Published in final edited form as:

Anal Bioanal Chem. 2017 November ; 409(29): 6781–6789. doi:10.1007/s00216-017-0633-y.

Required Hydrophobicity of Fluorescent Reporters for Phosphatidylinositol Family of Lipid Enzymes

Jarod Waybright¹, Weigang Huang¹, Angela Proctor², Xiaoyang Wang¹, Nancy L. Allbritton^{2,3}, and Qisheng Zhang¹

¹Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, 301 Pharmacy Lane, Chapel Hill, North Carolina 27599, USA

²Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA

³Department of Biomedical Engineering, University of North Carolina at Chapel Hill, Campus Box 3290, Chapel Hill, North Carolina 27599 and North Carolina State University, Raleigh, North Carolina 27695, USA

Abstract

The phosphatidylinositol (PtdIns) family of lipids plays important roles in cell differentiation, proliferation and migration. Abnormal expression, mutation or regulation of their metabolic enzymes has been associated with various human diseases such as cancer, diabetes and bipolar disorder. Recently, fluorescent derivatives have increasingly been used as chemical probes to monitor either lipid localization or enzymatic activity. However, the requirements of a good probe have not been well defined, particularly modifications on the diacylglycerol side chain partly due to challenges in generating PtdIns lipids. We have synthesized a series of fluorescent PtdIns(4,5)P₂ (PIP₂) and PtdIns (PI) derivatives with various length of side chains and tested their capacity as substrates for PI3K α and PI4KII α , respectively. Both capillary electrophoresis and thin layer chromatography were used to analyze enzymatic reactions. For both enzymes, the fluorescent probe with a longer side chain functions as a better substrate than that with a shorter chain, and works well in the presence of the endogenous lipid, highlighting the importance of hydrophobicity of side chains in fluorescent phosphoinositide reporters. This comparison is consistent with their interactions with lipid vesicles, suggesting that the binding of a fluorescent lipid with liposome serves as a standard for assessing its utility as a chemical probe for the corresponding endogenous lipid. These findings are likely applicable to other lipid enzymes where the catalysis takes place at the lipid-water interface.

Keywords

Fluorescent reporter; chemical cytometry; lipid enzyme; phosphatidylinositol

Correspondence to: Qisheng Zhang.

Compliance with Ethical Standards: The authors declare no conflicts of interest.

Introduction

The phosphatidylinositol (PtdIns) family of lipids constitutes approximately 1% of the phospholipids in the cell membranes[1]. The inositol head unit of PtdIns possesses five hydroxyl groups, with those at the C3, C4, and C5 positions selectively phosphorylated by multiple kinases to generate seven endogenous phosphatidylinositides (PIs). As one of the most versatile family of signaling molecules, PIs play important roles in cell signaling, cell motility, vesicle transport and development[2-6]. Abnormal levels of PIs have been associated with development of a wide range of diseases including cancer[7], myotubular myopathy[8, 9], and neurodegenerative disease[10].

The enzymes involved in PI metabolism are also important regulators of disease development. For example, constitutively active mutations in phosphatidylinositol 3-kinase (PI3K) are found in 15% of glioblastoma[11] and 30% of prostate cancer patients[12]. Mutation or homozygous deletion of phosphatase and tensin homolog (PTEN) is also found in 36% of glioblastoma and 42% of metastatic prostate cancer[11, 13]. Other PI metabolic enzymes have also been established as promising therapeutic targets including phosphatidylinositol 3,4,5-triphosphate 5-phosphatase 2 (SHIP2) for diabetes[14], inositol polyphosphate-4-phosphatase (INPP4) for metastatic prostate cancer[15], and phospholipase C (PLC) for cancer[16-18] and neuropathic pain[19]. Consequently, there is a great need to monitor lipid localization, metabolism and corresponding metabolic enzyme activity.

Several methods[20, 21] have been used to monitor PI metabolism, with radioactivity-based thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC) as the most commonly used techniques[22, 23]. Mass spectrometry (MS) in combination with an upstream HPLC separation has also been reported to analyze PIs in a complex mixture[24-26]. In addition, phosphoinositide-binding pleckstrin homology (PH) domains that are fused with green fluorescent protein (GFP) or environmentally sensitive fluorophores have been developed as biosensors for PtdIns(4,5)P₂ (PIP₂) and PtdIns(3,4,5)P₃ (PIP₃)[6, 27-31]. The relative changes in cellular localizations and intensities of these biosensors reflect the dynamic metabolism of the corresponding PIs, and have been used to monitor activity of PI metabolic enzymes. Recently, fluorescent PI derivatives have been coupled with capillary electrophoresis separation technique to develop a unique assay for PI signaling[32-34]. In this assay, the substrate and product forms of the fluorescent PI derivatives are separated and quantified to measure cellular enzyme activity. Because all PIs, including isomers, can be efficiently separated from each other in capillary electrophoresis (CE)[35], this technique can be used to measure activity of multiple PI metabolic enzymes simultaneously to profile PI metabolic flux. Furthermore, routine detection limits of laser-induced fluorescence detection are 10⁻²¹ moles corresponding to an intracellular concentration of 10 nM[36], making this method suitable for single cell analysis to assess cell-to-cell variation and heterogeneity in clinical samples.

