Signal propagation from membrane messengers to nuclear effectors revealed by reporters of phosphoinositide dynamics and Akt activity

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Edited by Lewis C. Cantley, Harvard Institutes of Medicine, Boston, MA, and approved August 25, 2005 (received for review April 7, 2005)

Among various second messengers, phosphatidylinositol 3,4,5-triphosphate (PIP3) and phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2] regulate a variety of cellular processes, such as cell survival, polarization, and proliferation. Many of these functions are achieved via activation of serine/threonine kinase Akt. To investigate the spatiotemporal regulation of these lipids, we constructed a genetically targetable phosphoinositide (PI) indicator and a nuclear-targeted Akt activity reporter revealing a gradual and sustained accumulation of Akt activity in the nucleus after rapid and transient production of PIP3 and PI(3,4)P2 by growth factor-induced activation of phosphatidylinositol 3-kinase (PI3K) resulted in a change in fluorescence resonance energy transfer (FRET) between the fluorescent proteins, increasing yellow to cyan emission ratios by 10–30%. This response can be reversed by inhibiting PI3K and abolished by mutating the critical residues responsible for PI binding. Differential dynamics of PIs were observed at plasma membrane of NIH 3T3 cells, stimulated by various growth factors. On the other hand, the nuclear targeted indicator showed no response within an hour after platelet-derived growth factor stimulation, suggesting that no appreciable amounts of accessible PIP3 and PI(3,4)P2 were produced in the nucleus. Furthermore, simultaneous imaging of a plasma membrane-targeted PI indicator and a nuclear-targeted Akt activity reporter revealed a gradual and sustained accumulation of Akt activity in the nucleus after rapid and transient production of PIP3 and PI(3,4)P2 at plasma membrane in the same cell. Thus, signal propagation from the lipid messengers at plasma membrane to the effectors in the nucleus is precisely controlled by kinases as well as lipid and protein phosphatases.

Materials and Methods

Reporter Construction. The Akt/PKB PH domain (1–164) was created by PCR with full-length Akt as the template. The pseudoligand peptide sequence VAEEEDDEEEEDDD was inserted between the C terminus of the PH domain and the N terminus of improved versions of yellow fluorescent protein (YFP), Citrine (16) or Venus (17). Double mutation R23A/R25A was incorporated by the QuikChange method (Stratagene) (Fig. 1). The constructs were first generated in pRSET B (Invitrogen) and subcloned into modified pcDNA3 (Invitrogen) behind a Kozak sequence for mammalian expression. For plasma membrane targeting of InPAkt (indicator for phosphoinositides based on Akt), the sequence KKKKSSSTTKCVIM was used. For nuclear targeting, the nuclear localization signal (NLS) PKB (protein kinase B), to the plasma membrane where phosphorylation at two sites, T308 and S473 (in Akt1), fully activates the kinase (6, 7). Upon activation Akt plays important roles in various cellular processes such as proliferation, differentiation, survival, and tumorigenesis (8). Because of the involvement of these PIs in such diverse functions, a cell must precisely control their spatial and temporal dynamics to avoid abnormalities. Thus, investigation of the regulation and functional roles of PIs largely relies on methods that allow measuring PI dynamics in real time in living cells.

Fortunately, nature has evolved various modules or protein domains to recognize and bind to PIs, such as pleckstrin homology (PH) domain (9) from Akt, and from general receptor for phosphoinositides 1 (GRP1). Previously, several groups have used these individual PH domains fused with GFP as indicators for PIs (10–13). For instance, fluorescence intensity change is tracked for GFP-PH (GRP1) where the clearing of the cytosolic milieu to overpopulate the plasma membrane indicates PIP3 production (11). These indicators have proven to be very useful, but their dependence on the translocation event makes it difficult to target them to subcellular organelles or signaling complexes. Such targetability is one of the most important advantages of genetically encoded reporters and is crucial to many of their applications (14, 15). Here, we report the development of a ratiometric indicator for PIP3 and PI(3,4)P2, which can be exclusively targeted to subcellular organelles by using various localization sequences, thus offering an additional advantage of monitoring specific pools of PIs.

Abbreviations: BKAR, B-kinase activity reporter; CFP, cyan fluorescent protein; IGF, insulin-like growth factor; InPAkt, indicator for phosphoinositides based on Akt; NLS, nuclear localization signal; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI, phosphoinositide; PI3K, phosphatidylinositol 3-kinase; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; YFP, yellow fluorescent protein.

