A direct fluorometric activity assay for lipid kinases and phosphatases

Jiachen Sun, Indira Singaram, Mona Hoseini Soflaee, and Wonhwa Cho

Department of Chemistry, University of Illinois at Chicago, Chicago, IL 60607

ORCID ID: 0000-0003-1410-2481 (W.C.)

Abstract  Lipid kinases and phosphatases play key roles in cell signaling and regulation, and are thus attractive targets for drug development. Currently, no direct in vitro activity assay is available for these important enzymes, which hampers mechanistic studies as well as high-throughput screening of small molecule modulators. Here, we report a highly sensitive and quantitative assay employing a ratiometric fluorescence sensor that directly and specifically monitors the real-time concentration change of a single lipid species. Because of its modular design, the assay system can be applied to a wide variety of lipid kinases and phosphatases, including class I phosphoinositide 3-kinase (PI3K) and phosphatase and tensin homolog (PTEN). When applied to PI3K, the assay provided detailed mechanistic information about the product inhibition and substrate acyl-chain selectivity of PI3K and enabled rapid evaluation of small molecule inhibitors. We also used this assay to quantitatively determine the substrate specificity of PTEN, providing new insight into its physiological function.

In summary, we have developed a fluorescence-based real-time assay for PI3K and PTEN that we anticipate could be adapted to measure the activities of other lipid kinases and phosphatases with high sensitivity and accuracy. —Sun, J., I. Singaram, M. H. Soflaee, and W. Cho. A direct fluorometric activity assay for lipid kinases and phosphatases. J. Lipid Res. 2020, 61: 945–952.

Supplementary key words  lipid phosphatases • high-throughput inhibitor screening • phosphoinositide 3-kinase/ phosphatase and tensin homolog • ratiometric sensor • real-time activity assay • enzyme kinetics

Lipids are ubiquitous regulatory molecules that control a wide variety of biological processes primarily by modulating the localization, structure, function, and activity of effector proteins (1–5). Specificity and fidelity of lipid-mediated cellular signal transduction and regulation critically depend on lipid-modifying enzymes, including lipid kinases, lipid phosphatases, and phospholipases, which interconvert different lipid species and thereby control their cellular levels. For instance, the cellular levels of phosphoinositides, which play pivotal roles in cell signaling and membrane trafficking, are tightly regulated by a panel of kinases and phosphatases in a spatiotemporally specific and stimulus-dependent manner (4, 5). Due to their crucial roles in health and disease, lipid kinases and phosphatases have been extensively studied in terms of structure, physiological function, and cellular regulation (6, 7). However, detailed studies of the enzymatic properties of these proteins, which are necessary for full understanding of their biological functions and development of specific small molecule modulators for them, have been hampered by lack of direct and quantitative continuous enzyme activity assays. Enzymatic activity of lipid kinases and phosphatases is typically measured by a radioactivity-based assay (8, 9), which is suited for neither quantitative and mechanistic enzyme studies nor small molecule modulator screening. To overcome these technical limitations, we developed a fluorescence-based real-time activity assay for lipid kinases and phosphatases. This new assay allows quantitative analysis of enzyme kinetics for these enzymes and rapid screening of their small molecule modulators.

Class I phosphoinositide 3-kinase (PI3K) converts phosphatidylinositol-4,5-bisphosphate (PI4,5P2) in the plasma membrane (PM) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) (10). PIP3 is a potent signaling lipid that activates myriad of cellular processes (10, 11). PIP3 carries out its signaling functions primarily by facilitating PM recruitment

Abbreviations:  eENTH, engineered epsin1 ENTH domain; E, initial enzyme concentration; LUV, large unilamellar vesicle; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI3K, class I phosphoinositide 3-kinase; PI3,4,5P3, phosphatidylinositol-3,4,5-trisphosphate; PI4,5P2, phosphatidylinositol-4,5-bisphosphate; PI4,5P3, phosphatidylinositol-4,5,3-trisphosphate; PM, plasma membrane; POPS, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoserine; PIP3, phosphatidylserine and tensin homolog; pY2, ESDGpYMDMDSPVMDLDMKDVIYKA; SAPI4,5P2, S-[(2S)-carboxyethyl]phosphine; V, initial rate.

1To whom correspondence should be addressed.
e-mail: wcho@uic.edu

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of cellular proteins with PIP₃-binding domains and motifs, most notably the pleckstrin homology (PH) domain (12). Dysregulated PI3K signaling has been linked to various human diseases, including cancer (13, 14) and inflammatory diseases (15), and thus PI3K signaling pathways are major targets for drug development (16). Despite numerous studies on PI3K signaling pathways, the enzymatic properties of PI3K have not been fully characterized largely due to the lack of a direct and continuous assay that allows thorough and systematic enzyme kinetic studies (8). The action of PI3K is counterbalanced by phosphatase and tensin homolog (PTEN), which converts PIP₃ to PI4,5P₂, thereby serving as a tumor suppressor (17, 18). PTEN is frequently deleted in cancer. It has been recently reported that there are multiple isoforms of PTEN with different subcellular localization and function (19, 20) and that PTEN may have promiscuous lipid phosphatase activity (21). As is the case with PI3K, the lack of an available direct activity assay has hampered full characterization of PTEN isoforms (9). Our new fluorescence-based activity assay, which enables direct quantitative analysis of enzyme kinetics for PI3K and PTEN through real-time quantification of their substrate and/or product, provides new mechanistic insight for these enzymes and also serves as a convenient tool for identification and characterization of enzyme modulators.

