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QUANTITATION OF PI[3,4,5]P3 DYNAMICS IN EGF-STIMULATED CARCINOMA CELLS: A comparison of PH domain-mediated versus immunological methods

Shu-Chin Yip^{1,2,4}, Robert J. Eddy², Angie M. Branch³, Huan Pang¹, Haiyan Wu¹, Ying Yan¹, Beth E. Drees³, Paul O. Neilsen³, John Condeelis², and Jonathan M. Backer¹

¹ Dept. of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY

² Dept. of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

³ Echelon Biosciences, Inc, Salt Lake City, UT

Abstract

Class IA phosphoinositide 3-kinases (PI3-kinase) generate the secondary messenger PI[3,4,5]P₃, which plays an important role in many cellular responses. The accumulation of PI[3,4,5]P₃ in cell membranes is routinely measured using GFP-labeled PH domains. However, the kinetics of membrane PI[3,4,5]P₃ synthesis and turnover as detected by PH domains has not been validated using an independent method. In the present study, we measured EGF-stimulated membrane PI[3,4,5]P₃ production using a specific monoclonal anti-PI[3,4,5]P₃ antibody, and compared the results to those obtained using PH domain-dependent methods. Anti-PI[3,4,5]P₃ staining rapidly accumulated at the leading edge of EGF-stimulated carcinoma cells. PI[3,4,5]P₃ levels were maximal at 1 min, and returned to basal levels by 5 min. In contrast, membrane PI[3,4,5]P₃ production, measured by the membrane translocation of either pSer473-Akt or an epitope-tagged PH domain from BTK (^{BTK}PH), remained approximately 2-fold above basal level throughout 4–5 min of EGF-stimulation. To determine the reason for this disparity, we measured the rate of PI[3,4,5]P₃ hydrolysis by measuring the decay of the PI[3,4,5]P₃ signal after LY294002 treatment of EGF-stimulated cells. LY294002 abolished anti-PI[3,4,5]P₃ membrane staining within 10 sec of treatment, suggesting that PI[3,4,5]P₃ turnover occurs within seconds of synthesis. In contrast, ^{BTK}PH membrane recruitment, once initiated by EGF, was relatively insensitive to LY294002. These data suggest that sequestration of PI[3,4,5]P₃ by PH domains may affect the apparent kinetics of PI[3,4,5]P₃ accumulation and turnover; consistent with this hypothesis, we found that GRP1 PH domains (which like BTK are specific for PI[3,4,5]P₃) inhibit PTEN dephosphorylation of PI[3,4,5]P₃ in vitro. These data suggest that anti-PI[3,4,5]P₃ antibodies are a useful tool to detect localized PI[3,4,5]P₃, and illustrate the importance of using multiple approaches for the estimation of membrane phosphoinositides.

Keywords

PI 3-kinase; phosphoinositides; PH domains; carcinoma cells; EGF; antibodies

Address Correspondence to: Dr. Jonathan M. Backer, Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, backer@aecom.yu.edu, Tel: 718-430-2153, Fax: 718-430-3749.

⁴Current address: Fox Chase Cancer Center, Philadelphia, PA

Introduction

Phosphoinositide 3-kinases (PI3-kinases) are key regulators of many cellular responses. The Class I PI 3-kinases, which include both receptor tyrosine kinase-activated (Class IA) and trimeric G-protein activated (Class IB) enzymes, produce primarily PI[3,4,5]P3 in intact cells. Production of this lipid in cellular membranes is thought to recruit and/or activate downstream signaling proteins, including serine/threonine protein kinases such as PDK1 and Akt [1], tyrosine kinase such as BTK [2], guanine nucleotide exchange factors such as Vav1, ARNO and GRP1 [3–5].

The analysis of phosphoinositide synthesis and localization has, in the past few years, been dominated by the use of eGFP-PH domain fusions. Most PH domains show low lipid binding specificity [6,7]. However, the few PH domains that show relatively high specificity, such as the PLC δ 1-PH domain for PI(4,5)P2 or the BTK and GRP1-PH domains for PI[3,4,5]P3 [8–10], have been used extensively as probes for phosphoinositide localization. However, these probes appear to provide a rather selective view of phosphoinositide localization. For example, as noted by Balla et al. [11], probes using the PLC δ 1-PH domain show almost exclusive localization to the plasma membrane, despite the fact that the presence of PI[4,5]P2 in the Golgi and in secretory vesicles is well established. Furthermore, Yu et al. note that in yeast, the phosphoinositide binding specificity of distinct PH domains is not predictive of their localization [7]. Finally, Varnai and Balla showed that despite the fact that the resynthesis of PI[4,5]P2 in ionomycin-treated cells is sensitive to micromolar concentrations of wortmannin, the localization of ^{PLC δ 1}PH-GFP is not. Thus, it is important to verify the results obtained with PH domain probes with other methods for the analysis of phosphoinositide localization.

