A direct fluorometric activity assay for lipid kinases and phosphatases

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Abstract  Lipid kinases and phosphatases play key roles in cell signaling and regulation, are implicated in many human diseases, 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 and phosphatases.

Abbreviations: eENTH, engineered epsin1 ENTH domain; Eo, initial enzyme concentration; LUV, large unilamellar vesicle; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI3K, class I phosphoinositide 3-kinase; PI3,4P2, phosphatidylinositol-3,4-bisphosphate; PI4,5P2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PM, plasma membrane; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PTEN, phosphatase and tensin homolog; pY2, ESDGPyMDMDSIAsWVPMLDMDKDIYK; SAPI4,5P2, 1-stearoyl-2-araehidonoyl derivative of phosphatidylinositol-4,5-bisphosphate; S0, initial substrate concentration; TCEP, tris(2-carboxyethyl)phosphate; V0, initial rate.

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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 PI3 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 (SAP14,5P2) were also from Avanti Polar Lipids. The 1,2-dipalmitoyl derivatives of PI4,5P2, phosphatidylinositol-3,4-bisphosphate (PI3,4P2), and PI2 were from Cayman Chemical Company. The cDNA for PTEN was purchased from OriGene (SC119965). The pY2 peptide (ES-DGGpYMDMSKDESIDpYVPMLDMKGDIKYA), derived from the 1-stearoyl-2-arachidonoyl derivatives of PI4,5P2 (SAPI4,5P2) were from Cayman Chemical Company. 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 b-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 (eMyoX-XpH) (24), and C-terminal PH domain of Tapp2 (eTapp2cPH), which have been employed as specific sensors for PI4,5P2 (23), PI3 (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 b-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 the Ni-NTA resin (Marvelgent Biosciences Inc.) for 2 h. The resin was then poured into a small column and washed with buffer A [20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 1 mM tris(2-carboxyethyl) phosphine (TCEP), and 1 mM phenylmethylsulphonyl fluoride (PMSF) (pH 7.9)] and eluted with 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 b-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.

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-Tn-5B1-4 (High Five) sus-
POPC/POPS/PI4,5P₂ (77:20:3). The ratio \((F_{470}/F_{530})\) 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 \(\mu\)l 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 \(\mu\)M 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 \(\mu\)l of buffer solution [20 mM Tris buffer (pH 7.4) with 0.16 M NaCl] containing PI3K\(β\) and lipid LUVs with the indicated concentrations, 500 nM lipid sensor and 10 \(\mu\)M 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 PI3K\(β\) and 50 \(\mu\)M lipid LUVs, 500 nM DAN-eENTH, 10 \(\mu\)M pY2 peptide, and varying concentrations of inhibitor (0–1 \(\mu\)M for GDC-0941 and wortmannin and 0–1 \(\mu\)M 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

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**Fig. 1.** Fluorescence emission spectra (A–C) of DAN-eENTH (PI4,5P₂ sensor), DAN-eMyoX-cPH (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 (or PI4,5P₂ and PI3,4P₂) (77/20/3) LUVs with varying total lipid concentrations [from 0, 10, 20, 30, 40, 50, 80, 100 to 155 \(\mu\)M 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 (\(ΔF\)) for each vesicle was normalized against the maximal fluorescence increase value (\(ΔF_{\text{max}}\)) 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 of fluorescence intensity at 470 nm versus that at 530 nm (\(F_{470}/F_{530}\)) for each vesicle was normalized against the maximal fluorescence increment value \(F_{470}/F_{530}\) (D) and DAN-eMyoX-cPH (E) and \(F_{470}/F_{530}\) for NR3-eTapp2-cPH (F), respectively, were calculated. Nonlinear least-squares analysis of the plots using the equation (e.g., for DAN-eMyoX-cPH): \(F_{470}/F_{530} = (F_{470}/F_{530})_{\text{max}} + (F_{250}/F_{530})_{\text{min}}/ (1 + K_d/ [P_I])\) yielded \(K_d\) \((F_{470}/F_{530})_{\text{max}}\) and \(F_{250}/F_{530}\) values and the calibration curves were constructed using these parameters. \(K_d\) \((F_{470}/F_{530})_{\text{max}}\) and \(F_{250}/F_{530}\) values are the equilibrium dissociation constant, the maximal \(F_{470}/F_{530}\), and the minimal \(F_{250}/F_{530}\), respectively. Data in D–F indicate mean ± SD from the triplicate measurements.
Kinetic data analysis

All fluorescence intensity ratios (F_{530}/F_{470} for DAN-eENTH and DAN-eMyoX-tPH and F_{600}/F_{750} 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, \( V_o = \frac{\kappa_{\text{cat}} E_o}{1 + K_m/S_o} \), where \( E_o \) and \( S_o \) are the molar concentrations of enzyme and substrate, respectively, and \( K_m \) and \( \kappa_{\text{cat}} \) are the turnover number and Michaelis constant, respectively. The enzyme inhibition data were analyzed by nonlinear least-squares analysis using a simple competitive inhibition equation, \( V_o = V_{\text{max}}/(1 + I_o/K_{I_o}) \), where \( V_{\text{max}} \), \( I_o \) and \( K_{I_o} \) are maximal \( V_o \), the initial inhibitor concentration, and the inhibitor concentration yielding half-maximal inhibition. All kinetics parameters were expressed as average ± SD from minimum of triplicate measurements.