MATERIALS AND METHODS

Materials

The POPC and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) were from Avanti Polar Lipids. The 1,2-dioleoyl and 1-stearoyl-2-arachidonoyl derivatives of PI4,5P₂ (SAPI4,5P₂) were also from Avanti Polar Lipids. The 1,2-dipalmitoyl derivatives of PI4,5P₂, phosphatidylinositol-3,4-bisphosphate (PI3,4P₂), and PIP₂ were from Cayman Chemical Company. The cDNA for PTEN was purchased from OriGene (SC119965). The pY2 peptide (ES-DGGpYMDMSKDESIDpYVPMLDMKGDIKYA), derived from the mouse platelet-derived growth factor (PDGF), was lyophilized, desalted, and characterized by MALDI-TOF.

Vesicle preparation

Lipid solutions were mixed according to the final lipid composition of vesicles and the solvent was evaporated under a stream of nitrogen gas. Tris buffer (pH 7.4; 20 mM) containing 0.16 M NaCl was added to the lipid film and the mixture was shaken for 0.5 h and then sonicated for 1 min. Large unilamellar vesicles (LUVs) with a 100 nm diameter were then prepared by extrusion using the Avanti Mini-Extruder with a 100 nm polycarbonate filter (Whatman).

Protein expression and purification

Two subunits of PI3Kβ, p110β and p85β, were coexpressed in insect cells as described previously with minor modifications (22). Recombinant baculoviruses for p110β and p85β were amplified in Spodoptera frugiperda (SF9) cells. BTI-Tn5B1-4 (High Five) suspension insect cells (2 × 10⁶ cells/ml) were then co-infected with p110β and p85β baculoviruses (MOI ratio = 1:3) for protein expression. Cells were harvested 72 h after infection. Cell pellets were suspended in a buffer containing 50 mM Tris, 300 mM NaCl, 10 mM imidazole, 1 mM tris(2-carboxyethyl) phosphine (TCEP), and 1 mM phenylmethylsulphonyl fluoride (PMSF) (pH 7.9) and lysed using a hand-held homogenizer (Tissue Grinder Size C; Thomas Scientific). After centrifugation of the homogenate, the supernatant was incubated with the Ni-NTA resin (Merck Millipore) for 2 h. The resin was then pored into a small column and washed with buffer A [20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole (pH 7.9)] and then buffer B [20 mM Tris-HCl, 300 mM NaCl, 40 mM imidazole (pH 7.9)]. The protein was eluted with the elution buffer [20 mM Tris-HCl, 160 mM NaCl, 300 mM imidazole, 1 mM PMSF, 0.5 mM TCEP, 50 mM arginine, 50 mM glutamic acid (pH 7.9)].

The cDNA for PTEN (OriGene) was subcloned into the pET-30 a (+) vector with a His₆-tag, which was then transfected into Escherichia coli BL21 RIL codon plus (Stratagene) cells. After the optical density of cell suspension reached 0.6–0.8, protein expression was induced overnight at room temperature with 0.2 mM (final concentration) isopropyl β-d-thiogalactopyranoside. Cell pellets were lysed by sonication and the supernatant was incubated with the Ni-NTA resin after centrifugation of the homogenate. The protein was purified as described above. The purity of the proteins was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Ratiometric lipid sensor preparation and characterization

The engineered epsin1 ENTH domain (eENTH) (23), tandem PH domains of myoxin X (eMyoxX-PH) (24), and C-terminal PH domain of Tapp2 (eTapp2c-PH), which have been employed as specific sensors for PI4,5P₂ (23), PIP₃ (24), and PI3,4P₂ (25), respectively, were expressed as glutathione-S-transferase-tagged proteins in BL21 RIL codon plus cells and purified as described previously. Protein expression was induced overnight at room temperature with 0.5 mM (final concentration) isopropyl β-d-thiogalactopyranoside when the optical density of the media reached 0.6–0.8. Cells were harvested and cell pellets were suspended in 20 mM Tris buffer (pH 7.4) with 160 mM NaCl, 1 mM TCEP, and 1 mM PMSF and then lysed by sonication. The supernatant was incubated with glutathione resin (GenScript) for 2 h. The resin mixture was then poured into a small column and washed with 20 mM Tris buffer (pH 7.4) containing 0.16 M NaCl. After the resin became clear, the buffer solution was replaced by 5 ml of labeling buffer [50 mM Tris-HCl, 300 mM NaCl, 1 mM TCEP, 50 mM arginine, 50 mM glutamic acid (pH 8.0)]. After adding 100 µl of a cysteine-reactive solvatochromic fluorophore (10 mg/ml in DMSO), acrylodan (Thermo Fisher), or a Nile Red dye, the mixture was gently shaken at room temperature for 2 h. The resin was then washed with 20 mM Tris buffer (pH 7.4) containing 0.16 M NaCl and 5% DMSO until the free dye was completely removed. The resin was then suspended in the digest buffer [20 mM Tris-HCl, 160 mM NaCl, 20 mM CaCl₂, 0.5 mM TCEP, 50 mM arginine, 50 mM glutamic acid (pH 7.4)] containing 40 U of bovine α-thrombin (Haematologic Technologies). After the overnight incubation at 4°C to remove the glutathione-S-transferase tag, the sensor was eluted from the column and any insoluble matter was removed by centrifugation at 4°C. The purity of the sensor was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the protein concentration was determined by the Bradford assay. The activity of the purified sensor was routinely checked by a quick three-point fluorometric measurement. For DAN-eENTH, for example, its fluorescence emission intensity at 470 nm (F₄₇₀) and at 530 nm (F₅₃₀) was measured with three LUVs, e.g., 10, 50, and 100 µM of...
POPC/POPS/PI4,5P₂ (77:20:3). The ratio \(F_{530}/F_{470}\) values from these measurements should lie within the 10% range of the standard calibration curves (see Fig. 1A) for the sensor to be qualified for the enzyme assay.