In the present study, we have used a recently developed anti-PI[3,4,5]P3 antibody (Echelon Biosciences) to analyze PI[3,4,5]P3 dynamics at the leading edge of EGF-stimulated carcinoma cells. This antibody shows highly specific binding to PI[3,4,5]P3 *in situ*. When we compared different methods of PI[3,4,5]P3 detection, we found that both antibody staining and PH domain-mediated methods showed similar kinetics of PI[3,4,5]P3 accumulation, but the persistence of the signal was much greater with the PH domain probe. Consistent with this finding, we observe that the half-life of PI[3,4,5]P3 measured by antibody staining is significantly shorter than that detected by PH domain localization, and we find that PH domains from GRP-1, which specifically binds to PI[3,4,5]P3 [12,13], inhibit PTEN dephosphorylation of PI[3,4,5]P3 *in vitro*. Our data point to pitfalls in the exclusive use of PH domain-mediated methods to detect PI[3,4,5]P3 levels, and show that immunostaining methods are a valuable additional approach for quantitation and localization of intracellular PI[3,4,5]P3.

EXPERIMENTAL

Materials

Monoclonal anti-PI[3,4,5]P3 antibodies were a generous gift from Echelon Bioscience Incorporated (Salt Lake City, UT). Anti-phospho-Ser473 antibodies were purchased from Upstate Biotechnology (Charlottesville, VA). Monoclonal anti-Myc antibodies (clone 4E-10; ATCC, Rockville, MD) were isolated from mouse ascites. Wortmannin and LY294002 were obtained from CalBiochem (San Diego, CA). A construct expressing GFP-PTEN (wild-type or catalytically inactive) was a gift from Dr. Peter Downes, Univ. of Dundee. Recombinant PH domains from GRP-1 were produced in house by Echelon Biosciences (Salt Lake City, UT). A construct for the dynamin PH domain was provided by Dr. Mark Lemmon, Univ. of Pennsylvania. The dynamin PH domain was produced in

bacteria as a GST fusion, purified with glutathione Sepharose beads, and cleaved from GST with thrombin.

Cell Culture and EGF Stimulation

The rat mammary adenocarcinoma breast cancer cell line, MTLn3, has been previously described [14]. Prior to EGF stimulation, MTLn3 cells were starved in L15 media (GIBCO BRL) supplemented with 0.35% BSA in a 37°C, non- CO₂ incubator for 3-4 hours. EGF stimulations used a final concentration of 5 nM EGF (Upstate Biotechnology) and were performed in a 37 °C water bath [14].

Constructs and Transient Transfection

The GFP/myc-tagged ^{BTK}PH construct was obtained from Dr. Skolnik (NYU Medical Center). Transient transfection of MTLn3 cells were performed using Lipofectamine Plus reagents (Invitrogen). After 48 hours, ^{BTK}PH transfected cells were quiesced, stimulated with EGF for various times, fixed and immunostained with monoclonal anti-Myc antibody.

Quantitative Fluorescence Microscopy

Cells were plated on collagen-coated coverslips 24 hours prior to the experiment. EGF-stimulated cells were rapidly fixed and permeabilized in 3.7% paraformaldehyde/0.1% glutaraldehyde/0.15 mg/ml saponin solution prepared in fix buffer (5 mM KCl, 137 mM NaCl, 4 mM NaHCO₃, 0.4 mM KH₂PO₄, 1.1 mM Na₂HPO₄, 2 mM MgCl₂, 5 mM PIPES pH 7.2, 2 mM EGTA, and 5.5 mM glucose) for 1 hour at 37°C. The fixed cells were incubated with primary antibodies for 1 hour, followed by secondary antibodies for 45 min, and mounted in 6 mg/ml of N-propyl gallate prepared in 50% glycerol-PBS (v/v). All fluorescence images were obtained using a 60X 1.4 NA Olympus objective and a cooled CCD camera. We have previously shown, using atomic force microscopy, that the height of the lamellipod in EGF-stimulated MTLn3 cells is approximately 600–800 nm [15]. Thus, the entire lamellipod is within one optical section and the use of confocal microscopy was not necessary. For fluorescence quantification, digital images were imported in NIH image software and analyzed using a previously described macro [16]. This macro collects pixel intensities from the perimeter of the cell in a 0.22 µm stepwise manner. The pixel intensities in the leading edge compartment, defined as the first 3 slices (0.66 µm) from the perimeter of the cells, were averaged and normalized to the edge intensity of non-stimulated cells.