Despite the unique and promising features of using fluorescent PI derivatives, the production of such reporters is a challenge. PIs have seven stereogenic centers and the hydroxyl groups around the inositol head unit have similar reactivity. In addition, PIs contain both the highly hydrophilic inositol phosphate head group and hydrophobic aliphatic side chains, making

them difficult to purify from the reaction mixtures. Consequently, PI synthesis is labor intensive and the research community highly relies on the very few commercial sources for fluorescent PI derivatives, which vary in the inositol head group but lack diversity in the hydrophobic side chain. For example, only one fluorescent PI is commercially available, which has a short alkyl chain (C₇H₁₅) in the diacylglycerol (DAG) unit. Fluorescent PIP₂ analogs are most readily available among different PIs, yet only two variations are in the side chain (C₆H₁₃ and C₁₆H₃₃). This is understandable considering short chain PIs are easier to synthesize, purify and analyze in assays. However, the effects of the acyl chain in these fluorescent PIs on their capacity as enzyme substrates are largely ignored.

PI3K and other PI modifying enzymes catalyze reactions at the lipid/water interface in a process known as interfacial catalysis[37]. Consequently, the hydrophobic side chains in fluorescent PI derivatives will likely play important roles in their capacity as enzyme substrates. The roles of the side chains in non-fluorescent substrates have been previously demonstrated. For example, a study of *B. cereus* PLC activity on phosphatidylcholine substrates revealed changes in the kinetic constants for the various substrates with a preference for long acyl chains[38]. Likewise, the acyl acid composition of PIP₂ has been shown to be important in its inhibition of actin-capping protein[39].

Towards the goal of using fluorescent PtdIns and PI derivatives to profile PI metabolism and measure activity of PI metabolic enzymes, we synthesized fluorescent PIP₂ and PI derivatives that contain varied degrees of hydrophobicity and investigated their ability to serve as reporters for PI3K α and PI4K α , respectively. This work represents the first study to investigate the effects of acyl side chains in fluorescent phosphoinositides on their applications in chemical cytometry. An ideal fluorescent reporter would effectively function as enzyme substrate in the presence of the endogenous lipids while being easy to handle during separation by CE.

Materials and Methods

Purified PI3K α enzyme was purchased from Invitrogen-Life Technologies. Purified PI4K α was purchased from Creative Biomart. Dynamic light scattering data were recorded on a Wyatt DynaPro dynamic light scattering plate reader. Endogenous PtdIns(4,5)P₂ (brain, porcine) and phosphatidylinositol (PI) 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol) were purchased from Avanti Polar Lipids. ATP and TLC plates with silica gel 60 were purchased from Sigma. All solvents were purchased from Fischer Scientific.

Soluble PI3K assay conditions

Fluorescent PIP₂ derivatives (10 μ M) were added to assay buffer composed of MOPS (50 mM, pH 6.7), NaCl (100 mM), MgCl₂ (10 mM), sodium cholate (0.5 mM), DTT (1 mM), followed by the addition of ATP (2 mM). The reaction was initiated by the addition of purified PI3K α enzyme to a final concentration of 1.2 ng/ μ L for PIP₂-C15 or 2.4 ng/ μ L for the other 3 probes and incubated at 37 °C. At the indicated time point, an aliquot of the reaction was removed and diluted in CHCl₃/MeOH (1:1) for analysis. All assays were performed in duplicate for three times.

Vesicle PI3K assay conditions

Liposomes were prepared to contain 10 μM total PIP_2 composed of $\text{PIP}_2\text{-C15}$ and endogenous substrate with 10 μM phosphatidylserine (PS) as a carrier lipid[40, 41] in final assay conditions. Lipid stocks in CHCl_3 or water were added to a 1:1 $\text{MeOH}/\text{H}_2\text{O}$ mixture followed by solvent removal by speed-vac and drying under vacuum for at least 1 h. The lipids were then re-suspended in 5 $\times$ MOPS (250 mM, pH 6.7) and 5 $\times$ NaCl (500 mM) followed by sonication in a bath for 5 min to form lipid vesicles. The vesicles were then used to prepare a mixture containing a final $\text{PtdIns}(4,5)\text{P}_2$ concentration of 10 μM in MOPS (50 mM, pH 6.7), NaCl (100 mM), DTT (1 mM), MgCl_2 (10 mM) and ATP (2 mM). The reaction was initiated by adding purified PI3K enzyme (0.6 ng/ μL) and then incubated at 37 $^\circ\text{C}$. At the indicated time point, an aliquot of the reaction mixture was removed and diluted in 1:1 $\text{CHCl}_3/\text{MeOH}$ for TLC analysis. In the case of the 10% $\text{PIP}_2\text{-C15}$ liposome, the reaction was first stopped by the addition to methanol, followed by concentration and addition of $\text{CHCl}_3/\text{MeOH}$ to achieve the desired concentration of 0.2 μM FL- $\text{PtdIns}(4,5)\text{P}_2$ substrate as direct dilution results in phase separation. All experiments were performed using a single batch of liposome in duplicate for three times.