Spectrofluorometric activity assay

All cuvette-based continuous activity assays were performed with the FluoroLog3 spectrofluorometer at 37°C in a 1 ml quartz cuvette (Hellma Analytics). For the PI3K activity assay, 874 μl of buffer solution [20 mM Tris buffer (pH 7.4) containing 0.16 M NaCl] containing 10 nM PI3K and lipid LUVs with the indicated concentrations, 500 nM lipid sensor and 10 μM pY2 peptide were added to each well. After a 5 min incubation, the reaction was triggered by adding ATP (final concentration: 0.1 mM) to the mixture and the fluorescence emission intensity was simultaneously measured at two wavelengths (470 nm and 530 nm) for DAN-based sensors with the excitation set at 380 nm. The PTEN assay was performed in a similar manner except that ATP and pY2 were absent in the reaction mixture. For NR3-eTapp2-cPH, the emission intensity was measured at 600 nm and 675 nm with the excitation set at 560 nm.

Fluorescence plate reader assay

Enzyme reactions were also monitored with the BioTek Synergy Neo HTS multi-mode plate reader using nontreated black polystyrene 96-well plates (Corning Inc.). For the PI3K activity assay, 200 μl of buffer solution [20 mM Tris buffer (pH 7.4) with 0.16 M NaCl] containing PI3Kβ and lipid LUVs with the indicated concentrations, 500 nM lipid sensor and 10 μM pY2 peptide were added to each well. After a 5 min incubation, the reaction was triggered by adding ATP (final concentration: 0.1 mM) to the mixture and the fluorescence emission intensity was simultaneously measured at two wavelengths (470 nm and 530 nm) for DAN-based sensors with the excitation set at 380 nm. The PTEN assay was performed in a similar manner except that ATP and pY2 were absent in the reaction mixture. For NR3-eTapp2-cPH, the emission intensity was measured at 600 nm and 675 nm with the excitation set at 560 nm.

P13K inhibition assay

Two hundred microliters of buffer solution [20 mM Tris buffer (pH 7.4) with 0.16 M NaCl] containing 10 nM PI3Kβ and 50 μM lipid LUVs, 500 nM DAN-eENTH, 10 μM pY2 peptide, and varying concentrations of inhibitor (0–1 μM for GDC-0941 and wortmannin and 0–1 μM for LY294002) were added to each well. After a 10 min incubation, the reaction was triggered by adding ATP (final concentration: 0.1 mM) to the mixture and the fluorescence intensity was monitored at 675 nm with the excitation wavelength set at 560 nm.

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Fig. 1. Fluorescence emission spectra (A–C) of DAN-eENTH (PI4,5P₂ sensor), DAN-eMyoX-tPH (PIP₃ sensor), and NR3-eTapp2-cPH (PI3,4P₂ sensor) as a function of the lipid concentration and resulting ratiometric calibration curves (D–F). A–C: POPC/POPS/PI4,5P₂ (77:20:3; 400 μg/ml) LUVs with varying total lipid concentrations [from 0, 10, 20, 30, 40, 50, 80, 100 to 155 μM (1–100 μM for NR3-eTapp2-cPH)] from bottom to top] were added to each sensor (500 nM) and fluorescence emission spectra were monitored with the excitation wavelength set at 380 nm for DAN-labeled sensors and 560 nm for NR3-labeled sensors, respectively. The spectra of the sensors without lipid vesicles are indicated by arrows. The change in fluorescence emission intensity \(\Delta F\) for each vesicle was normalized against the maximal fluorescence increase value \(\Delta F_{\text{max}}\) observed for each sensor. Each spectrum is from a single representative scan selected from triplicate measurements \((n = 3)\) that showed essentially the same patterns. D–F: From the emission spectra, the ratio \(F_{530}/F_{470}\) for DAN-eENTH (D) and DAN-eMyoX-tPH (E) and NR3-eTapp2-cPH (F), respectively, were calculated. Nonlinear least-squares analysis of the plots using the equation (e.g., for DAN-eMyoX-tPH): \(F_{530}/F_{470} = (F_{530}/F_{470})_{\text{max}} + \left(F_{530}/F_{470}\right)_{\text{max}}/(1 + F_{530}/F_{470})\), yielded \(K_d\) \((F_{530}/F_{470})_{\text{max}}\) and \((F_{530}/F_{470})_{\text{min}}\) values and the calibration curves were constructed using these parameters. \(K_d\) \((F_{530}/F_{470})_{\text{max}}\) and \((F_{530}/F_{470})_{\text{min}}\) values are the equilibrium dissociation constant, the maximal \(F_{530}/F_{470}\) value, and the minimal \(F_{530}/F_{470}\) value, respectively. Data in D–F indicate mean ± SD from the triplicate measurements.
emission intensity was simultaneously measured at 470 nm and 530 nm with the excitation set at 380 nm.