Analysis of endogenous PI[3,4,5]P₃ production

MTLn3 cells were cultured in insitol-free medium for four days, then labeled [³H]myo-inositol (Perkin-Elmer) for two days. The cells were starved in serum-free media for 4h, then then stimulated with EGF for various times. Lipids were extracted and deacylated as described by Stephens et al. [17], and glycerol phosphoinositides were analyzed by anion exchange HPLC using a Partisil-SAX column as described by Auger et al. [18]. PI[4,5]P₂ and PI[3,4,5]P₃ standards were prepared by deacylation of [³H]-labeled PI[4,5]P₂ and PI[3,4,5]P₃, the latter produced in vitro with recombinant p85/p110.

PI3-kinase inhibition assays

MTLn3 cells were stimulated with EGF for 1 min, then rapidly washed with warm media containing either 0.1% DMSO or 20µM LY294002 in the continued presence of 5 nM EGF. After various times of drug treatment, the cells were permeabilized, fixed and stained as described above.

PTEN assays

Di-C8 PI(3,4,5)P3 substrate (1,500 pmol/point) was diluted in assay buffer (Tris-buffered saline containing 31 mM DTT) then combined with the indicated concentrations of purified PH domain protein and incubated for 15 minutes at room temperature on an orbital plate shaker. PTEN reactions were initiated by adding 110 ng of recombinant PTEN, mixing for 1 min, then incubating at 37 °C for an additional 14 min. Reactions were terminated by the addition of Malachite Green solution and the color developed for 15 min before reading absorbance at 620 nm on a SPECTRAmax plate reader. Background (no PTEN) values were subtracted from each measurement. Each protein concentration was assayed in duplicate, and the experiment was repeated 5 times with similar results using comparable controls.

RESULTS

Specific Detection of EGF-stimulated PI[3,4,5]P3 Production in MTLn3 cells

We used a monoclonal anti- PI[3,4,5]P3 antibody to measure the spatial and the temporal distribution of endogenous PI[3,4,5]P3 in EGF-stimulated rat adenocarcinoma cells (MTLn3). To confirm the specificity of the anti-PI[3,4,5]P3 antibody, we pre-absorbed the antibody with either PI, PI[4,5]P2 or PI[3,4,5]P3 lipid vesicles at 4°C for 1 hr prior to cell staining. We found that incubation of the PI[3,4,5]P3 antibody with PI[3,4,5]P3 – containing vesicles abolished membrane staining of EGF-stimulated MTLn3 cells (Figure 1A, bottom left panel, and Figure 1B). In contrast, incubation with PI or PI[4,5]P2 vesicles had no effect on PI[3,4,5]P3 staining (Figure 1A, top left and right panels respectively, and Figure 1B). This is a particularly important control, as PI[4,5]P2 levels in cells are 200-fold higher than PI[3,4,5]P3 levels [19]. Quantification of PI[3,4,5]P3 staining after pre-absorption with various mono- and di-phosphoinositides showed that the anti- PI[3,4,5]P3 antibody was unaffected by PI, PI[3]P, PI[4]P or PI[4,5]P2 vesicles (Fig. 1B). While incubation with PI[3,4]P2 vesicles slightly reduced the PI[3,4,5]P3 staining, the reduction was not statistically significant (Figure 1B). PI[3,4,5]P3 staining was also abolished in wortmannin-treated cells (Figure 1A, bottom right panel, and Figure 1B). Finally, anti-PI[3,4,5]P3 staining was markedly diminished in cells overexpressing GFP-tagged PTEN (Fig. 1C); expression of a catalytically inactive mutant of PTEN had no effect on PI[3,4,5]P3 staining (data not shown). Our results show that the anti-PI[3,4,5]P3 antibody is specific, and has minimal cross reactivity with either PI or PI[4,5]P2, which are the most abundant phosphoinositides in mammalian cells.