TLC analysis of PI3K and PI4K reactions

TLC plates (Merck, Silica Gel-60) were pre-treated with a solution of 1.2% potassium oxalate and 1.2 mM EGTA in MeOH/water ($v:v = 2:3$) and heated at 110 $^\circ\text{C}$ for 20 min before use. Reaction mixtures at indicated time points were diluted in $\text{CHCl}_3/\text{MeOH}$ ($v:v = 1:1$) and spotted on a TLC plate directly. The TLC plate was then developed in $\text{CHCl}_3\text{:acetone:MeOH:AcOH:water}$ ($v:v:v:v:v = 80:30:26:24:14$) and scanned on a Typhoon 9400 Variable Mode Imager ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 488\text{ nm}/520\text{ nm}$). The fluorescence intensity of various spots on the TLC plate was quantified with ImageQuant software (V.5.0). The substrate conversion or % $\text{PtdIns}(3,4,5)\text{P}_3$ was calculated as product fluorescence intensity divided by total fluorescence intensity.

CE analysis of lipid analytes

Capillary electrophoresis coupled with laser induced fluorescence detection (CE-LIF, 488 nm excitation) was performed on a custom-built system mounted to the stage of an inverted microscope, described previously in detail[42]. Fused silica capillaries were 38 cm long with a 20.5 cm effective length [30 μm inner diameter and 360 μm outer diameter (Polymicro Technologies; Phoenix, AZ)] and were conditioned prior to use by rinsing for 1 h in DI H_2O , 12 h in 0.1 M NaOH, 1 h in DI H_2O , 6 h in 0.1 M HCl, and 12 h in DI H_2O . Prior to each run, capillaries were rinsed with 1 M NaOH and DI H_2O for 5 min each and with electrophoretic buffer for 10 min by application of pressure to the capillary outlet. Buffer at the capillary inlet and outlet was completely refreshed prior to each electrophoretic run. The composition of the electrophoretic buffer was 80 mM NaH_2PO_4 , pH 6.8 containing 15% 2-propanol and a field strength of 210 V cm^{-1} was used for all separations. Internal standards and samples were hydrodynamically loaded by raising the inlet 3 cm relative to the outlet and holding the capillary inlet in the sample for 10 sec. The inlet was then lowered to the height of the outlet and electrophoresis was initiated by application of a negative voltage to

the outlet while grounding the inlet. Electropherograms were plotted and analyzed utilizing OriginLab 9.0 (OriginLab Corporation; Northampton, MA).

Fluorescent lipids/liposome interaction

The liposome mixture was prepared from stock solutions of lipids in CHCl_3 for a final composition of 45% PC, 25% PE, 15% cholesterol, 10% PI, and 5% PS. The solvent was blown off under a stream of N_2 followed by drying under vacuum for at least 1 h. The lipid film was then suspended in buffer composed of MOPS (50 mM, pH 6.7), NaCl (100 mM), DTT (1 mM), and MgCl_2 (10 mM) to a concentration of 2 mM. Liposomes were extruded through a 0.03 μm pore size polycarbonate filter membrane for at least 11 times back and forth. Fluorescent PIP_2 or PI (10 μM) and liposomes (1 mM) were incubated in buffer at room temperature for 5 min in a total volume of 150 μL . The suspension was adjusted to 30% sucrose by the addition of 100 μL of 75% w/v sucrose in buffer followed by mixing. Buffer (200 μL) containing 25% w/v sucrose was then overlaid on the high-sucrose suspension followed by 50 μL of buffer containing no sucrose. The sample was centrifuged at 55,000 r.p.m in a Beckman swing rotor (TLS 55) for 1 h at 4 $^\circ\text{C}$. The bottom 350 μL and top 150 μL were manually collected using a syringe and analyzed for fluorescent PI or PIP_2 content by both TLC and CE. A fluorescence image of the tubes was also taken prior and following centrifugation using a UV light source and a CCD camera in front of the samples.

PI4K reaction

Fluorescent PI derivatives were added to assay buffer composed of MOPS (50 mM, pH 6.7), NaCl (100 mM), MgCl_2 (10 mM), sodium cholate (0.5 mM), DTT (1 mM), followed by the addition of ATP (2 mM). The reaction was initiated by the addition of purified PI4KII α enzyme to a final concentration of 1.0 ng/ μL and incubated at 37 $^\circ\text{C}$. At the indicated time point, an aliquot of the reaction was removed and diluted to 0.2 μM in $\text{CHCl}_3/\text{MeOH}$ (1:1) for analysis. All assays were performed in duplicate for 3 times.