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. As sensors for PI4,5P2 and PIP3, we selected DAN-eENTH (23) and DAN-eMyoX-tPH (25), respectively, which have been fully characterized and successfully used for in situ quantification of cellular PI4,5P2 and PIP3. Spectrofluorometric properties of these sensors and their ratiometric lipid titration curves are shown in Fig. 1. Briefly, these solvatochromic sensors displayed a hypsochromic shift (or blue shift) of the fluorescence emission peak from 530 to 470 nm upon membrane lipid binding, and the intensity at 470 nm was increased proportionally to the increase in the concentration of their cognate lipid (Fig. 1A, B). Data in Fig. 1 were collected by varying the total lipid concentration of vesicles with a fixed PI4,5P2 (PIP3 or PI3,4P3) composition [e.g., POPC/POPS/PI4,5P2 (77:20:3 in mole percent)], but similar results were obtained when the PI4,5P2 (PIP3 or PI3,4P3) content in the vesicles was varied (e.g., POPC/POPS/PI4,5P2 = 80:x:20, x = 0–10 mol%) with the fixed total lipid concentration (not shown). The ratio of fluorescence intensity at 470 nm versus that at 530 nm (F_{530}/F_{470}) showed hyperbolic dependence of the lipid concentration (Fig. 1D, E). These ratiometric calibration curves allowed direct conversion of F_{470}/F_{530} values into lipid concentrations, thereby enabling quantitative real-time monitoring of changes in the substrate or product concentration and thus robust kinetic analysis of the reaction.

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 PI3K α can be activated in vitro by a pY-containing peptide derived from PDGFβ (pY2) (22). Addition of PI3K β 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_{470}/F_{530} (data not shown). Conversion of F_{470}/F_{530} 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_{470}/F_{530}, 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 PI3Kβ 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 [PI4,5P$_2$]$_o$), respectively. According to the Michaelis-Menten kinetics [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 [PI4,5P$_2$]$_o$ was kept at 50 μM. Also, $V_o$ showed typical hyperbolic dependence on [PI4,5P$_2$]$_o$ in the range of 0–50 μM with $E_o = 10$ nM (Fig. 3C, D). The $V_o$ versus [PI4,5P$_2$]$_o$ plot was successfully fit by nonlinear least-squares analysis using the Michaelis-Menten equation (Fig. 3D) and the analysis yielded $k_{cat} (= 50 ± 5$ s$^{-1}$) and $K_m$ (36 ± 6 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 PIP$_3$ reached only 60% of PI4,5P$_2$, 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 PIP$_3$ in the PI4,5P$_2$-containing vesicles [i.e., POPC/POPS/PI4,5P$_2$/PIP$_3\ (77/20/3/x; x = 0–3$ mol%)]. As shown in Fig. 3E, the initial rate decreased as a function of pre-added PIP$_3$ and essentially reached an undetectable

![Fig. 3. Kinetic analysis of PI3K reactions.](image-url)
level when the equimolar PIP3 and PI4,5P2 were present in the same vesicles. These results support the notion that PIP3 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 PI3Kβ-catalyzed phosphorylation of various commercially available PI4,5P2 with different acyl chains, including SAPI4,5P2, 1,2-dipalmitoyl-PI4,5P2, and 1,2-dioleoyl-PI4,5P2. Briefly, we determined $k_{cat}$ and $K_m$ values for these PI4,5P2 molecules from their $V_o$ versus $[\text{PI4,5P}2]$ 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 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 PI3Kβ, 50 μM of POPC/POPS/PI4,5P2 (77:20:3) LUVs, and 500 nM DAN-ENTH, 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, $F_{470}/F_{530}$ was monitored for 3 min and converted into PI4,5P2 concentration, from which $V_o$ 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/PIP3 (77:20:3) LUVs and DAN-eMyoX-tPH resulted in a rapid decrease in $F_{470}/F_{530}$ (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, $V_o$ was linearly proportional to $k_{cat}$ in the range of 0–40 nM when [PIP3]0 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
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 \( \mu \text{M} \) with \( E_o = 10 \text{nM} \) (Fig. 5D). The non-linear least-squares analysis of the \( V_o \) versus \([\text{PIP}_3]_o\) plot using the Michaelis-Menten equation yielded \( k_{\text{cat}} (= 150 \pm 20 \text{s}^{-1}) \) and \( K_m (82 \pm 10 \mu \text{M}) \) values.

\( \text{PIP}_3 \) generated by PI3K is subsequently converted to \( \text{PI3,4P}_2 \) by lipid phosphatases, including SHIP (25) and INPP5 (10), and \( \text{PI3,4P}_2 \) plays unique signaling roles (34, 35). It has been reported that PTEN regulates \( \text{PI3,4P}_2 \) signaling 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 \( \text{PIP}_3 \) and \( \text{PI3,4P}_2 \) by simultaneously measuring the \( \text{PIP}_3 \) and \( \text{PI3,4P}_2 \) dephosphorylation. To this end, we employed a well-established \( \text{PI3,4P}_2 \) sensor, NR3-eTapp2-cPH (25), which is spectrally orthogonal to the DAN-eMyoX-tPH (see Fig. 1C, F), thereby enabling direct simultaneous monitoring of \( \text{PIP}_3 \) and \( \text{PI3,4P}_2 \) 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/\( \text{PI3,4P}_2 \) (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 \( \text{PIP}_3 \) disappearance (Fig. 5E); however, the decrease in \( F_{600}/F_{675} \), which reflects the dephosphorylation of \( \text{PI3,4P}_2 \), 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 \( \text{PI3,4P}_2 \) than toward \( \text{PIP}_3 \) and suggest that the reported activity of PTEN to regulate \( \text{PI3,4P}_2 \) signaling might not derive from its catalytic action on \( \text{PI3,4P}_2 \). 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 \( \text{PI3,4P}_2 \) 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 phosphatasas as well as rapid screening and testing of small molecule modulators of pharmacologically important lipid kinases and phosphatasas.

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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