The kinetics of EGF-induced PI[3,4,5]P3 production at the leading edge of the lamellipod

We have recently examined the kinetics of PI[3,4,5]P3 production in EGF-stimulated MTLn3 cells using the anti-PI[3,4,5]P3 antibody [20]. These cells respond to EGF by producing a broad flat ruffle-free lamellipod, whose height is approximately 0.5 μ m [15]. Protrusion is maximal by 3 min, and is driven by the PI 3- kinase-dependent polymerization of actin filaments at the leading edge of the protruding cell [14,21,22]. In quiescent MTLn3 cells, membrane associated PI[3,4,5]P3 is not detected (Figure 2A left panel). However, after EGF stimulation, the anti-PI[3,4,5]P3 antibody detected a narrow band of punctate staining at the cell periphery (Figure 2A, right panel). As previously noted [20], quantification of the edge fluorescence intensity (0.66 μ m from the periphery of the cell) showed that EGF-stimulated PI[3,4,5]P3 production showed a sharp peak at 1 min. PI[3,4,5]P3 staining at the edge was still detectable at 3 min, when the protrusion of the lamellipod is maximal [14,21]. Anti- PI[3,4,5]P3 staining was markedly reduced at 4 min and returned to basal level by 5 min. Our results show that EGF-stimulated PI[3,4,5]P3 production is rapid, with maximal levels at 1 min, and declines to basal levels by 4–5 min, after the peak of MTLn3 protrusion [14,20]. A similar profile was obtained when PI[3,4,5]P3 production was measured by HPLC analysis of deacylated lipids extracted from

[3H]-myo-inositol-labeled cells, with PI[3,4,5]P3 levels maximal at 1 min of EGF stimulation. We were unable to detect the increased PIP3 at 3 and 5 min, presumably due to the lesser sensitivity of detection by metabolic labeling versus the antibody-based method.

To measure PI[3,4,5]P3 production at the leading edge of EGF-stimulated cells using another independent method, MTLn3 cells were transfected with a GFP/myc-tagged PH domain from BTK (^{BTK}PH), which has high binding specificity for PI[3,4,5]P3 [8,9]. The cells were stimulated, fixed and immunostained with anti-Myc antibody. In quiescent MTLn3 cells, anti-Myc staining was cytosolic and largely excluded from the cell periphery (Figure 2C, left panel). ^{BTK}PH was rapidly recruited to the leading edge of EGF-stimulated cells, where the maximal edge intensity occurred at 50 sec after stimulation (Figure 2C & D). However, unlike the data obtained with anti-PI[3,4,5]P3 staining or metabolic labeling, which show a sharp peak of PIP3 followed by a decline to basal levels, a sustained elevation of ^{BTK}PH at the cell edge was observed throughout 5 min of EGF stimulation (Figure 2C & D).

Kinetics of PI[3,4,5]P3 hydrolysis

The production of PI[3,4,5]P3 at the leading edge of EGF-stimulated carcinoma cells is detected within 1 min, and persists up to 3 min or greater than 5 minutes, depending on the detection method used. This could reflect the timecourse of PI 3-kinase activity in MTLn3 cells. Alternatively, persistent PI[3,4,5]P3 levels could be due to slow hydrolysis of PI[3,4,5]P3. To distinguish these possibilities, a reversible PI3-kinase inhibitor, LY294002, was used to inhibit PI3-kinase activity in EGF-stimulated cells. While LY294002 is less specific than wortmannin [23], it was chosen for these experiments for its rapid onset of inhibition, and because the experiment focuses directly on PI[3,4,5]P3, rather than on downstream signaling. MTLn3 cells were stimulated with EGF for 1 min, a time when PI[3,4,5]P3 production is maximal. The medium was then rapidly replaced with warm media containing EGF plus LY294002 or carrier. At various times, the cells were then fixed, and PI[3,4,5]P3 production was measured by staining with anti-PI[3,4,5]P3 antibodies.

Leading edge anti-PI[3,4,5]P3 staining in carrier-treated cells (Figure 3B, left panels) was similar to that in untreated cells (Figure 3A). In contrast, the intensity of anti-PI[3,4,5]P3 staining decreased rapidly after LY294002 treatment (Figure 3B, right panels). Higher magnification of these images showed that treatment of cells with LY294002 significantly attenuated leading edge PI[3,4,5]P3 levels within 10 sec (Figure 3C). Quantification of the leading edge fluorescence intensity showed that membrane PI[3,4,5]P3 levels were reduced to ~18% of maximal after 10 sec of LY294002 treatment, whereas membrane PI[3,4,5]P3 in carrier-treated cells remained within ~80-100% of maximal. After 60 sec of LY294002 treatment, leading edge PI[3,4,5]P3 staining was completely abolished (Figure 3D), whereas PI[3,4,5]P3 levels in carrier-treated cells were at ~30-40% of maximal. This latter value is consistent with the kinetics of EGF-stimulated PI[3,4,5]P3 production in untreated cells (Figure 2B). These results indicate that EGF-stimulated membrane PI[3,4,5]P3 turnover is extremely fast, and that the sustained elevation of leading edge PI[3,4,5]P3 in untreated cells requires persistent PI3-kinase activity.