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PKKKRKVEDA was attached to the C terminus of the Venus-containing construct.

**Cell Culture.** HEK 293 and NIH 3T3 cells were plated onto sterilized glass coverslips in 35-mm dishes and grown to 50–90% confluency in DMEM (10% FBS) at 37°C with 5% CO₂. Cells were transfected with FuGENE 6 (Roche) then serum-starved for 24–36 h before imaging. Colocalization studies were performed by incubating transfected cells with Hoechst 33342 cell-permeable dyes (Molecular Probes) for staining nucleic acids.

**Imaging.** Cells were washed twice with Hanks’ balanced salt solution buffer and maintained in the dark at room temperature with the addition of 50 ng/ml rat platelet-derived growth factor (PDGF) (Sigma), 100 ng/ml bovine insulin (Calbiochem), 50 nM IGF-1 (Sigma), or 20 μM LY294002 (Sigma) as described. Cells were imaged on a Zeiss Axiovert 200M microscope with a cooled charge-coupled device camera (MicroMAX BFT512, Roper Scientific, Trenton, NJ) controlled by METAFLUOR 6.2 software (Universal Imaging, Downingtown, PA). Dual emission ratio imaging used a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters [475DF40 for cyan fluorescent protein (CFP) and 535DF25 for YFP] alternated by a filter-changer Lambda 10-2 (Sutter Instruments, Novato, CA). Exposure time was 50–500 ms, and images were taken every 15–30 s. Fluorescence images were background-corrected by subtracting the autofluorescence intensity of untransfected cells (or background with no cells) from the emission intensities of cells expressing fluorescent reporters. The ratios of yellow-to-cyan emissions were then calculated at different time points.

**Results**

**Development of a FRET-Based Phosphoinositide Indicator.**

**Design of the reporter.** To measure the spatiotemporal dynamics of PIs in living cells, we sought for a general molecular design in which binding of PIs in the “sensing unit” of the indicator can be translated into a change in the “reporting unit.” A pair of fluorescent proteins that can undergo FRET, enhanced CFP, and improved versions of YFPs, namely Citrine (16) or Venus (17), is used as the reporting unit (Fig. 1 and b). These two fluorophores can be genetically fused to a conformationally responsive element, the conformational change of which alters the relative distance and/or orientation of the two fluorophores and generates a change in the emission ratio. Recently, many such reporters have been developed for measuring signaling molecules like Ca^{2+}, cGMP, cAMP, or signaling events such as protein phosphorylation (14, 15).
For the sensing component, we chose the PH domain of Akt that binds specifically to two of the major PI3K products, namely PIP3 and PI(3,4)P2 (18, 19). Crystal structure of this PH domain complexed to soluble inositol (1,3,4,5)-tetrakisphosphate (IP3) (20) shows that this motif forms a bowl-like structure lined with basic residues into which the highly negatively charged head-group is accommodated.

To convert the PI binding to a conformational change, we engineered a “pseudoligand” sequence to associate with the basic patch of amino acids responsible for PI binding. It consists of a series of acidic residues taken from nucleolin 1, which was shown to bind to PH domain of insulin receptor substrate 1 (21). In the absence of PIs, the pseudoligand is expected to interact with the basic residues in the PH domain. Once the natural ligand is accumulated, the pseudoligand is competed off, unblocking the PH domain and generating a conformational change (Fig. 1a). This conformational change is relayed to the FRET pairs, thus yielding a change in FRET as readout to monitor PI dynamics.

Cellular response. When this construct was expressed in NIH 3T3 cells, the fluorescence was uniformly distributed throughout the cell (Fig. 1d Left). A similar expression pattern was seen in HEK 293 cells (data not shown). To verify that our reporter was expressed in full length, lysates of HEK 293 cells expressing this chimera were separated on SDS/PAGE and probed with anti-GFP antibody. This chimeric protein was of the expected molecular weight showing no proteolysis (Fig. 1c).

