. Perform this procedure in a fume hood to minimize exposure to aerosols. Also, be careful not to overdry the lipids, as they will become impossible to resuspend—the lipids should be dried until the liquid just disappears.
7. When resuspending lipids, scrape the sides of the microcentrifuge tube with a pipette tip to remove any bound lipid.
8. Suitable shielding and personal protection must be used when working with ^{32}P. Dispose of waste according to local guidelines. Ensure that the microcentrifuge tubes used have tight-fitting lids that do not leak when vortexed.
9. If necessary, the 5-phosphatase assays can be stopped with 100 µl of 1 M HCl and then stored at 4°C overnight (with appropriate shielding). The extraction and separation can then be continued the next day.
10. Use a hairdryer to warm the plate, but do not put it too close to the plate as too much airflow over the plate can make loading the samples very difficult and blow them across the plate.
11. The timeline for the *in vitro* PtdIns(3,4,5)P_3 5-phosphatase assays is as follows. Day 1: Transfect COS-1 cells. Day 2: Split transfected cells 1:2. Day 3: Prepare cell lysates, immunoprecipitate protein of interest, perform PtdIns(3,4,5)P_3 labeling reaction, prepare TLC tank, oxalate-treat plates. Day 4: Extract PtdIns(3,4,5)P_3 substrate, perform PtdIns(3,4,5)P_3 5-phosphatase assays.
12. Depending on the antibody, this incubation can be performed at room temperature for 1 h.

13. Primary antibodies can be reused several times. Following incubation with the membrane, transfer the antibody to a 10-ml tube and add sodium azide to a final concentration of 0.1% (this is only necessary following the first use). Sodium azide is toxic and should be handled in a fume hood. Store the diluted antibody at 4°C. Additional antibody can be added after three uses to boost the signal strength.
14. The washes can be extended to 10 minutes each if necessary to remove background signals. Tween 20 can also be added to a final concentration of 0.1% to decrease the background.
15. The cells must not be more than ~50% confluent when harvested; otherwise, contact inhibition will prevent the cells from being properly stimulated by the growth factor treatment. The cell density may need to be adjusted to ensure that the cells do not become overconfluent.
16. Various concentrations of EGF can be used to stimulate the cells. In addition, other methods of cell stimulation can be used.
17. 4% formaldehyde is also a suitable fixative as an alternative to paraformaldehyde. To make 4% formaldehyde, mix 5 ml of a 40% formaldehyde solution (BDH), 5 ml of 10x PBS, and 40 ml of water.
18. Washing steps to remove growth media, fixative, and permeabilizing solution do not require an incubation period. Simply apply the PBS and aspirate.
19. The volume of antibody required for immunostaining can be reduced by using a staining/Petri dish. To prepare the dish, two pieces of gel blotting paper are placed in the bottom of an agar plate and dampened with water. The blotting paper is then covered with a piece of Parafilm (or equivalent) and the coverslips placed on top of the Parafilm, cell side facing up. This setup can be reused, but the gel blotting paper needs to be dampened before each use.
20. Washing steps to remove unbound antibody require a 30-second incubation period of the coverslips with the PBS. To further minimize nonspecific staining with either primary or secondary antibodies, the incubation period during the PBS washes can be extended to anywhere from 5–30 minutes per wash.
21. Prior to mounting the coverslip on the glass slide, gently dab the edge of the coverslip onto a Kimwipe to remove excess PBS. Avoid trapping air bubbles under the coverslips: Slowly apply the coverslip to the mounting media by gently guiding it onto the media using fine forceps or a needle.
22. The selection of appropriate cells to an image is critical. A wide variation in the level of expression of the fusion proteins will be apparent. Avoid imaging cells with high levels of protein expression, which can cause aberrant cell behavior and reduced responsiveness to hormonal or GF stimulation [7]. It is also preferable to choose separated cells to minimize the influence of cell-cell contact on cellular responses to hormonal or GF stimulation. Laser attenuation should be set at levels such that the signal is not saturating.
23. The ImageJ program can be downloaded from rsb.info.nih.gov/ij/.
24. The level of pixel fluorescence intensity should be fairly uniform throughout the cytosol. Higher cytosolic fluorescence observed near the center of the cell compared to the periphery may indicate that the cell has not completely spread due to high expression levels of the GFP-fusion protein and interference with normal cell adhesion processes.

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