Results and discussion

Design and synthesis of fluorescent PIP_2 derivatives

To investigate the effects of the acyl side chain on the capacity of fluorescent PI derivatives as enzyme substrates, we have developed four PIP_2 analogs $\text{PIP}_2\text{-C}_6$, $\text{PIP}_2\text{-C}_9$, $\text{PIP}_2\text{-C}_{12}$ and $\text{PIP}_2\text{-C}_{15}$ (Fig. 1A) with different alkyl groups C_6H_{13} , C_9H_{19} , $\text{C}_{12}\text{H}_{25}$, and $\text{C}_{15}\text{H}_{31}$, respectively, at the *sn*-2 position. Fluorescein has been chosen as the fluorophore because of our prior success in separating fluorescein-tagged PIs by both thin layer chromatography (TLC) and capillary electrophoresis (CE).

The synthesis of the fluorescent PIP_2 probes is shown in Scheme 1. Carboxylic acids 1 with various length of alkyl chain coupled with the alcohol 2. Subsequently, the *p*-methoxybenzyl (PMB) group was removed to form 3. The primary alcohol in 3 was then converted to a phosphoramidite, which was coupled to the protected inositol 4 previously synthesized according to literature protocols[43, 44]. After oxidation with *t*-butyl hydroperoxide, compound 5 was formed and the carboxybenzyl (Cbz) and benzyl (Bn) groups were removed by hydrogenolysis. Deprotection of the methoxymethyl (MOM) groups then

provided the primary amine 6, which reacted with the NHS ester of fluorescein 7 to yield fluorescent PIP₂ derivatives.

Biophysical and kinetic analysis of fluorescent PIP₂ analogs

The critical micelle concentration (CMC) of the fluorescent PIP₂ derivatives was calculated by measuring light scattering of various concentrations of the reporters in H₂O at 25 °C (see Electronic Supplementary Material (ESM) Fig. S1). As expected, the PIP₂ derivative with a longer acyl chain, and thereby higher hydrophobicity, has lower CMC value than that with a shorter acyl chain, with the CMC values for PIP₂-C6, PIP₂-C9, PIP₂-C12 and PIP₂-C15 measured as 196 ± 1 , 134 ± 4 , 26 ± 6 , and 13 ± 1 mM, respectively. In comparison, the endogenous PtdIns(4,5)P₂ has a reported CMC of 10 μ M[32].

We chose to use soluble assay conditions with sodium cholate to enhance solubility of lipids to measure kinetic parameters of different fluorescent PIP₂ probes as substrates of PI3K α considering their wide range of CMC values. Reaction times and enzyme concentrations were varied to ensure all measurements were taken under initial velocity conditions (data not shown). The assay was initiated by the addition of purified PI3K enzyme to assay buffer containing individual fluorescent PIP₂ derivative and ATP and stopped by the addition of a mixture of CHCl₃/MeOH (1:1). The reaction mixture was analyzed by TLC or CE for production of PtdIns(3,4,5)P₃ as previously described [32]. The concentration of substrate was varied to give a set of initial velocity values and these data were fit to the Michaelis-Menten equation to calculate the apparent K_m and V_{max} values of the reporters (Figs. 1A and 1B).

Under these conditions, no detectable amount of the corresponding PtdIns(3,4,5)P₃ was formed for the PIP₂-C6 probe, suggesting that PIP₂-C6 was not an effective substrate for PI3K α . The plots of PI3K activity versus probe concentration for PIP₂-C9, PIP₂-C12 and PIP₂-C15 are shown in Fig. 1B. The apparent K_m values for the three probes did not show significant changes (Fig. 1A), suggesting that the acyl side chains in the probes played a smaller role in their interactions with PI3K α compared to the inositol phosphate head group. In contrast, the apparent V_{max} for PIP₂-C12 (0.182 pmol/ng/min) was approximately 2.5 fold that for PIP₂-C9 (0.077 pmol/ng/min) and 0.5 fold that for PIP₂-C15 (0.332 pmol/ng/min). These results suggest the importance of hydrophobicity in the fluorescent probes as effective PI3K α substrate, likely through modulating turnover efficiency. Importantly, PIP₂-C15 was still efficiently separated from its corresponding PI3K α product, either by TLC (ESM Fig. S2) or CE (Fig. 1D).

