Rapid Turnover Rate of Phosphoinositides at the Front of Migrating MDCK Cells

Teruko Nishioka,* Kazuhiro Aoki,* Kazuhiro Hikake,* Hisayoshi Yoshizaki,[†]
Etsuko Kiyokawa,* and Michiyuki Matsuda*

*Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan; and [†]Department of Structural Analysis, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan

Submitted March 25, 2008; Revised July 14, 2008; Accepted July 29, 2008
Monitoring Editor: Mark H. Ginsberg

Phosphoinositides (PtdInss) play key roles in cell polarization and motility. With a series of biosensors based on Förster resonance energy transfer, we examined the distribution and metabolism of PtdInss and diacylglycerol (DAG) in stochastically migrating Madin-Darby canine kidney (MDCK) cells. The concentrations of phosphatidylinositol (4,5)-bisphosphate, phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), phosphatidylinositol (3,4)-bisphosphate, and DAG were higher at the plasma membrane in the front of the cell than at the plasma membrane of the rear of the cell. The difference in the concentrations of PtdInss was estimated to be less than twofold between the front and rear of the migrating MDCK cells. To decode the spatial activities of PtdIns metabolic enzymes from the obtained concentration maps of PtdInss, we developed a one-dimensional reaction diffusion model of PtdIns metabolism. In this model, the activities of phosphatidylinositol monophosphate 5-kinase, phosphatidylinositol 3-kinase, phospholipase C, and PIP₃ 5-phosphatases were higher at the plasma membrane of the front than at the plasma membrane of the rear of the cell. This result suggests that, although the difference in the steady-state level of PtdInss is less than twofold, PtdInss were more rapidly turned over at the front than the rear of the migrating MDCK cells.

INTRODUCTION

Cell migration is an important event during early development, inflammatory responses to infection, and wound healing, and it is an important pathological event during tumor invasion and metastasis. Despite morphological and functional differences, different migratory cells share a conserved set of polarity signals. Kinases for phosphoinositides (PtdInss), Rho GTPases, and the actin and microtubule cytoskeletons play key roles in signaling polarity in cells ranging from

Dictyostelium discoideum (Charest and Firtel, 2006) to neurons (Luo, 2000; Aoki *et al.*, 2007) and neutrophils (Van Keymeulen *et al.*, 2006; Wong *et al.*, 2006).

PtdInss are a family of phospholipids containing *myo*-inositol as their head group (reviewed in Takenawa and Itoh, 2001). Despite a relatively low abundance in biological membranes, PtdInss have been reported to regulate a myriad of cellular processes. Among them, phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P₂] is the major PtdInss at the inner leaflet of the plasma membrane. On growth factor stimulation, intracellular second messengers such as inositol (1,4,5)-trisphosphate (IP₃), diacylglycerol (DAG), and phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) are generated from PI(4,5)P₂.

The discovery that the pleckstrin homology (PH) domain in the signaling proteins recognizes specific phosphoinositides revealed that stimulated proteins translocate to specific regions of the membrane to participate in signaling events via interaction with these lipids (reviewed in Di Paolo and De Camilli, 2006). A series of experiments indicated that the PH domains from different proteins recognize different PtdInss and led to the development of a novel tool for detecting the localization of these lipids in living and fixed cells by fusing the green fluorescent protein (GFP)-tag to the PH domain (reviewed in Di Paolo and De Camilli, 2006). The drawback of these GFP-tagged probes is that the images obtained with them are substantially affected by the ratio of the surface area of the plasma membrane to the cytosolic volume (surface-to-volume ratio [SVR]) (Craske *et al.*, 2005). For example, by using a GFP-tagged PH domain, a location with a high SVR, such as the membrane ruffles, provides pseudopositive signals even when the PtdIns distribution is uniform. Therefore, GFP-tagged probes require appropriate

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-03-0315>) on August 6, 2008.