We next checked the ability of this chimera to detect changes in 3’ PIs in response to agonist stimulation. NIH 3T3 cells expressing this construct were serum-starved, then stimulated with 50 ng/ml PDGF. Stimulation of endogenous PDGF receptor (PDGFR) generated a FRET increase, resulting in an increase in the ratio of yellow-to-cyan emissions (Fig. 1d and e; also see Movie 1, which is published as supporting information on the PNAS web site), which was detectable within several seconds and reached a plateau of 25.4 ± 3.7% (average ± SD, n = 9) within minutes (t1/2 = 3.45 ± 0.65 min). The FRET change occurred in the plasma membrane and was accompanied by the translocation of the reporter (Fig. 1d), reiterating that 3’ PIs are mainly produced at the plasma membrane (22). To verify that the FRET response is caused by binding of PIP3 and PI(3,4)P2, we generated a variant of the reporter in which two critical residues in the PH domain, Arg-23 and Arg-25, which mainly contribute to D-3 phosphate recognition (20), are mutated. As shown in Fig. 1c, incorporating the mutations R23A/R25A into the chimeric protein completely abolished the FRET change.

To determine whether production of 3’ PIs via PI3K activation is responsible for the FRET change, we pretreated cells with PI3K inhibitor LY294002 (20 μM). In the presence of the inhibitor, no emission ratio change was observed upon PDGF stimulation, but removal of the inhibitor recovered the response (Fig. 1f). In addition, this construct was shown to respond to microinjected dioctanoyl PIP3 and its soluble headgroup IP4 (Fig. 5, which is published as supporting information on the PNAS web site), suggesting the FRET change is caused by ligand binding.

We next tested whether the FRET response was reversible. Addition of LY294002 resulted in a decrease in emission ratios (Fig. 1g), presumably because of the degradation of PIP3 and PI(3,4)P2 by lipid phosphatases. Similarly, another PI3K inhibitor, wortmannin, when added at 500 nM, could reverse the PDGF-stimulated response (data not shown). Thus, this reporter is useful in monitoring not only the production of PI3K products but also their degradation, and is designated as the indicator for phosphoinositides based on Akt (InPAkt).

Differential Dynamics Stimulated by Different Growth Factors. Although PI3K is a crucial regulatory component shared by various growth factor pathways, their differential coupling to PI3K isoforms (23) and distinct modes of negative regulation (24) could lead to significant difference in PI dynamics, thereby differentiating downstream signaling. To compare the accumulation of PIP3 and PI(3,4)P2 induced by the activation of different tyrosine kinase receptor pathways, we applied three different ligands, namely insulin, insulin-like growth factor 1 (IGF-1), and PDGF, to serum-starved NIH 3T3 cells expressing InPAkt. As shown above, PDGF stimulation produced an acute response that reached a plateau of 25.4 ± 3.7% ratio increase in 5–9 min (t1/2 = 3.45 ± 0.65 min) (Fig. 2a). In contrast, addition of 50 nM IGF-1 to activate insulin-like growth factor receptor (IGF-IR) produced a more gradual response of 6.5 ± 0.8% (n = 5) in 10–12 min (t1/2 = 5.4 ± 0.4 min) (Fig. 2a). Finally, stimulation with 100 ng/ml insulin did not generate any discernable response, possibly because of low copies of insulin receptor (IR) in NIH 3T3 cells (25). Thus, the magnitude of the responses differed significantly in the increasing order of insulin << IGF-1 < PDGF.

To determine whether the mechanisms that account for the suboptimal responses stimulated by insulin or IGF-1 could affect the PDGF-stimulated responses, we applied three agonists sequentially with sufficient time intervals in between. First, the addition of insulin did not generate any appreciable change in the emission ratio, as shown above. When IGF-1 was added to

![Comparison of the cellular responses stimulated by various growth factors](image-url)
activate IGF-IR, the emission ratios increased by 6.8 ± 0.9% in 10–12 min (t1/2 = 5.2 ± 0.1 min; n = 3) and reached a plateau, indicating that the production and degradation of PIP3 and PI(3,4)P2 reached an equilibrium. Lastly, when these fibroblasts were stimulated with PDGF; the emission ratio further increased by 14.6 ± 1.4% in 6–7 min (n = 4; t1/2 = 3.2 ± 0.2 min) (Fig. 2 b and c), indicating that the balance between PI3K and phosphatases shifted more toward production of PIs and subsequently reached a new equilibrium. Conversely, when we reversed the order of the ligand addition, with PDGF addition first, followed by IGF-1, PDGF stimulated a full response of 24.2 ± 1.5% in 7–9 min (n = 2; t1/2 = 3.9 ± 0.2 min) that was not enhanced by the addition of IGF-1 (data not shown).