To further compare the relative capacity of different fluorescent PIP₂ probes as PI3K α substrates, a 1:1 mixture of PIP₂-C9 and PIP₂-C15 was used in the enzymatic reaction. As controls, the PI3K reactions with equimolar PIP₂-C9 or PIP₂-C15 alone under the same assay conditions were also carried out. PIP₂-C9, PIP₂-C15 and their corresponding PtdIns(3,4,5)P₃ products were efficiently separated by CE (Fig. 1D). As shown in Fig. 1C, the conversion of PIP₂-C9 in the presence of PIP₂-C15 was decreased by approximately 5-fold compared to when it was the only PI3K substrate. In contrast, the conversion of PIP₂-C15 remained consistent with or without the presence of PIP₂-C9. These results again highlighted the importance of the acyl side chain in a fluorescent PIP₂ probe, especially

when competing substrates were present- a scenario when the probe was used as a reporter in live cells.

In vitro membrane association of fluorescent PIP₂ derivatives

The acyl side chain in a fluorescent PIP₂ probe likely contributes to its cellular localization. However, the non-invasive method[45] of using histone as a carrier protein to deliver fluorescent PIP₂ derivatives into cells resulted in very low efficiency in our hands. Consequently, we utilized a membrane association assay[46] that was developed for protein-lipid interactions to investigate likely membrane localization of fluorescent PIP₂ probes. In this assay, liposomes and the associated cargo are recovered by centrifugation atop a dense sucrose gradient. Liposomes were formulated to mimic mammalian membranes with a final composition of phosphatidylcholine (45%), phosphatidylethanolamine (25%), phosphatidylserine (5%), phosphatidylinositol (10%), and cholesterol (15%). The average radius of liposome prepared as described is around 33 nm with narrow distribution (ESM Fig. S3). Fig. 2A shows representative images of the assay mixtures after centrifugation. The top lipid layer and bottom sucrose layers were then collected and each layer quantified by CE for the presence of fluorescent PIP₂. Approximately 91% of the PIP₂-C15 probe was incorporated into the liposome layer (Fig. 2A). The percentage of incorporation decreased dramatically to 35% for PIP₂-C12. Only 12% and 9% of the probe was incorporated into liposome for PIP₂-C9 and PIP₂-C6, respectively (Fig. 2A). These results demonstrated that only the PIP₂-C15 probe with a long acyl side chain could be efficiently incorporated into liposome that mimics cellular membranes where the endogenous PIs reside.

To further assess whether PIP₂-C15 functions as an effective reporter in the presence of endogenous substrate, the PI3K-catalyzed reaction was run on lipid vesicles, an environment that closely mimic cellular presentation of lipid substrates, in the presence or absence of endogenous PIP₂. Liposomes composed of a carrier lipid, phosphatidylserine (PS), PIP₂-C15 and endogenous PIP₂ at different ratios, were formulated for enzymatic reactions[41]. The total concentration of PIP₂-C15 and endogenous PIP₂ was 10 μ M, which is close to that of the cellular environment. The conversion of PIP₂-C15 to its corresponding PtdIns(3,4,5)P₃ product were then measured (Fig. 2B). The rate of conversion decreased by approximately 27% when the ratio of PIP₂-C15 to endogenous PIP₂ was 1:1, and approximately 50% when the ratio was 1:9, compared to 100% PIP₂-C15. These results suggested that PIP₂-C15 was a similar, albeit slightly less effective PI3K substrate than the endogenous PIP₂, and can function as a reporter of PIP₂.

Required hydrophobicity for PI4K substrate

Like PI3K, PI4K catalyzes reactions at the lipid/water interface. Consequently, what was observed for PI3K likely applies to PI4K. There is only one commercial fluorescent PI derivative, PI-C7 (Fig. 3A) with a short acyl side chain. We thus designed PI-C15 (Fig. 3A) with a longer acyl side chain to assess its capacity as PI4K substrate. The BODIPY-tagged PI-C15 was synthesized in a similar manner as PIP₂-C15 (Scheme 2). Briefly, DAG phosphoramidite 9 containing palmitic acid at the *sn*-2 position was synthesized as described in Scheme 1 and was coupled with protected inositol derivative 8 followed by oxidation with

t-BuOOH to generate compound 10. Removal of all the protective groups resulted in 11, which reacted with BODIPY-NHS to provide fluorescent PI-C15.

Next, both PI-C15 and PI-C7 were tested as substrates of PI4KII α under soluble assay conditions (Fig. 3B). Consistent with what we observed with fluorescent PIP₂ derivatives, the PtdIns(4)P product of the fluorescent PI with a longer acyl side chain PI-C15 was efficiently generated. In contrast, no detectable amount of product was formed for PI-C7 containing a shorter acyl side chain (Fig. 3B). Likewise, over 99% of PI-C15 (not detectable in the aqueous layer) but only approximately 15% of PI-C7 participated into the lipid layer in the *in vitro* membrane association assay (Fig. 3C).