**Kinetic data analysis**

All fluorescence intensity ratios ($F_{530}/F_{500}$ for DAN-eENTH and DAN-eMyoX-tPH and $F_{600}/F_{570}$ for NR3-eTapp2-cPH) at different time points were converted into total PI4,5P2 (PIP3 or PI3,4P2) concentrations by Excel using respective ratiometric calibration curves (Fig. 1D–F) to yield full enzyme reaction curves. The initial rate ($V_o$) of enzyme reaction was then calculated from the initial linear part of the reaction curves. Apparent Michaelis-Menten kinetic parameters were calculated by nonlinear least-squares analysis using the Michaelis-Menten equation, $V_o = \frac{V_{max}E}{1 + Km/S}$.

**RESULTS AND DISCUSSION**

**Assay strategy**

We recently developed a fluorescence-based ratiometric imaging analysis that allows accurate in situ quantification of cellular lipids in live cells (23–27). This method utilizes a ratiometric fluorescence sensor prepared from a genetically engineered lipid binding domain that is chemically labeled on a single site with a solvatochromic fluorophore that exhibits a large change in fluorescence emission upon lipid binding. After in vitro calibration of the lipid sensor using lipid vesicles with the varying lipid composition, the sensor is delivered to cells for in situ lipid quantification with high spatiotemporal resolution and accuracy. In this work, we applied the same lipid quantification technology to the in vitro real-time activity measurement for lipid kinases and phosphatases. For instance, we directly measured the enzymatic kinetics of PI3K through real-time spectrofluorometric quantification of either its substrate, PI4,5P2, or its product, PIP3. Likewise, we monitored the enzyme activity of its counterbalancing enzyme, PTEN, by following the kinetics of the PIP3 decrease or the PI4,5P2 increase.

**Fig. 2.** PI3K activity monitored by DAN-eENTH (A) and DAN-eMyoX-tPH (B). A: Kinetics of PI3Kβ-catalyzed PI4,5P2 phosphorylation. DAN-eENTH (500 nM) was incubated with 50 μM POPC/POPS/PI4,5P2 (77/20/3) LUVs for 1 min, and then 10 nM PI3Kβ, 10 μM pY2 peptide, and 100 μM ATP were added in different order as indicated. The time course of $F_{530}/F_{500}$ was then converted into the total PI4,5P2 concentration using the calibration curve (see Fig. 1D). B: Kinetics of PI3Kβ-catalyzed PIP3 formation. The same as in A except that DAN-eMyoX-tPH was employed in place of DAN-eENTH. Notice that the order of addition of reagents did not affect the kinetic curves. The data are representative sets from quadruple independent measurements (n = 4).

**Conditions and efficiency of the PI3K activity assay**

The cellular activation PI3K, which is composed of two subunits, p110 (catalytic subunit) and p85 (regulatory subunit), involves binding of two SH2 domains in the p85 to phosphotyrosines (pY) in an activating protein, such as a receptor tyrosine kinase, which relieves p110 from its inhibitory tethering by p85 (22). It has been shown that PI3Kβ can be activated in vitro by a pY-containing peptide derived from PDGFβ (pY2) (22). Addition of PI3Kβ and cofactors to the mixture of POPC/POPS/PI4,5P2 (77:20:3 in mole percent) LUVs and DAN-eENTH resulted in a rapid decrease in $F_{530}/F_{500}$ (data not shown). Conversion of $F_{530}/F_{500}$ into the total PI4,5P2 concentration by the calibration curve (see Fig. 1D) yielded a kinetic curve of PI4,5P2 disappearance (Fig. 2A). The order of addition of different reagents did not affect the kinetic curve (Fig. 2A). When the PIP3 sensor (DAN-eMyoX-tPH) was employed in place of the PI4,5P2 sensor, the reaction led to a rapid increase in $F_{530}/F_{500}$, which was converted into the total PIP3 concentration, yielding the kinetic curve of PIP3 appearance (Fig. 2B). Throughout the reaction, the sum of the PI4,5P2 and PIP3 concentrations remained constant (Fig. 2A, B), verifying that our assay faithfully monitors the conversion of PI4,5P2 to PIP3 by PI3K. The reaction could be monitored with either a cuvette-based spectrofluorometer or a plate reader.
Kinetic analysis of PI3K reaction

It has been shown that the reaction catalyzed by interfacial enzymes, most notably phospholipases, follows complex mechanisms involving interfacial binding/unbinding of the enzyme, which often makes it difficult to analyze interfacial enzyme kinetics by the conventional Michaelis-Menten kinetics (28, 29). To determine whether the reaction catalyzed by PI3KΔ8 could be analyzed by the Michaelis-Menten kinetics, we measured the initial rate \( (V_o) \) as a function of total enzyme concentration \( (E_o) \) and substrate concentration \( (S_o) \) or \([\text{PI4,5P}_2]_o\) respectively. According to the Michaelis-Menten kinetics \([\text{i.e., } V_o = k_{cat} E_o/(1 + K_m/S_o)\] where \( k_{cat} \) and \( K_m \) are turnover number and Michaelis constant, respectively, \( V_o \) should be linearly proportional to \( E_o \) at a given \( S_o \) and show hyperbolic dependence on \( S_o \) at a given \( E_o \). As shown in Fig. 3A and B, \( V_o \) was linearly proportional to \( E_o \) in the range of 0–25 nM when \([\text{PI4,5P}_2]_o \) was kept at 50 μM. Also, \( V_o \) showed typical hyperbolic dependence on \([\text{PI4,5P}_2]_o \) in the range of 0–50 μM with \( E_o = 10 \) nM (Fig. 3C, D). The \( V_o \) versus \([\text{PI4,5P}_2]_o \) plot was successfully fit by nonlinear least-squares analysis using the Michaelis-Menten equation (Fig. 3D) and the analysis yielded \( k_{cat} (= 50 \pm 5 \text{ s}^{-1}) \) and \( K_m (36 \pm 6 \text{ μM}) \) values. These results indicate that although the PI3K-catalyzed reaction might involve more steps than the conventional homogenous enzyme catalysis, our activity assay allows robust determination of (apparent) kinetic parameters by the straightforward Michaelis-Menten analysis and that these parameters can be used to quantitatively assess the effects of diverse factors, including PI3K mutations and variation of the substrate structure, on the PI3K enzyme activity.