**Phosphoinositide Dynamics Within Subcellular Compartments. Plasma membrane.** Taking advantage of the targetability of InPAkt, we constructed several fusions of InPAkt with various specific targeting motifs (Fig. 3a) to monitor PIP3 dynamics at different subcellular locations inside cells. Attaching the plasma membrane-targeting sequence of small guanosine triphosphatase K-ras4B to the C terminus of the InPAkt localized the reporter to the plasma membrane (Fig. 3b). Upon PDGF stimulation, we observed a ratio change of 9.25 ± 0.4% (n = 4) in 6–8 min (t1/2 = 3.9 ± 0.7 min) (Fig. 3c). Compared with the responses from the untargeted reporter, (Figs. 3c and 1g), the change in emission ratio was lesser for the membrane targeted InPAkt. Furthermore, upon addition of LY294002, we observed that the emission ratio decreased dramatically and reached below the initial ratio in the resting state. These data suggest the existence of basal levels of PIP3 and PI(3,4)P2 at the plasma membrane maintained by a critical balance between lipid kinase and phosphatase activities. Indeed, inhibition of PI3K led to a decrease in emission ratio in nonstimulated cells (Fig. 6, which is published as supporting information on the PNAS web site).

**Nucleus.** Although PI signaling research mainly has been focused on events at the plasma membrane, recent experimental evidence suggested the presence of PIP3 in the nucleus. For instance, it has been reported that PI3K translocates from the cytoplasm to the nucleus (26), and the presence of estrogen in cells containing a transmembrane intracellular estrogen receptor resulted in the accumulation of PIP-binding PH domain in the nucleus (27). To directly visualize the presence and dynamics of PIP3 and PI(3,4)P2 in the nucleus, we targeted InPAkt to the nucleus by attaching a NLS (Fig. 3a), shown by colocalization with the nuclear staining with Hoechst dye (Fig. 3b). Upon stimulation with PDGF, the nuclear-targeted InPAkt did not elicit any response in NIH 3T3 cells (Fig. 3d). Furthermore, treatment with LY294002 did not produce any detectable change in FRET. On the other hand, microinjection of dioctanoyl PIP3 and IP3 into the nuclei of cells expressing the same construct generated emission ratio increases of 5.4 ± 2.2% (n = 3) and 9.1 ± 0.42% (n = 2), respectively (Fig. 7, which is published as supporting information on the PNAS web site), indicating that the nuclear-localized InPAkt was functional. Furthermore, surface plasmon resonance (SPR) analysis showed that attaching NLS did not significantly alter the binding affinity (Fig. 8, which is published as supporting information on the PNAS web site). Hence, results from our reporter indicate that there may not be any appreciable amounts of accessible PIP3 generated in the nucleus upon PDGF stimulation, in other words, these 3′-PIs, if produced, may be accumulated in specific subnuclear compartments or complexed with other cellular components.

**Simultaneous Imaging of Phosphoinositide Dynamics and Akt Phosphorylation.** Increasing evidence has suggested that Akt, an immediate downstream effector of 3′-PIs, is active within the nucleus (8). In the absence of accessible PIP3 and PI(3,4)P2 in the nucleus, how is nuclear Akt activity generated and maintained?

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**Fig. 3.** Fusions of InPAkt targeted to various subcellular locations. (a) Domain structure of the fusion constructs. (b) Localization of plasma membrane targeted InPAkt (pm InPAkt) is shown in the fluorescence image (YFP). Pseudocolor images show colocalization of nuclear targeted reporter (NLS InPAkt) with a cell-permeable DNA dye, Hoechst 3342. (c) A representative emission ratio time course from four independent experiments showing the response of plasma membrane targeted InPAkt stimulated with 50 ng/ml PDGF (9.25 ± 0.4%), followed by addition of 20 μM LY294002. (d) A representative emission ratio time course from three different trials for NLS InPAkt in NIH 3T3 cells stimulated with PDGF.

It has been suggested that Akt gets activated at the plasma membrane by up-regulated PIP3 and subsequently translocates to the cytosol and, at a later time point, to the nucleus (28). To directly assess the signal propagation from the plasma membrane to the nucleus, we used two different FRET reporters: InPAkt to monitor the production and degradation of PIP3 and PI(3,4)P2 at the membrane, and a nuclear-targeted B kinase activity reporter (BKAR) to monitor Akt activity in the nucleus.