Conclusions

In summary, we have synthesized a series of fluorescent PIP₂ derivatives with varied levels of hydrophobicity and investigated their capacity as PI3K substrates. The probe with a longer acyl side chain functions as a better substrate for PI3K and has a higher affinity with liposome compared to that with a shorter acyl side chain. Strikingly, when the side chain is sufficiently short and thereby has low hydrophobicity, the fluorescent PIP₂ is no longer an effective PI3K substrate to generate detectable amount of enzymatic product, highlighting the required hydrophobicity of fluorescent PIP₂ derivatives. Likewise, the fluorescent PI derivative with a long acyl side chain functions as an effective PI4K substrate, while that with a short acyl side chain does not generate detectable amount of enzymatic product. These results suggest that the required hydrophobicity of a fluorescent probe, which can be assessed through its interaction with liposomes, is likely essential for phosphatidylinositol metabolic enzymes. Although not entirely surprising, these data clearly demonstrate limitations of existing fluorescent probes and potential misleading results when they are used to mimic endogenous substrates of lipid enzymes. A systematic study on effects of side chains on fluorescent phosphatidylinositol probes has to be performed before using them in biological assays.

PIP₂-C15 has shown similar properties as the endogenous PIP₂ in regard to CMC, kinetic parameters and tendency for membrane association. Unlike the endogenous PIP₂, PIP₂-C15 is easy to handle both in chemical synthesis and CE separation. Similarly, PI-C15 has also been efficiently synthesized and separated from its enzymatic products. These results demonstrate that PIP₂-C15 and PI-C15 may strike the right balance between required hydrophobicity and practical handling during chemical synthesis and separation. Consequently, they are likely effective fluorescent probes that can be used to profile metabolism of PIPs when coupled with CE separation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the National Institutes of Health (GM086558 to Q.Z.; CA177993 to N.A.), the American Foundation for Pharmaceutical Education (fellowship to J.W.), and the Eshelman Institute for Innovation (award to W.H.) for funding this work.

References

1. Balla T. Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol Rev.* 2013; 93:1019–137. [PubMed: 23899561]
2. Skwarek LC, Boulianne GL. Great expectations for PIP: phosphoinositides as regulators of signaling during development and disease. *Dev Cell.* 2009; 16:12–20. [PubMed: 19154715]
3. Vicinanza M, D'Angelo G, Di Campli A, De Matteis MA. Phosphoinositides as regulators of membrane trafficking in health and disease. *Cell Mol Life Sci.* 2008; 65:2833–41. [PubMed: 18726176]
4. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. *Nature.* 2006; 443:651–7. [PubMed: 17035995]
5. Vicinanza M, D'Angelo G, Di Campli A, De Matteis MA. Function and dysfunction of the PI system in membrane trafficking. *EMBO J.* 2008; 27:2457–70. [PubMed: 18784754]
6. Downes CP, Gray A, Lucocq JM. Probing phosphoinositide functions in signaling and membrane trafficking. *Trends Cell Biol.* 2005; 15:259–68. [PubMed: 15866030]
7. Weber G. Down-regulation of increased signal transduction capacity in human cancer cells. *Adv Enzyme Regul.* 2005; 45:37–51. [PubMed: 16054198]
8. Tronchere H, Laporte J, Pendaries C, Chaussade C, Liaubet L, Pirola L, et al. Production of phosphatidylinositol 5-phosphate by the phosphoinositide 3-phosphatase myotubularin in mammalian cells. *J Biol Chem.* 2004; 279:7304–12. [PubMed: 14660569]
9. Takasuga S, Sasaki T. Phosphatidylinositol-3,5-bisphosphate: metabolism and physiological functions. *J Biochem.* 2013; 154:211–8. [PubMed: 23857703]
10. Ferguson CJ, Lenk GM, Meisler MH. PtdIns(3,5)P₂ and autophagy in mouse models of neurodegeneration. *Autophagy.* 2009; 6:170–1.
11. Network CGAR. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* 2008; 455:1061–8. [PubMed: 18772890]
12. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. *Cancer Cell.* 2010; 18:11–22. [PubMed: 20579941]
13. Suwa A, Kurama T, Shimokawa T. SHIP2 and its involvement in various diseases. *Expert Opin Ther Targets.* 2010; 14:727–37. [PubMed: 20536411]
14. Suwa A, Kurama T, Shimokawa T. SHIP2 and its involvement in various diseases. *Expert Opin Ther Targets.* 14:727–37.
15. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. *Cancer Cell.* 18:11–22.
16. Shepard CR, Kassiss J, Whaley DL, Kim HG, Wells A. PLC- γ contributes to metastasis of insitu-occurring mammary and prostate tumors. *Oncogene.* 2007; 26:3020–6. [PubMed: 17130835]
17. Bertagnolo V, Benedusi M, Brugnoli F, Lanuti P, Marchisio M, Querzoli P, et al. Phospholipase C-b2 promotes mitosis and migration of human breast cancer-derived cells. *Carcinogenesis.* 2007; 28:1638–45. [PubMed: 17429106]
18. Sala G, Dituri F, Raimondi C, Previdi S, Maffucci T, Mazzeo M, et al. Phospholipase C- γ 1 is required for metastasis development and progression. *Cancer Res.* 2008; 68:10187–96. [PubMed: 19074886]
19. Shi TJ, Liu SX, Hammarberg H, Watanabe M, Xu ZQ, Hokfelt T. Phospholipase C-b3 in mouse and human dorsal root ganglia and spinal cord is a possible target for treatment of neuropathic pain. *Proc Natl Acad Sci U S A.* 2008; 105:20004–8. [PubMed: 19066214]
20. Rusten TE, Stenmark H. Analyzing phosphoinositides and their interacting proteins. *Nat Methods.* 2006; 3:251–8. [PubMed: 16554828]
21. Idevall-Hagren O, De Camilli P. Detection and manipulation of phosphoinositides. *Biochim Biophys Acta.* 2015; 1851:736–45. [PubMed: 25514766]
22. Hokin LE, Hokin MR. Phosphoinositides and Protein Secretion in Pancreas Slices. *Journal of Biological Chemistry.* 1958; 233:805–10. [PubMed: 13587496]