We next examined the rate of PI[3,4,5]P3 hydrolysis as detected by the PH-domain membrane translocation assays. ^{BTK}PH-transfected cells were stimulated with EGF for 1 min, then treated with LY294002 or carrier in the presence of continuing EGF stimulation. ^{BTK}PH was rapidly recruited to the plasma membrane of EGF-stimulated cells (Figure 3E). Unlike immunostaining of leading edge PI[3,4,5]P3 production, membrane localization of ^{BTK}PH was relative insensitive to LY294002 (Figure 3F, right panels). Higher magnification of the membrane ^{BTK}PH showed that the fluorescence intensity of ^{BTK}PH at the leading edge was only slightly reduced after 60 sec of LY294002 treatment

(Figure 3G). After 120 sec of LY294002 treatment, the translocation of ^{BTK}PH was reduced to 10% of maximal, whereas the carrier-treated cells remained at ~60% of maximal levels (Figure 3H). Consistent with previous findings [24], our results indicated that the half-life of membrane PI[3,4,5]P3 as detected in the PH-domain-dependent assay was approximately 60 sec.

Overexpression of ^{BTK}PH delays membrane PI[3,4,5]P3 hydrolysis

To determine the reason for the relatively slow rate of decay of membrane-targeted ^{BTK}PH in LY294002-treated cells, we measured the rate of PI[3,4,5]P3 turnover by anti-PI[3,4,5]P3 immunostaining method in cells transfected with the ^{BTK}PH construct. The cells were stimulated with EGF for 1 min, treated with LY294002 or carrier in the continued presence of EGF for various times, and fixed and stained with anti-PI[3,4,5]P3 antibody. The maximal signal in ^{BTK}PH-transfected after 1 min of EGF stimulation was higher than in untransfected cells (Figure 4A), consistent with the sequestration of PI[3,4,5]P3 by the overexpressed PH domain. Furthermore, anti-PI[3,4,5]P3 staining in cells transfected with ^{BTK}PH was relatively insensitive to LY294002 treatment (Figure 4B). This was in marked contrast to non-transfected cells, where leading edge PI[3,4,5]P3 staining was virtually undetectable within 10 sec of LY294002 treatment. Quantification of leading edge fluorescence showed that PI[3,4,5]P3 levels in ^{BTK}PH transfected cells remained ~60–70% above basal levels after treatment with either DMSO or LY294002, whereas membrane PI[3,4,5]P3 levels in non-transfected cells dropped to ~10% after 10 sec of LY 294002 treatment. These results suggest that overexpression of ^{BTK}PH alters the kinetics of PI[3,4,5]P3 hydrolysis.

To test the hypothesis that the binding of ^{BTK}PH domains to PI[3,4,5]P3 could inhibit PTEN-mediated hydrolysis *in vivo*, we measured the PTEN-catalyzed hydrolysis of short-chain (Di-C8) PI[3,4,5]P3 in the presence of recombinant PH domain from GRP-1, which like BTK binds to specifically PI[3,4,5]P3 [12,13], or from dynamin, which preferentially binds to PI[4,5]P2 [9]. Whereas ^{dynamin}PH had little effect on PTEN activity, significant inhibition was observed with ^{GRP-1}PH (Fig. 5). Thus, PH domains that bind PI[3,4,5]P3 inhibit the hydrolysis of this lipid both *in vitro* and in cells.

DISCUSSION

We have examined the kinetics of PI[3,4,5]P3 synthesis and turnover in EGF-stimulated MTLn3 cells, where Class IA PI3-kinases are required for barbed end formation and protrusion [22]. Membrane PI[3,4,5]P3 levels increase rapidly after EGF stimulation and remain elevated for up to 3 min in EGF-stimulated cells. Furthermore, we show that membrane PI[3,4,5]P3 turnover is extremely rapid, with a half-life of less than 10 sec. Given the speed of PI[3,4,5]P3 turnover, the kinetics of PI[3,4,5]P3 levels in the plasma membrane directly reflect the kinetics of PI 3-kinase activity. Thus, the sustained elevation of leading edge PI[3,4,5]P3 production in EGF-stimulated cells requires persistent PI3-kinase activation at the leading edge of protruding cells.