Interestingly, the concentration of PIP3 reached only 60% of PI4,5P2, even with the saturating concentration of PI3K (i.e., >50 nM; see also Fig. 3A). To explore the possibility that this was due to product inhibition, we carried out the PI3K reaction in the presence of varying concentrations of PIP3 in the PI4,5P2-containing vesicles \([\text{i.e., POPC/POPS/PI4,5P}_2/\text{PIP}_3 (77/20/3/x; x = 0-3 \text{ mol%})}]\). As shown in Fig. 3E, the initial rate decreased as a function of pre-added PIP3 and essentially reached an undetectable

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**Fig. 3.** Kinetic analysis of PI3K reactions. A: The kinetics of PI4,5P2 phosphorylation as a function of the increasing PI3K concentration. DAN-eENTH (500 nM) was incubated with 50 μM POPC/POPS/PI4,5P2 (77/20/3) LUVs for 1 min, and then 0–25 nM (0, 3, 5, 8, 10, 12, 15, 18, 20, and 25 nM from top to bottom) PI3KΔ8, 10 μM pY2 peptide, and 100 μM ATP were added to initiate the reaction. Notice that the reaction did not go to completion even with the increasing concentrations of PI3KΔ8. B: The initial rate of PI4,5P2 phosphorylation as a function of the PI3KΔ8 concentration. The plot was analyzed by linear regression using the equation: \( V_o = k_{cat} E_o/(1 + K_m/S_o) \). C: The kinetics of PI4,5P2 phosphorylation as a function of the increasing PI4,5P2 concentration. DAN-eENTH (500 nM) was incubated with 0–50 μM (0, 5, 10, 15, 20, 25, 30, 35, 40, and 50 μM from top to bottom) POPC/POPS/PI4,5P2 (77/20/3) LUVs for 1 min, and then 10 nM PI3KΔ8, 10 μM pY2 peptide, and 100 μM ATP were added to initiate the reaction. Notice that the reaction did not go to completion even with the increasing concentrations of PI3KΔ8. D: The initial rate of PI4,5P2 phosphorylation as a function of the PI3KΔ8 concentration. The plot was analyzed by nonlinear least-squares fit using the equation: \( V_o = k_{cat} E_o/(1 + K_m/S_o) \). E: The kinetics of PI4,5P2 phosphorylation as a function of the increasing product (PIP3) concentration added to the reaction mixture. DAN-eENTH (500 nM) was incubated with 50 μM POPC/POPS/PI4,5P2/PIP3 (77–x/20/3/x; x = 0, 0.5, 1, 1.5, 2, and 3 mol% from top to bottom) LUVs for 1 min, and then 10 nM PI3KΔ8, 10 μM pY2 peptide, and 100 μM ATP were added to initiate the reaction. F: Relative activity of PI3KΔ8 (expressed in terms of \( k_{cat}/K_m \)) toward PI4,5P2 with different acyl chains, including SAPi4,5P2 \([1.2 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{s}^{-1}]\), 1,2-dipalmitoyl-PI4,5P2 \([1.4 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{s}^{-1}]\), and 1,2-dioleoyl-PI4,5P2 \([1.1 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}]\). The \( k_{cat} \) and \( K_m \) for these PI4,5P2 molecules were determined from the respective \( V_o \) versus \([\text{PI4,5P}_2]_o \) plots. The data in A–E are representative sets from quadruplicate independent measurements \((n = 4)\). Data in B–D indicate mean ± SD from the measurements. The data in E are a representative set from triplicate independent measurements, whereas the data in F indicate the mean ± SD from triplicate measurements.
level when the equimolar PIP₃ and PI₄,5P₂ were present in the same vesicles. These results support the notion that PI₃K inhibits the PI₃K reaction. This feedback inhibition mechanism might also contribute to the regulation of cellular PI₃K activity under physiological conditions. In fact, our recent in situ quantification showed that stimulation of PI₃K by growth factors converts only about 60% of PI₄,5P₂ into PIP₃ at the PM of PTEN-null mammalian cells (25).

**Acyl chain specificity of PI₃K**

It has been well documented that the PI₄,5P₂ present in the PM of mammalian cells mainly contains the stearoyl group in the sn-1 position and the arachidonoyl group in the sn-2 position (30). This raises the question as to whether PI₃K has evolved to specifically recognize 1-stearoyl-2-arachidonoyl-PI₄,5P₂ (SAPI₄,5P₂). To check the potential PI₄,5P₂ acyl chain selectivity of PI₃K, we determined the kinetic parameters for the PI₃K-catalyzed phosphorylation of various commercially available PI₄,5P₈ with different acyl chains, including SAPI₄,5P₂, 1,2-dipalmitoyl-PI₄,5P₂, and 1,2-dioleoyl-PI₄,5P₂. Briefly, we determined the k_cat and K_m values for these PI₄,5P₂ molecules from their V_max versus [PI₄,5P₂], plots (see for example, Fig. 3D) and compared the relative activity of PI₃Kβ for them in terms of k_cat/K_m. As shown in Fig. 3F, PI₃Kβ could not distinguish among SAPI₄,5P₂, 1,2-dipalmitoyl-PI₄,5P₂, and 1,2-dioleoyl-PI₄,5P₂ beyond the experimental error range. These results indicate that PI₃Kβ does not have the pronounced specificity for SAPI₄,5P₂ and would act on various PI₄,5P₂ with different acyl chains.