Address correspondence to: Etsuko Kiyokawa (kiyokawa@lif.kyoto-u.ac.jp).

Abbreviations used: PtdInss, phosphoinositides; PI(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate; PIP, phosphatidylinositol monophosphate; IP₃, inositol (1,4,5)-trisphosphate; DAG, diacylglycerol; PIP₃, phosphatidylinositol (3,4,5)-trisphosphate; PH, pleckstrin homology; GFP, green fluorescent protein; FRET, Förster resonance energy transfer; PI(3,4)P₂, phosphatidylinositol (3,4)-bisphosphate; Pippi, phosphatidylinositol phosphate indicator; PLC, phospholipase C; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; PDGF, platelet-derived growth factor; Digda, diacylglycerol indicator; OAG, 1-oleoyl-2-acetylgllycerol; PI(4)P, phosphatidylinositol (4)-monophosphate; PAO, phenylarsine oxide; PI 4-kinase, phosphatidylinositol 4-kinase; FKBP, FK506-binding protein; 5ptase, phosphoinositide 5-phosphatase; FRB, fragment of mammalian target of rapamycin; PI3-kinase, phosphatidylinositol 3-kinase; PA, phosphatidic acid; PI(4)P 5-kinase, phosphatidylinositol (4)-monophosphate 5-kinase; PIP₂ 5-Pase, PIP₂ 5-phosphatase; PTEN, phosphatase and tensin homologue; PIP₃ 5-Pase, PIP₃ 5-phosphatase.

negative controls and quantitative analysis (Yoshizaki *et al.*, 2007).

Förster (or fluorescence) resonance energy transfer (FRET) is a process by which a fluorophore (donor) in an excited state nonradiatively transfers its energy to a neighboring fluorophore (acceptor), thereby causing the acceptor to emit fluorescence at its characteristic wavelength. Variants of GFP have provided genetically encoded fluorophores that serve as donors and/or acceptors in FRET (Pollok and Heim, 1999; Miyawaki *et al.*, 2003). Using these GFP variants and FRET technology, FRET biosensors have been reported for PIP_3 , $\text{PI}(3,4)\text{P}_2$, and DAG (Sato *et al.*, 2003; Sato *et al.*, 2006; Yoshizaki *et al.*, 2007). Here, we extended this technique to develop FRET biosensors for $\text{PI}(4,5)\text{P}_2$, $\text{PI}(4)\text{P}$, and DAG, and we analyzed the distribution of PtdIns in migrating MDCK cells. In addition, we developed a kinetic simulation model of PtdIns metabolism to clarify the mechanism of the polarized distribution of PtdIns in migrating MDCK cells.

MATERIALS AND METHODS

FRET Biosensors and Plasmids

Plasmids for FRET-based monitors were constructed essentially as described previously (Mochizuki *et al.*, 2001; Sato *et al.*, 2003). The FRET biosensor for $\text{PI}(4,5)\text{P}_2$, Phosphatidylinositol phosphate indicator (Pippi)- $\text{PI}(4,5)\text{P}_2$, consisted of cyan fluorescent protein (CFP) (amino acids [aa] 1–237), a spacer (Glu-Ala-Ala-Ala-Arg)₆, the PH domain of phospholipase C (PLC) δ (aa 18–130), a spacer (Glu-Ala-Ala-Ala-Arg)₃-Gly-Gly-(Glu-Ala-Ala-Ala-Arg)₃, yellow fluorescent protein (YFP) (aa 1–237), a spacer (Glu-Ala-Ala-Ala-Arg)₇, and the C-terminal region of Ki-Ras4B (aa 169–188) or H-Ras (aa 170–189, called as HRas-CT, hereafter). For the FRET monitors for DAG and $\text{PI}(4)\text{P}$, the lipid-binding domain of Pippi- $\text{PI}(4,5)\text{P}_2$ was replaced by the C1 domain of protein kinase C (PKC) βII (aa 37–140) and the PH domain of FAPP1 (aa 1–101), respectively. Other FRET biosensors, Pippi- PIP_3 and Pippi- $\text{PI}(3,4)\text{P}_2$, have been described previously (Sato *et al.*, 2003, 2006; Aoki *et al.*, 2005). The plasmid coding mRFP-FKBP-5-Ptase-dom was a gift from Dr. Tamas Balla, and pERed-NLS-LDR (Lyn₁₁-targeted FRB) was described previously (Aoki *et al.*, 2007).