Recent advances in defining the mechanisms that drive chemotaxis have focused on the asymmetric production of PI[3,4,5]P3 at the leading edge. These studies have widely used the PH-domain-containing proteins to detect membrane localization of phosphoinositide lipids [25,26]. While the use of GFP-linked PH-domain probes is clearly advantageous for live-image microscopy, our data suggest that the kinetics of PI[3,4,5]P3 accumulation in live cell PH-domain assays may be inaccurate. When we directly compared measurements of PI[3,4,5]P3 turnover using different methods, we found that PH-domain-dependent assays showed a slow rate of PI[3,4,5]P3 turnover, with a half-life of approximately 60 sec. This is consistent with a previous study in which the half-life of PI[3,4,5]P3 turnover in insulin-

stimulated 3T3-L1 cells was estimated to be 1 min, based on the membrane translocation of an ^{AKT}PH-CFP construct [24]. Our data suggest that PH-domain-dependent assays overestimate the lifetime of PI[3,4,5]P3 relative to anti-PI[3,4,5]P3 immunostaining assays. This is likely to be the explanation for the fact that both pAkt and PH-domain-based assays show that PI[3,4,5]P3 levels remain elevated for over 5 min in EGF-stimulated cells, whereas anti-PI[3,4,5]P3 staining shows that PI[3,4,5]P3 returns to basal levels by 5 min. Interestingly, the ^{BTK}PH probe responded to EGF-stimulation with similar initial kinetics to that seen in anti-PI[3,4,5]P3 immunostained cells (Figure 2). These results suggest that even though the protective effects of the PH-domain probes are likely to distort the decay rates of PI[3,4,5]P3 signaling, these probes may accurately measure initial rates of PI[3,4,5]P3 accumulation. It should be noted that these problems do not apply to the recently described use of PH domains for electron microscopic analysis of phosphoinositide localization and abundance [27–29].

While expression of PH domains may alter the apparent kinetics of lipid accumulation/degradation, they may be a less perturbing and more accurate method of analyzing the spatial distribution of membrane phosphoinositides. As noted by Downes, Lucocq and coworkers, membrane lipids are less likely to be immobilized than membrane proteins by aldehyde-based fixation methods [27]. This raises the question of potential lateral diffusion of membrane-bound PI[3,4,5]P3 using the antibody-based method. Furthermore, the fixation method used here requires the presence of saponin (0.15 mg/ml). Triton concentrations as low as 0.0025% induce clustering of PI[4,5]P2 detectable by FRET, with visible clustering observed at 0.005% Triton [30]. However, aldehyde fixation would certainly block membrane trafficking and changes in cell spreading, as well as production of new PI[3,4,5]P3. In the kinetic studies in this paper, PI[3,4,5]P3 was quantitated by measuring total fluorescence intensity in 0.2 μ m concentric slices in from the cell perimeter. While detergent-induced lipid clustering might affect measurements from a subjectively defined region of interest, it would be unlikely to affect the total fluorescence in concentric slices, which sample the entire cell periphery. Furthermore, samples at 1 min (the peak of PI[3,4,5]P3 accumulation) are subjected to the same treatment as samples from later times, suggesting that the rapid loss of PI[3,4,5]P3 staining in LY294002-treated cells is not due to diffusion of the lipid during processing.

A final difference between the two methods is the availability of the membrane PI[3,4,5]P3 to the probe. If membrane PI[3,4,5]P3 was rapidly bound by effector proteins, this could limit its detection by anti-PI[3,4,5]P3 antibodies. Intracellular PH domain probes would presumably be less subject to this constraint, as they would compete with signaling molecules for PI[3,4,5]P3 during signal generation. However, immuno-detection of membrane PI[3,4,5]P3 after 1 min of EGF stimulation is robust, even though this is also the time of peak PH domain recruitment (based on the kinetics of ^{BTK}PH and endogenous Akt translocation). Furthermore, maximal anti-PI[3,4,5]P3 staining is increased, not decreased, in cells expressing ^{BTK}PH. These data would suggest that antibody binding is not limited by effector binding. Presumably, PH domain-associated-PI[3,4,5]P3 can be detected by subsequent anti-PI[3,4,5]P3 staining, perhaps due to structural alterations in the PH domain during fixation that lead to a reduced affinity for PI[3,4,5]P3.