**PI₃K inhibition assay**

PI₃K is one of the most frequently mutated proteins in cancer and has thus been an attractive cancer drug target (16). Having established the conditions for the rapid plate reader-based PI₃K assay, we tested to determine whether the assay could be used to screen molecules for PI₃K-modulating activity. As a proof of principle, we measured the inhibitory activity of three well-characterized PI₃K inhibitors, GDC-0941, LY294002, and wortmannin. GDC-0941 is a potent class I-selective PI₃K inhibitor targeting their ATP-binding pocket with a reported IC₅₀ value of 33 nM for PI₃Kβ (31). LY294002 is a nonselective inhibitor of PI₃K with a reported IC₅₀ of 1.4 μM (32), whereas wortmannin is an irreversible inhibitor of PI₃K with the reported IC₅₀ value of 1.9 nM (33). Increasing concentrations (0–500 nM) of each of these molecules were added to each row of a 96-well plate containing 10 nM of PI₄,5P₂, 50 μM of POPC/POPS/PI₄,5P₂ (77:20:3) LUVs, and 500 nM DAN-eENTH, and the mixture was incubated for 10 min at room temperature. After the reaction was initiated by adding 10 μM pY² peptide and 100 μM ATP, F₄70/F₅30 was monitored for 3 min and converted into PI₄,5P₂ concentrations, from which V_max was calculated. The data represent the mean ± SD from triplicate measurements.

**PTEN activity assay**

A PTEN-catalyzed reaction was followed by monitoring the time-dependent decrease of PIP₃ or the time-dependent increase of PI₄,5P₂. The assay condition for PTEN was simpler than that for PI₃K because PTEN is not known to require cofactors for activity as long as the reaction medium is kept under reducing conditions (9). Addition of recombinant PTEN to the mixture of POPC/POPS/PI₄,5P₂ (77:20:3) LUVs and DAN-eMyoX-tPH resulted in a rapid decrease in F₄70/F₅30 (data not shown), which was converted to a kinetic curve of PIP₃ disappearance (Fig. 5A). The use of PI₄,5P₂ sensor (DAN-eENTH) in place of the PIP₃ sensor yielded the kinetic curve of PI₄,5P₂ formation (Fig. 5B). As was the case with the PI₃K activity assay, V_max was linearly proportional to E_in in the range of 0–40 nM when [PI₃], was kept at 50 μM (data not shown). Unlike the case with PI₃K, however, the PTEN-catalyzed reaction reached near full conversion of PIP₃ to PI₄,5P₂ and did not exhibit product.

**Fig. 4.** Determination of inhibitory activity of GDC-0941 (open triangles), LY294002 (open circles), and wortmannin (open squares). Increasing concentrations (0–500 nM) of each of these molecules were added to each row of a 96-well plate containing 10 nM of PI₄,5P₂, 50 μM of POPC/POPS/PI₄,5P₂ (77:20:3) LUVs, and 500 nM DAN-eENTH, and the mixture was incubated for 10 min at room temperature. After the reaction was initiated by adding 10 μM pY² peptide and 100 μM ATP, F₄70/F₅30 was monitored for 3 min and converted into PI₄,5P₂ concentrations, from which V_max was calculated. The data represent the mean ± SD from triplicate measurements.
Fig. 5. Kinetics of PTEN reactions. A: Kinetics of PTEN-catalyzed PIP₃ dephosphorylation. DAN-eMyoX-tPH (500 nM) was incubated with 50 μM POPC/POPS/PIP₃ (77/20/3) LUVs for 1 min, and then 10 nM PTEN were added (arrow) to initiate the reaction. The time course of $F_{470}/F_{530}$ was then converted into the total PIP₃ concentration. B: Kinetics of PTEN-catalyzed PI4,5P₂ formation. The same as in A except that DAN-ENTH was employed in place of DAN-eMyoX-tPH. C: PTEN activity monitored by DAN-eENTH with multiple addition of PTEN. The final concentration of PTEN after three equimolar additions was 90 nM. The arrows indicate the time points of enzyme addition. D: The kinetics of PIP₃ dephosphorylation as a function of the increasing PIP₃ concentration. DAN-eENTH (500 nM) was incubated with 0–50 μM POPC/POPS/PIP₃ (77/20/3) LUVs for 1 min, and then 10 nM PTEN were added to initiate the reaction. The plot was analyzed by non-linear least-squares fit using the equation: $V_0 = k_{cat} E_o/(1 + K_m/S_o)$. E: Relative activity of PTEN for PIP₃ and PI3,4P₂. DAN-eMyoX-tPH (500 nM) and NR3-eTapp2-cPH (500 nM) were mixed with 50 μM POPC/POPS/PIP₃ (77/20/3) and 50 μM POPC/POPS/PI3,4P₂ (77/20/3) LUVs for 1 min, and then 10 nM PTEN were added to the mixture to initiate the reaction. $F_{470}/F_{530}$ and $F_{600}/F_{675}$ were monitored to simultaneously track the dephosphorylation of PIP₃ and PI3,4P₂, respectively. PTEN activity against POPC/POPS/PI3,4P₂ (77:20:3) LUVs was separately measured using DAN-eENTH. The data in A–C and E are representative sets from quadruple independent measurements (n = 4). The data in D represent the mean ± SD from triplicate measurements.

inhibition as witnessed by the uninhibited activity of PTEN added after the significant accumulation of PI4,5P₂ (Fig. 5C). Also, $V_o$ showed hyperbolic dependence on $[\text{PIP}_3]_o$ in the range of 0–50 μM with $E_o = 10$ nM (Fig. 5D). The non-linear least-squares analysis of the $V_o$ versus $[\text{PIP}_3]_o$ plot using the Michaelis-Menten equation yielded $k_{cat} (= 150 ± 20 \text{ s}^{-1})$ and $K_m$ (82 ± 10 μM) values.