Cell Culture, Transfection, and Immunoblotting

NIH 3T3 cells were purchased from the RIKEN Gene Bank (Wako-shi, Japan) and maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). MDCK cells were from Dr. Yoshimi Takai (Kobe University, Kobe, Japan). For transient expression studies, cells were transfected using Polyfect (QIAGEN, Valencia, CA) or 293fectin (Invitrogen, Carlsbad, CA). Cells were analyzed at 24 h after transfection. For immunoblotting, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane, followed by detection with the antibodies described below. The bound antibodies were detected by an ECL chemiluminescence detection system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom), and binding was quantified with the aid of an LAS-1000 image analyzer (Fuji-Film, Tokyo, Japan). Anti-PLC γ and -phospho PLC (pTyr-783) antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Lipids and Thin Layer Chromatography (TLC)

NIH3T3 cells were labeled with $^{32}\text{P}_i$ (0.4 mCi/ml) for 4 h and stimulated with platelet derived growth factor (PDGF)-BB (50 ng/ml) for the indicated periods. Lipids were extracted with CHCl_3 :methanol: H_2O (1:1:1, vol/vol), separated by TLC (separating solvent: CHCl_3 :methanol:ammonium: H_2O [45:35:6:4, vol/vol]), and quantified by BAS2000 (Fuji-Film). To mark the region for phosphatidylcholine and phosphatidylinositol, the standard lipids were separated by the same TLC plate and chemically detected.

FRET Imaging

FRET imaging was performed essentially as described previously (Yoshizaki *et al.*, 2003). Briefly, cells plated on a collagen- or fibronectin-coated 35-mm-diameter glass-base dish (Asahi Techno Glass, Chiba, Japan) were imaged every 1 min on an IX81 inverted microscope (Olympus, Tokyo, Japan) equipped with a laser-based autofocus system, IX2-ZDC, and an automatically programmable XY stage, MD-XY30100T-Meta, which allowed us to obtain the time-lapse images of several view fields in a single experiment. For dual-emission ratio imaging of the intramolecular FRET biosensors, we used previously described filter sets and obtained images for CFP and FRET. After background subtraction was carried out, the FRET/CFP ratio was depicted using MetaMorph software (Molecular Devices, Sunnyvale, CA), and this

image was used to represent FRET efficiency. The filters used for the dual-emission ratio imaging were purchased from Omega Optical (Brattleboro, VT): an XF1071 (440AF21) excitation filter, an XF2034 (455DRLP) dichroic mirror, and two emission filters, XF3075 (480AF30) for CFP and XF3079 (535AF26) for FRET. Cells were illuminated with a 75-W xenon lamp through a 6% ND filter (Olympus) and a 60 $\times$ oil immersion objective lens (PlanApo 60 $\times$ /1.4). The exposure time was 0.3 s when the binning of the cooled charge-coupled device camera Cool SNAP-HQ (Roper Scientific, Trenton, NJ) was set to 4 $\times$ 4. The ratio image of FRET/CFP was created with MetaMorph software and was used to represent the efficiency of the FRET.

Immunostaining

MDCK cells were fixed with 3.7% paraformaldehyde, followed by permeabilization with 0.2% TritonX-100. After having been soaked for 30 min in phosphate-buffered saline (PBS) containing 10% FBS, cells were incubated for 1 h at room temperature with anti-Giantin antibody (Covance Research Products, Princeton, NJ), followed by incubation with Alexa-568 conjugated anti-rabbit IgG at 4°C for overnight. After washing with PBS, cells were imaged with an FV-1000 confocal microscope.