What is the mechanism for the prolonged half-life of PI[3,4,5]P3 as detected by ^{BTK}PH membrane recruitment? The simplest explanation is that PH-domain binding interferes with the accessibility of membrane-bound PI[3,4,5]P3 to lipid phosphatases. This model, in which the inositol head group of PI[3,4,5]P3 is sequestered by the PH domain, is consistent with our in vitro data showing that PH domains from GRP-1 inhibit PTEN dephosphorylation of PI[3,4,5]P3. This is in contrast to experiments using the PLC δ PH domain to measure plasma membrane PI[4,5]P2 levels; in this case, the probe was shown to

rapidly cycle on and off the membrane, suggesting that it was unlikely to sequester its lipid target [31]. The published binding affinities of the PLC δ PH domain for PI[4,5]P₂ and the BTK PH domain for PI[3,4,5]P₃ (K_D 1.7 vs. 0.8 μ M) are similar, although the measurements were made using different methods [8,32]. Thus, based on lipid binding affinity alone, one would predict similarly fast on/off rates for the BTK PH domain. It is possible that for the BTK PH domain, hydrophobic residues of the PH-domain that interact with the membrane may increase the effective binding affinity for PI[3,4,5]P₃ in the plasma membrane [11].

An alternative explanation for slower PI[3,4,5]P₃ turnover as measured by PH^{BTK} is that the *in vitro* phosphoinositide binding by the PH domains may not correlate with their behavior *in vivo* [11]. For example, the recombinant SOS PH domain binds selectively to PI[4,5]P₂ lipid vesicles in an *in vitro* assay, but the membrane localization of the SOS PH domain appears to be PI[4,5]P₂ independent *in vivo* [33]. Similarly, while a critical role for PI[4,5]P₂ in the secretory pathway is well established biochemically, PLC δ 1 PH- GFP probes fail to recognize PI[4,5]P₂ in the Golgi and secretory vesicles [11]. Finally, it is important to emphasize that PH domains are capable of protein-protein interactions [34]. Mutagenesis studies of PH-domains have shown that the inhibitory effect of PH-domain expression can be independent of their lipid binding properties, suggesting that PH-domains might interact with other proteins [34,35].

Interestingly, our results show that overexpression of BTK^{PH} delays membrane PI[3,4,5]P₃ hydrolysis in both the BTK^{PH} translocation (Figure 3E-H) and anti- PI[3,4,5]P₃ staining (Figure 4) assays. Expression of BTK^{PH} is higher than that of endogenous PH domain-containing proteins, and therefore could lead to the sequestration of PI[3,4,5]P₃. Alternatively, we cannot rule out the possibility that overexpression of BTK^{PH} in cells in fact alters the hydrolysis rate of endogenous PI[3,4,5]P₃ by interfering with EGF signaling, leading to an inhibition of cellular lipid phosphatase activity or expression. Consistent with this latter explanation, a recent study showed that overexpression of isolated PH domains can disrupt PI[3,4,5]P₃-regulated cellular processes [35]. Similarly, overexpression of the dynamin-1 PH domain suppresses the process of rapid endocytosis [10], and expression of the BTK PH domain inhibits AKT activation [35].

In summary, the use of anti-PI[3,4,5]P₃ immunostaining for measuring PI[3,4,5]P₃ production has both advantages and disadvantages relative to live cell PH-domain-mediated techniques. Immunofluorescence analysis of PI[3,4,5]P₃ using anti- PI[3,4,5]P₃ antibodies eliminates the possibility of perturbing effects of PH domain overexpression, and may more accurately reflect the kinetics of endogenous PI[3,4,5]P₃ levels. We have used this method to define the kinetics of PI[3,4,5]P₃ accumulation in EGF-stimulated carcinoma cells, and we have demonstrated an extremely rapid turnover rate for PI[3,4,5]P₃. On the other hand, the possibility of lipid diffusion in fixed cells, and detergent-induced lipid clustering, may limit the spatial resolution of anti-PI[3,4,5]P₃ staining. Nonetheless, our data support the use of this method as a valuable addition to analyses using PH domains and other lipid binding domains.

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Abbreviations

BSA	bovine serum albumin
BTK	Bruton Tyrosine Kinase
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
GFP	green fluorescent protein
PKC	Phosphoinositide dependent kinase 1
PH domain	Pleckstrin homology domain
PI[3,4,5]P3	phosphatidylinositol 3,4,5-P3
PLC	phospholipase C