PIP₃ generated by PI3K is subsequently converted to PI3,4P₂ by lipid phosphatases, including SHIP (25) and INPP5 (10), and PI3,4P₂ plays unique signaling roles (34, 35). It has been reported that PTEN regulates PI3,4P₂ by converting it to phosphatidylinositol-4-phosphate (21). To investigate the enzymatic basis of this finding, we rigorously determined the relative activity of PTEN toward PIP₃ and PI3,4P₂ by simultaneously measuring the PIP₃ and PI3,4P₂ dephosphorylation. To this end, we employed a well-established PI3,4P₂ sensor, NR3-eTapp2-cPH (25), which is spectrally orthogonal to the DAN-eMyoX-tPH (see Fig. 1C, F), thereby enabling direct simultaneous monitoring of PIP₃ and PI3,4P₂ dephosphorylation. As a negative control, we separately checked the activity of PTEN against POPC/POPS/PI4,5P₂ (77:20:3) LUVs (Fig. 5E). Addition of PTEN to the mixture containing POPC/POPS/PIP₃ (77:20:3) LUVs, POPC/POPS/PI3,4P₂ (77:20:3) LUVs, DAN-eMyoX-tPH, and NR3-eTapp2-cPH resulted in a rapid decrease in $F_{470}/F_{530}$ (data not shown), which was converted to a kinetic curve of PIP₃ disappearance (Fig. 5E); however, the decrease in $F_{600}/F_{675}$, which reflects the dephosphorylation of PI3,4P₂, was only slightly above the negative control under the same conditions (Fig. 5E). These results show that PTEN has much lower intrinsic enzymatic activity toward PI3,4P₂ than toward PIP₃ and suggest that the reported activity of PTEN to regulate PI3,4P₂ signaling might not derive from its catalytic action on PI3,4P₂. It should be noted that our study was performed with the bacterially expressed PTEN and that one cannot preclude the possibility that posttranslational modification in mammalian cells might confer enhanced activity for PI3,4P₂ on PTEN.
CONCLUSIONS

We have developed a new fluorescence-based real-time assay for PI3K and PTEN. The main advantages of this direct quantitative assay are high sensitivity, accuracy, speed, and a high degree of flexibility in assay design. Although the present study was confined to a single form of PI3K and PTEN, respectively, the assay is universally applicable to the kinetic analysis of any lipid kinase and phosphatase, as long as a sensor specific for its lipid substrate or product can be prepared. Our straightforward kinetic analysis of PI3K and PTEN produced the new mechanistic information about these enzymes, and our pilot study demonstrates feasibility for high-throughput screening of small molecules for their PI3K-modulating activity. The new method will facilitate further mechanistic studies on other lipid kinases and phosphatases as well as rapid screening and testing of small molecule modulators of pharmacologically important lipid kinases and phosphatases.

Data availability

All data are contained within the article. The raw data will be shared upon request: contact Wonhwa Cho (University of Illinois at Chicago, e-mail: wcho@uic.edu)