Microinjection and Spectrometry Measurement

Microinjections were performed under an inverted microscope (Carl Zeiss, Jena, Germany) equipped with a micromanipulator 5171 (Eppendorf, Hamburg, Germany) by using an automated FemtoJet (Eppendorf) and Femtotip glass microcapillaries. Pippi- PIP_3 -expressing NIH3T3 cells were plated on fibronectin-coated 35-mm glass-bottomed dishes and microinjected with 10 μM PIP_3 -3-phosphothioate (Echelon, Salt Lake City, UT) and 0.1 μM Alexa568-hydrazide (Invitrogen). The injection parameters were kept as follows: P_i , 30 hPa; T_i , 0.1 s; and P_e , 30 hPa. Immediately after microinjection, cells were observed with a FV-1000 confocal microscope for the fluorescence spectrum analysis. We used an LD405 laser for the excitation, and acquired the emission profiles from 430 to 600 nm in a lambda scan mode. Similarly, the spectrometry of Digda-expressing NIH3T3 cells was obtained in presence and absence of 50 μM 1-oleoyl-2-acetylgllycerol (OAG).

Modeling and Simulation

Kinetic reactions were described with mass action kinetics. All reactions and the kinetic constants used in this study are shown in Supplemental Material 2. For the modeling of FRET biosensor reactions, CellDesigner (version 4.0a; The Systems Biology Institute, Tokyo, Japan) was used for modeling and solving the ordinary differential equations (Kitano *et al.*, 2005).

In the one-dimensional model, the reaction-diffusion problem was solved by numerical integration with a commercially available solver, COMSOL multiphysics version 3.4 with a Chemical-Engineering module and COMSOL Reaction Engineering Lab1.4 (Comsol, Los Angeles, CA) running on a Workstation PC (Dell Precision 390; Dell, Round Rock, TX). The reaction model generated by CellDesigner was imported into the one-dimensional model. See the Supplemental Materials 2 for details.

RESULTS

Development of a $\text{PI}(4,5)\text{P}_2$ Indicator, Pippi- $\text{PI}(4,5)\text{P}_2$

To investigate the spatiotemporal dynamics of $\text{PI}(4,5)\text{P}_2$, we developed a FRET-based monitor for $\text{PI}(4,5)\text{P}_2$, named Pippi- $\text{PI}(4,5)\text{P}_2$, essentially as described previously (Figure 1A) (Sato *et al.*, 2003; Aoki *et al.*, 2005). In this biosensor, when the PH domain of PLC δ binds to $\text{PI}(4,5)\text{P}_2$ on the cell membrane, the CFP is brought in proximity to the YFP, increasing the level of FRET. As an index of the level of FRET, we used the FRET/CFP ratio as described in the *Materials and Methods*. To evaluate Pippi- $\text{PI}(4,5)\text{P}_2$, we visualized the FRET/CFP change in NIH3T3 cells, in which PDGF-BB (hereafter PDGF) induces rapid activation of PLC and concomitant decrease in $\text{PI}(4,5)\text{P}_2$. As expected, in NIH3T3 cells expressing Pippi- $\text{PI}(4,5)\text{P}_2$, the level of FRET/CFP was decreased rapidly at the plasma membrane (Figure 1, B and C). After this rapid and diffuse decrease in FRET/CFP, at 15–20 min after stimulation, we observed the restoration of FRET/CFP over a diffuse area. Interestingly, the increase was most prominent at the peripheral membrane protrusion. Such changes in FRET/CFP was not observed in cells expressing Pippi- $\text{PI}(4,5)\text{P}_2$ -R37D/R38E, which harbored two amino-acid substitutions within the critical lipid binding motif of the PH domain, demonstrating that the change in FRET/CFP depended on the PH domain binding