23. Whitman M, Downes CP, Keeler M, Keller T, Cantley L. Type-I Phosphatidylinositol Kinase Makes a Novel Inositol Phospholipid, Phosphatidylinositol-3-Phosphate. *Nature*. 1988; 332:644–6. [PubMed: 2833705]
24. Wenk MR, Lucast L, Di Paolo G, Romanelli AJ, Suchy SF, Nussbaum RL, et al. Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat Biotechnol*. 2003; 21:813–7. [PubMed: 12808461]
25. Ogiso H, Taguchi R. Reversed-phase LC/MS method for polyphosphoinositide analyses: changes in molecular species levels during epidermal growth factor activation in A431 cells. *Anal Chem*. 2008; 80:9226–32. [PubMed: 19551943]
26. Pettitt TR, Dove SK, Lubben A, Calaminus SD, Wakelam MJ. Analysis of intact phosphoinositides in biological samples. *J Lipid Res*. 2006; 47:1588–96. [PubMed: 16632799]
27. Balla T. Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. *J Cell Sci*. 2005; 118:2093–104. [PubMed: 15890985]
28. Hardie RC, Liu CH, Randall AS, Sengupta S. In vivo tracking of phosphoinositides in *Drosophila* photoreceptors. *J Cell Sci*. 2015; 128:4328–40. [PubMed: 26483384]
29. Weber S, Wagner M, Hilbi H. Live-cell imaging of phosphoinositide dynamics and membrane architecture during *Legionella* infection. *MBio*. 2014; 5:e00839–13. [PubMed: 24473127]
30. Yoon Y, Lee PJ, Kurilova S, Cho W. In situ quantitative imaging of cellular lipids using molecular sensors. *Nat Chem*. 2011; 3:868–74. [PubMed: 22024883]
31. Liu SL, Sheng R, O'Connor MJ, Cui Y, Yoon Y, Kurilova S, et al. Simultaneous in situ quantification of two cellular lipid pools using orthogonal fluorescent sensors. *Angew Chem Int Ed Engl*. 2014; 53:14387–91. [PubMed: 25345859]
32. Huang W, Jiang D, Wang X, Wang K, Sims CE, Allbritton NL, et al. Kinetic analysis of PI3K reactions with fluorescent PIP2 derivatives. *Anal Bioanal Chem*. 2011; 401:1881–8. [PubMed: 21789487]
33. Wright BD, Simpson C, Stashko M, Kireev D, Hull-Ryde EA, Zylka MJ, et al. Development of a High-Throughput Screening Assay to Identify Inhibitors of the Lipid Kinase PIP5K1C. *J Biomol Screen*. 2015; 20:655–62. [PubMed: 25534829]
34. Jiang DC, Sims CE, Allbritton NL. Single-cell analysis of phosphoinositide 3-kinase and phosphatase and tensin homolog activation. *Faraday Discuss*. 2011; 149:187–200. [PubMed: 21221426]
35. Wang K, Jiang D, Sims CE, Allbritton NL. Separation of fluorescently labeled phosphoinositides and sphingolipids by capillary electrophoresis. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2012; 907:79–86.
36. Meredith GD, Sims CE, Soughayer JS, Allbritton NL. Measurement of kinase activation in single mammalian cells. *Nat Biotechnol*. 2000; 18:309–12. [PubMed: 10700147]
37. Barnett SF, Ledder LM, Stirdivant SM, Ahern J, Conroy RR, Heimbroke DC. Interfacial catalysis by phosphoinositide 3'-hydroxykinase. *Biochemistry*. 1995; 34:14254–62. [PubMed: 7578025]
38. Martin SF, Pitzer GE. Solution conformations of short-chain phosphatidylcholine. Substrates of the phosphatidylcholine-preferring PLC of *Bacillus cereus*. *Biochim Biophys Acta*. 2000; 1464:104–12. [PubMed: 10704924]
39. Kim K, McCully ME, Bhattacharya N, Butler B, Sept D, Cooper JA. Structure/function analysis of the interaction of phosphatidylinositol 4,5-bisphosphate with actin-capping protein: implications for how capping protein binds the actin filament. *J Biol Chem*. 2007; 282:5871–9. [PubMed: 17182619]
40. Klink TA, Kleman-Leyer KM, Kopp A, Westermeyer TA, Lowery RG. Evaluating PI3 kinase isoforms using Transcreeper (TM) ADP assays. *J Biomol Screen*. 2008; 13:476–85. [PubMed: 18566477]
41. Carpenter CL, Duckworth BC, Auger KR, Cohen B, Schaffhausen BS, Cantley LC. Purification and Characterization of Phosphoinositide 3-Kinase from Rat-Liver. *Journal of Biological Chemistry*. 1990; 265:19704–11. [PubMed: 2174051]
42. Proctor A, Herrera-Loeza SG, Wang Q, Lawrence DS, Yeh JJ, Allbritton NL. Measurement of protein kinase B activity in single primary human pancreatic cancer cells. *Anal Chem*. 2014; 86:4573–80. [PubMed: 24716819]