Briefly, BKAR is a genetically encoded fluorescent reporter (29) that monitors Akt activity. BKAR is comprised of enhanced CFP and Citrine connected by a phosphoamino acid-binding domain (PBD) and an Akt-specific substrate sequence, where phosphorylation of the substrate and its subsequent binding to PBD lead to a change in FRET, resulting in an increase in the...
observed an immediate InPAkt response (phase I in Fig. 4a) when Akt was coexpressed, the spatial separation of the fluorescent sensors for monitoring PI dynamics. The first one, “fllip” (34), used PH domain of GRP1 and required membrane anchoring to facilitate a PI binding-induced conformational change via rotation of rigid linkers around a diglycine hinge engineered within the construct, which limits the targetability of the receptor and generalizability of the design. The second sensor, termed “CAY,” is based on a peptide from Listeria protein ActA that undergoes a random coil to helix transition upon lipid binding (35). CAY is a sensor for polyphosphorylated PIs showing some preference to PIP3 in cells.

InPAkt is a genetically targetable reporter for PIP3 and PI(3,4)P2, and offers dual readout of FRET change and translocation in its untargeted form. The conformational change does not rely on any membrane anchoring, which provides the flexibility of targeting the reporter to different subcellular regions or tethering it to signaling components to track specific pools of PIs. Furthermore, it may offer a generalizable design that relies on the binding of PIs to compete off a concatenated pseudoligand to generate the conformational change that leads to the FRET change.

Responses of InPAkt at the Plasma Membrane. The responses from the plasma membrane-targeted InPAkt upon activation or inhibition of PI3K indicated the presence of basal levels of PIP3 and PI(3,4)P2 at the plasma membrane. Thus, some of the reporter molecules were presaturated with PIs, and activation of PI3K generated a moderate increase; yet subsequent PI3K inhibition allowed phosphatases to act on both stimulated and basal PIs, leading to a larger decrease. The basal levels of these PIs may be involved in constitutively activating the plasma membrane-targeted Akt (m/pAkt). In fact, it was shown that m/pAkt was still subject to inhibition by PI3K inhibitors (28), supporting our finding that the basal levels of PIP3 and PI(3,4)P2 are maintained by balanced activities of PI3K and phosphatases.

We observed distinct patterns of PI dynamics in response to activation of three different receptor tyrosine kinases (RTK), namely IR, IGF-IR, and PDGF receptor (PDGFR), in NIH 3T3 cells, where PDGFR activation produces the largest response with fastest kinetics, consistent with data obtained by using radioactive labeling (36). On the other hand, insulin induced a larger and more sustained response from PI3K activation than PDGFR in 3T3 L1 adipocytes (37), one of the most insulin-responsive cells expressing high levels of insulin receptors. However, the observed difference in responses stimulated by various growth factors cannot be accounted for solely by the difference in the total receptor numbers, because the numbers of PDGFR and IGF-IR per cell were similar.
Although it is possible that small amounts of PIP₃ and PI(3,4)P₂ total PIP₃ measured by Tanaka et al. (28) are present yet below the detection limit of InPAkt, it is also plausible that these 3’ PI₃s are involved in maintaining the basal levels of 3’ PI₃s and in shaping the stimulated transient response of the lipid second messenger, protein phosphatase activities are kept low to allow Akt-mediated phosphorylation to sustain in the nucleus for 30–50 min or longer.

In conclusion, InPAkt is a genetically targetable reporter that is useful in monitoring PIP₃ and PI(3,4)P₂ dynamics globally or locally in a living cell. It offers a simple generalizable design and dual readout capability in its untargeted form. Dual FRET imaging (29, 32, 43) using targeted BKAR and InPAkt revealed high-resolution spatiotemporal dynamics of signal propagation from lipid messengers PIP₃ and PI(3,4)P₂ to nuclear Akt. The use of these genetically targetable reporters should offer great opportunities to delineate the molecular mechanisms of PI3K/Akt signaling.

We thank Drs. Daniel Raben, Susan Craig, Peter Devreotes, and Pablo Iglesias for help and/or discussions. This work was supported by the Johns Hopkins University School of Medicine (J.Z.), the W. M. Keck Center (B.A. and J.Z.), and the Young Clinical Scientist Award Program of the Flight Attendant Medical Research Institute (J.Z.).