Figure 1. Development of a FRET biosensor for PI(4,5)P₂. (A) Schematic representation of a monitor for PI(4,5)P₂ [Pippi-PI(4,5)P₂]. YFP and CFP denote a yellow-emitting mutant of GFP and cyan-emitting mutant of GFP, respectively. K-Ras4B-CT and PH indicate the C-terminal region of Ki-Ras4B and the pleckstrin homology domain of PLCδ, respectively. (B) NIH3T3 cells were transfected with pPippi-PI(4,5)P₂. Twenty-four hours later, cells were serum starved, stimulated with PDGF (50 ng/ml), and observed by fluorescent microscopy. CFP (excitation 433 nm/emission 475 nm) and FRET (excitation 433 nm/emission 530 nm) images were obtained every 1 min with a time-lapse epifluorescent microscope. After recording, the ratio FRET/CFP was calculated by MetaMorph software. At the same time, differential interference contrast (DIC) images were obtained. The images shown here are of a representative cell stimulated with PDGF for 0, 3, 10, 15, and 20 min. The calculated FRET efficiency was colored in intensity-modulated display (IMD) modes as shown at the right. The upper and lower limits of the ratio range are shown at the right of the panel. At least 20 similar images were obtained. Bar, 20 μm. (C) The net intensities of CFP and FRET in each cell were measured to calculate the averaged emission ratio (FRET/CFP). The FRET/CFP ratio was normalized by the average value before stimulation. The results obtained using the wild-type PH domain are shown in red (n = 6). The biosensor containing amino acid substitution in the PH domain (R37D/R38E) was used as a negative control (shown in blue, n = 3). Bars indicate the SD. The bar graph shows the averaged FRET/CFP values at 5 min after PDGF addition. The asterisk indicates the significance by *t* test analysis: **p* < 0.05 (vs. cells expressing the mutant biosensor stimulated for the same period). (D) NIH3T3 cells were labeled with ³²P_i (0.4 mCi/ml) for 4 h and stimulated with PDGF (50 ng/ml) for the indicated periods. Lipids were extracted with CHCl₃:methanol:H₂O (1:1:1, vol/vol), separated by TLC (separating solvent: CHCl₃:methanol:ammonium:H₂O [45:35:6:4, vol/vol]), and quantified by BAS2000 (Fuji-Film). To mark the region for phosphatidylcholine and phosphatidylinositol (denoted as PC and PI at the left, respectively), the standard lipids were separated by the same TLC plate and chemically detected. (E) PIP₂, PC, and PI in D were quantitated and normalized to the basal level (time point 0 min; i.e., before stimulation). The results shown here are the averages from three independent experiments. Bar, SD. The asterisk indicates the significance of *t* test: **p* < 0.01 (vs. that of before stimulation). (F) NIH3T3 cells were stimulated with PDGF and subjected to SDS-PAGE and Western blotting by using the antibodies against phospho-PLCγ1 (pY783, top, denoted as p-PLCγ1) and PLCγ1 (bottom). (G) The intensity of the phospho-PLCγ and total PLCγ1 was measured, calculated by phospho-PLC/PLC, and normalized by the value at the time point 5 min. Results are the average from three independent experiments. Bar, SD.

to PI(4,5)P₂. We also confirmed that mock stimulation did not change the FRET/CFP ratio (Supplemental Figure S1, A and B).