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FIGURE 1. Specific staining of EGF-stimulated PI[3,4,5]P3 at the leading edge of lamellipod
 Quiescent MTLn3 cells were stimulated with 5nM EGF for 1min prior to fixation. **A.** Cells were stained with anti-PI[3,4,5]P3 antibody pre-absorbed with either PI, PI[4,5]P2, or PI[3,4,5]P3 lipid vesicles as indicated. Alternatively, cells were treated with 100 nM wortmannin prior to EGF stimulation, fixation and anti-PI[3,4,5]P3 staining (lower right). **B.** Quantified edge fluorescence intensity of unstimulated or EGF-stimulated cells stained with anti-PI[3,4,5]P3 antibody that had been preabsorbed with the indicated lipid vesicles. Data are the mean $\pm$ SEM from three separate experiments. **C.** MTLn3 cells were transfected the eGFP-PTEN. Quiescent cells were stimulated as above, fixed and stained with anti-PI[3,4,5]P3 antibodies. Left panels show GFP, right panels show anti-PI[3,4,5]P3. [17]

FIGURE 2. Kinetics of PI[3,4,5]P3 production in EGF-stimulated cells using both PH-domain-dependent and -independent assays

A. Unstimulated or EGF-stimulated (3 min) MTLn3 cells were stained with anti-PI[3,4,5]P3 antibodies. **B.** Cells were stimulated with EGF for various times, fixed and stained with anti-PI[3,4,5]P3 antibodies. Fluorescence intensity of anti-PI[3,4,5]P3 staining at the cells edge (0.66 μ m thickness) was quantified as described in Experimental Procedures. The data show the mean $\pm$ SD from two experiments. **C.** MTLn3 cells were labeled with [3H]-myo-inositol for 2 days, then stimulated with EGF as indicated. Lipids were extracted and deacylated, and glycerophosphoinositides were analyzed by anion-exchange HPLC. Left-hand panel: typical profile showing the PIP2/PIP3 region of the chromatograms from cells stimulated with EGF for 0, 1, 3 or 5 min. Right-hand panel: PIP3 levels in EGF-stimulated cells. The data are the mean $\pm$ SEM from three experiments. **D.** Unstimulated or EGF-stimulated (3 min) BTKPH-transfected MTLn3 cells were stained with anti-myc antibodies. **E.** BTKPH-transfected MTLn3 cells were stimulated with EGF for various times, fixed and stained with anti-myc antibodies. Staining was quantified as in (B). Data show mean values $\pm$ SEM from four independent experiments.

Figure 3. Comparison of membrane PI[3,4,5]P3 hydrolysis as measured by anti-PI[3,4,5]P3 staining versus BTKPH translocation

PI[3,4,5]P3 hydrolysis was measured in control MTLn3 cells (AD) or cells transfected with BTKPH (E–H). **A, E.** Representative images of unstimulated cells (right panel) and cell stimulated with EGF for 1 min (left panel), stained with either anti-PI[3,4,5]P3 antibody (A) or Anti-Myc antibody (E). **B, F.** Cells were stimulated with EGF for 1 min and then treated with DMSO or LY294002 for various times. The cells were fixed and stained with either anti-PI[3,4,5]P3 antibody (B) or Anti-Myc antibody (F). **C, G.** Magnification of the leading edge PI[3,4,5]P3 in DMSO or LY294002-treated cells as measured by anti-PI[3,4,5]P3 staining (C) or anti-Myc staining (E). **D, H.** Quantification of leading edge PI[3,4,5]P3 levels as measured by anti-PI[3,4,5]P3 staining (D) versus anti-Myc staining (H). The data

are mean $\pm$ SEM from three independent experiments, and are presented as % of maximal edge intensity, which is observed at 1 min of EGF stimulation. The image scale bars represent 10 μ m in all panels.

Figure 4. Overexpression of BTKPH decreases the apparent rate of PI[3,4,5]P3 hydrolysis
Control or Myc-BTKPH-transfected cells were stimulated with EGF for 1 min, treated with DMSO or LY294002 for various times, then fixed and stained with anti-PI[3,4,5]P3 antibodies. **A.** Representative images of BTKPH-transfected cells stimulated with EGF for 1 min. Left panel: Anti-myc staining identify transfected cells. Right panel: anti PI[3,4,5]P3 staining. **B.** Quantification of leading edge PI[3,4,5]P3 in BTKPH-transfected cells (BTK-PH) versus non-transfected cells (Ctl), treated with DMSO carrier or LY294002. The data are mean + SEM from three independent experiments, and are presented as % of maximal edge intensity, which is observed at 1 min of EGF stimulation under each condition.

Figure 5. PTEN is inhibited by PH domains from GRP-1

PTEN activity was measured in the presence of recombinant PH domains from GRP-1 or dynamin, using the Malachite Green assay kit from Echelon Biosciences (Salt Lake City, UT).