43. Chen J, Profit AA, Prestwich GD. Synthesis of Photoactivatable 1,2-O-Diacyl-sn-glycerol Derivatives of 1-L-Phosphatidyl-D-myo-inositol 4,5-Bisphosphate (PtdInsP(2)) and 3,4,5-Trisphosphate (PtdInsP(3)). *J Org Chem.* 1996; 61:6305–12. [PubMed: 11667472]
44. Kubiak RJ, Bruzik KS. Comprehensive and uniform synthesis of all naturally occurring phosphorylated phosphatidylinositols. *J Org Chem.* 2003; 68:960–8. [PubMed: 12558421]
45. Ozaki S, DeWald DB, Shope JC, Chen J, Prestwich GD. Intracellular delivery of phosphoinositides and inositol phosphates using polyamine carriers. *Proc Natl Acad Sci U S A.* 2000; 97:11286–91. [PubMed: 11005844]
46. Bigay J, Casella JF, Drin G, Mesmin B, Antonny B. ArfGAP1 responds to membrane curvature through the folding of a lipid packing sensor motif. *EMBO J.* 2005; 24:2244–53. [PubMed: 15944734]

Figure 1. Biophysical and kinetic analysis of fluorescent PtdIns(4,5)P₂ analogs

A. Chemical structures of PtdIns(4,5)P₂ derivatives. **B.** Kinetic analysis of PI3K reaction with fluorescent PtdIns(4,5)P₂ derivatives. Soluble PI3K assays were used to calculate the kinetic constant of the various fluorescent derivatives. Kinetic constants for the C6 reporter were not obtained due to the lack of production of the corresponding PtdIns(3,4,5)P₃. **C.** Fluorescent PtdIns(4,5)P₂ Substrate Competition. Soluble PI3K assay conditions were used to analyze the effects of multiple reporters in solution. Production of PtdIns(3,4,5)P₃ was monitored from reactions that contained a single fluorescent PtdIns(4,5)P₂ derivative (20 μ M), short chain C9 or long chain C15, as well as production of each product in reactions that contained both reporter in equimolar (20 μ M) concentrations. **D.** Representative chromatogram of separation of PIP₂-C9, PIP₂-C15, PIP₃-C9 and PIP₃-C15 by capillary electrophoresis (CE).

Figure 2. Competition with endogenous PtdIns(4,5)P₂ in the presence of liposome

A. An *in vitro* flotation assay was utilized to analyze the effects of substrate hydrophobicity on membrane association using liposomes formulated to mimic the lipid content of the plasma membrane. Fluorescence was analyzed before and after centrifugation and the various layers analyzed by capillary electrophoresis (CE). **B.** Liposomes were formulated to contain 10 μ M total PtdIns(4,5)P₂ using fluorescent C15 PtdIns(4,5)P₂ derivative or endogenous PtdIns(4,5)P₂ and 10 μ M of PS as a carrier lipid. Production of PtdIns(3,4,5)P₃ was monitored to analyze the ability of the reporter to be metabolized in the presence of endogenous substrate.

Figure 3. Biophysical and kinetic analysis of fluorescent PtdIns analogs

A. Chemical structures of PtdIns derivatives. **B.** Kinetic analysis of PI4K reaction with fluorescent PtdIns derivatives. **C.** Representative images of fluorescent PtdIns derivatives interacting with liposome. The presentation of the fluorescent lipids in either liposome or aqueous layer was quantified by CE.

Scheme 2.
Synthesis of PtdIns derivative PI-C15.