To confirm that the FRET/CFP ratio in Pippi-PI(4,5)P₂-expressing cells correlated with the amount of PI(4,5)P₂, we quantitated the amount of PI(4,5)P₂ by TLC by using NIH3T3 cells labeled with ³²P_i for 4 h and stimulated with PDGF for the indicated periods (Figure 1, D and E). Similarly to the change in FRET/CFP, the amount of PI(4,5)P₂ was decreased rapidly and restored to the basal state within 20 min. To further demonstrate the correlation between the FRET/CFP ratio and amount of PI(4,5)P₂ within the cells, we examined the activity of PLCγ1, which hydrolyzes PI(4,5)P₂ to DAG and IP₃ and plays the major role in decreasing the amount of PI(4,5)P₂ in PDGF-stimulated cells (Berridge *et al.*, 1984). Because the enzymatic activity of PLCγ1 correlates with phosphorylation at Tyr⁷⁶³ (Kim *et al.*, 1991), the activity of PLCγ1 was monitored by Western blotting with anti-phosphorylated Tyr⁷⁶³ of PLCγ1 (Figure 1, F and G). Again, we observed rapid phosphorylation of PLCγ1 within 1–5 min, followed by dephosphorylation to the basal state in PDGF-stimulated NIH3T3 cells. These observations supported the idea that Pippi-PI(4,5)P₂ could be used as a biosensor for PI(4,5)P₂ on the plasma membrane.

Development of a Diacylglycerol Indicator, Digda

Similarly to Pippi-PI(4,5)P₂, we developed a FRET-based monitor for DAG named Digda (for Diacylglycerol indicator; Figure 2A). The PH domain of Pippi-PI(4,5)P₂ was replaced with the C1 domain of PKCβII, which binds specifically to DAG. Thus, an elevated level of FRET is expected when DAG is increased at the cell membrane. In agreement with this prediction, when NIH3T3 cells expressing Digda were treated with 50 μM OAG, an analogue of DAG, the FRET/CFP value was elevated rapidly (Figure 2, B and C). Fluorescence spectra of Digda-expressing cells are shown in Supplemental Figure S1C. Digda-C67/132S having a two amino-acid substitution in the critical DAG-binding domain did not show such a response to OAG, demonstrating that the C1 domain binding to OAG was responsible for the increased FRET/CFP value. On PDGF stimulation of NIH3T3 cells, a transient increase in FRET/CFP was observed diffusely at the plasma membrane, suggesting that DAG was rapidly produced at the plasma membrane (Figure 2D). Notably, a remarkable increase in FRET/CFP was observed at the nascent lamellipodia induced by PDGF. Ten to 20 minutes after stimulation, the level of FRET/CFP gradually returned to the basal level. Again, we did not observe such changes in FRET/CFP in PDGF-stimulated cells expressing Digda-C67/132S or mock-stimulated cells expressing Digda (Supplemental Figure S1, D and E). Interestingly, there was a clear discrepancy in the time courses of PDGF-stimulated changes of PI(4,5)P₂ and DAG. Although the concentrations of both PI(4,5)P₂ and DAG reached their nadir or zenith within 5 min, the concentration of PI(4,5)P₂ returned to the basal level significantly faster than did the concentration of DAG.

Development of a PI(4)P Indicator, Pippi-PI(4)P

During the course of experiments that will be described later, it became necessary to determine the level of phosphatidylinositol (4)-monophosphate [PI(4)P] in the plasma membrane as a precursor of PI(4,5)P₂. Although PI(4)P has been reported to localize mainly at the Golgi apparatus, a small amount of PI(4)P localizes at cytoplasmic vesicles and the plasma membrane (Balla *et al.*, 2005). Thus, we developed another biosensor for PI(4)P at the plasma membrane,