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REFERENCES

1. Cho, W., and R. V. Stahelin. 2005. Membrane-protein interactions in cell signaling and membrane trafficking. Annu. Rev. Biophys. Biomol. Struct. 34: 119–151.
2. Cho, W. 2006. Building signaling complexes at the membrane. Sci. STKE 2006: p7.
3. van Meer, G., D. R. Voelker, and G. W. Feigenson. 2008. Membrane lipids: where are they and how they behave. Nat. Rev. Mol. Cell Biol. 9: 112–124.
4. Balla, T. 2013. Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol. Rev. 93: 1019–1137.
5. Schink, K. O., K. W. Tan, and H. Semmken. 2016. Phosphoinositides in control of membrane dynamics. Annu. Rev. Cell Dev. Biol. 32: 143–171.
6. Burke, J. E. 2018. Structural basis for regulation of phosphoinositide kinases and their involvement in human disease. Mol. Cell. 71: 653–675.
7. Sasaki, T., S. Takasuga, J. Sasaki, S. Kofugi, S. Eguchi, M. Yamazaki, and A. Suzuki. 2009. Mammalian phosphoinositide kinases and phosphatases. Prog. Lipid Res. 48: 307–343.
8. Ciracolo, E., F. Gullumi, and E. Hirsch. 2014. Methods to measure the enzymatic activity of PI3Ks. Methods Enzymol. 543: 115–140.
9. Spinelli, L., and N. R. Leslie. 2015. Assaying PI3K catalysis in vitro. Methods. 77–78: 51–57.
10. Vanhaesebroeck, B., L. Stephens, and P. Hawkins. 2012. PI3K signalling: the path to discovery and understanding. Nat. Rev. Mol. Cell Biol. 13: 195–203.
11. Cantley, L. C. 2002. The phosphoinositide 3-kinase pathway. Science. 296: 1655–1657.
12. DiNitto, J. P., and D. G. Lambright. 2006. Membrane and juxtamembrane targeting by PH and PTB domains. Biochim. Biophys. Acta. 1761: 850–867.
13. Thorpe, L. M., H. Yuzugullu, and J. J. Zhao. 2015. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. Nat. Rev. Cancer. 15: 7–24.
14. Fruman, D. A., and C. Rommel. 2014. PI3K and cancer: lessons, challenges and opportunities. Nat. Rev. Drug Discov. 13: 140–156.
15. Hawkins, P. T., and L. R. Stephens. 2015. PI3K signalling in inflammation. Biochim. Biophys. Acta. 1851: 882–897.
16. Mayer, I. A., and C. L. Arteaga. 2016. The PI3K/akt pathway as a target for cR. Rev. Pharmacol. 56: 128–141.
17. Song, M. S., L. Salmena, and P. P. Pandolfi. 2012. The functions and regulation of the PTEN tumour suppressor. Nat. Rev. Mol. Cell Biol. 13: 283–296.
18. Worby, C. A., and J. E. Dixon. 2014. PI3K. Annu. Rev. Biochem. 83: 641–669.
19. Bassi, C., J. Ho, T. Srikumar, R. J. Dowling, C. Gorrini, S. J. Miller, T. W. Mak, B. G. Neel, B. Raughli, and V. Stambolic. 2013. Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress. Science. 341: 395–399.
20. Hopkins, B. D., B. Fine, N. Steinbach, M. Dendy, Z. Rapp, J. Shaw, K. Pappas, J. S. Yu, C. Hodasoki, S. Mense, et al. 2013. A secreted PTEN phosphatase that enters cells to alter signaling and survival. Science. 341: 399–402.
21. Malek, M., A. Kielkowska, T. Chessa, K. F. Anderson, D. Barneda, P. Pir, H. Nakanshi, S. Eguchi, A. Koizumi, J. Sasaki, et al. 2017. PTEN regulates PI(3,4)P2 signaling downstream of class I PI3K. Mol. Cell. 68: 566–580.e10.
22. Zhang, X., O. Vadis, O. Perisic, K. F. Anderson, J. Clark, P. T. Hawkins, L. R. Stephens, and R. L. Williams. 2011. Structural and functional analysis of lipid kinase p110beta/p85beta elucidates an unusual SH2-domain-mediated inhibitory mechanism. Mol. Cell. 41: 567–578.
23. Yoon, Y., P. J. Lee, S. Kuriolova, and W. Cho. 2011. In situ quantitative imaging of cellular lipids using molecular sensors. Nat. Chem. 3: 868–874.
24. Liu, S. L., R. Sheng, M. J. O’Connor, Y. Cui, Y. Suron, K. Kuriolova, D. Lee, and W. Cho. 2014. Simultaneous in situ quantification of two cellular lipid pools using orthogonal fluorescent sensors. Angew. Chem. Int. Ed. Engl. 53: 14387–14391.
25. Liu, S. L., Z. G. Wang, Y. Hu, Y. Xin, I. Singaram, S. Gorai, X. Zhou, Y. Shim, J. H. Min, L. W. Gong, et al. 2018. Quantitative lipid imaging reveals a new signaling function of phosphatidylinositol-3,4-bisphosphate: isoform- and site-specific activation of Akt. Mol. Cell. 71: 1092–1104.e5.
26. Liu, S. L., R. Sheng, J. H. Jung, L. Wang, E. Stec, M. J. O’Connor, S. Song, R. K. Bikkavilli, R. A. Winn, D. Lee, et al. 2017. Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol. Nat. Chem. Biol. 13: 268–274.
27. Cho, W., Y. Yoon, S. L. Liu, K. Back, and R. Sheng. 2017. Fluorescence-based in situ quantitative imaging for cellular lipids. Methods Enzymol. 588: 19–33.
28. Wu, S. K., and W. Cho. 1993. Use of polymerized mixed liposomes to study interactions of phospholipase A2 with membranes. Biochemistry. 32: 13902–13908.
29. Berg, O. G., M. H. Gelb, M. D. Tsai, and M. K. Jain. 2001. Interface enzymology: the secreted phospholipase A2-paradigm. Chem. Rev. 101: 2613–2654.
30. Harayama, T., and H. Riezman. 2018. Understanding the diversity of membrane lipid composition. Nat. Rev. Mol. Cell Biol. 19: 281–296.
31. Folkes, A. J., K. Ahmadi, W. K. Alderton, S. Alix, S. J. Baker, G. Box, I. S. Chuckowree, P. A. Clarke, P. Depledge, S. A. Eccles, et al. 2008. The identifcation of 2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-1-piperazin-1-ylmethyl)-4-morpholin-4-1 hieno[3,2,d]pyrimidine (DGC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer. J. Med. Chem. 51: 5522–5532.
32. Vlahos, C. J., W. F. Matter, K. Y. Hui, and R. F. Brown. 1994. A specific inhibitor of phosphatidylinositol-3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. 269: 5241–5248.
33. Arcaro, A., and M. P. Wymann. 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem. J. 296: 297–301.
34. Li, H., and A. J. Marshall. 2015. Phosphatidylinositol (3,4) bisphosphate-specific phosphatases and effector proteins: A distinct branch of PI3K signalling. Cell. Signal. 27: 1789–1798.
35. Hawkins, P. T., and L. R. Stephens. 2016. Emerging evidence of signalling roles for PI(3,4)P2 in class I and II PI3K-regulated pathways. Biochem. Soc. Trans. 44: 307–314.