named Pippi-PI(4)P, by using the PH domain of the FAPP1 protein (Figure 2F). For the validation of this biosensor, we used phenylarsine oxide (PAO) at a concentration of 0.1 μM, which inhibits type-IIIα phosphatidylinositol 4-kinase (PI4K) and thereby reduces the amount of PI(4)P at the plasma membrane. As expected, treatment with PAO rapidly and diffusely reduced the FRET/CFP level of MDCK cells expressing Pippi-PI(4)P (Figure 2G). In one experiment, we used a negative control biosensor, the Ras and interacting protein chimeric unit (Raichu)-PAK-RhoA, in which the Rac-binding domain of PAK and RhoA were inserted between YFP and CFP. Because PAK does not bind to RhoA, this biosensor is used to negate the nonspecific change in FRET/CFP (Yoshizaki *et al.*, 2003). As expected, the change in the FRET/CFP ratio was not observed in cells expressing Raichu-PAK-RhoA (Supplemental Figure S1F). Next, we examined whether Pippi-PI(4)P could monitor the increase in PI(4)P by using a rapamycin-inducible membrane translocation system (Varnai *et al.*, 2006; Aoki *et al.*, 2007). FKBP-5ptase-dom is a fusion protein consisting of the catalytic domain of phosphoinositide 5-phosphatase (5ptase) and the FK506-binding protein (FKBP), a ligand for rapamycin. On treatment with rapamycin, FKBP-5ptase-dom is recruited to the plasma membrane, where another rapamycin ligand, a fragment of the mammalian target of rapamycin (FRB), is localized by the attached myristylation-moiety (Figure 2H). The translocation of FKBP-5ptase-dom dephosphorylates PI(4,5)P₂ to yield PI(4)P at the plasma membrane (Varnai *et al.*, 2006). Intriguingly, the increase in PI(4)P was not apparent at the plasma membrane; however, the biosensor was accumulated at perinuclear vesicular structures concomitant with an increase in FRET (Figure 2H). We interpreted this result as follows: On FKBP-5ptase-dom translocation to the plasma membrane and the resulting increase in PI(4)P, the excess amount of PI(4)P might have accelerated the rate of endocytosis. The Pippi-PI(4)P bound to PI(4)P might have been cotransported to these endosomes and exhibited a high FRET/CFP area.

To confirm that the high FRET/CFP ratio at the perinuclear region was not due to the aggregation of the FRET biosensor, we constructed biosensors fused to HRas-CT, which locates the probes not only at the plasma membrane but also at the Golgi apparatus via the exocytic pathway (Apolloni *et al.*, 2000). The Golgi localization of the probes was confirmed by coimmunostaining with a Golgi protein, Giantin (Supplemental Figure S1G). As shown in Figure 2I, the FRET/CFP ratio at the Golgi area was lower than that at the plasma membrane in cells expressing Pippi-PI(4,5)P₂-HRas-CT. In contrast, in cells expressing Pippi-PI(4)P-HRas-CT, the FRET/CFP ratio at the Golgi area was higher than that at the plasma membrane, negating the possibility that the high FRET/CFP ratio at Golgi area was caused by the accumulation of the probe. Notably, this observation is consistent with the previous report that the PI(4)P is enriched at the intracellular compartment including the Golgi apparatus (Balla *et al.*, 2005).

Polarized Distribution of PtdInss and DAG in Migrating MDCK Cells

Many biochemical events, including cell-matrix contact turnover and cytoskeletal restructuring during cell migration, are under the control of PtdInss. Using the FRET biosensors described above and reported previously, we examined the distribution of phosphoinositides and DAG at the plasma membrane of migrating MDCK cells. Typically, the migrating MDCK cells consist of a lamellar front half and a dome-shaped back half due to the presence of the nucleus

Figure 2. Characterization of the FRET biosensor for DAG and PI(4)P. (A) Schematic representation of the monitor for DAG (Digda). YFP and CFP denote a yellow-emitting mutant of GFP and cyan-emitting mutant of GFP, respectively. The C1 domain of PKC β II was used as the DAG-binding domain. In this biosensor design, the FRET level will increase when the biosensor recognizes DAG. (B) An NIH3T3 cell expressing Digda was stimulated with 50 μ M of OAG, an analog of DAG, and the FRET/CFP image was processed as in Figure 1B. (C) The net intensities of CFP and FRET in each cell were measured and the averaged emission ratio (FRET/CFP) was calculated as in Figure 1C. Digda-expressing cells were stimulated with OAG at the time point 0 min as indicated by the arrow. The results were obtained from the wild-type (shown in red; $n = 13$) and a mutant (C67/132S; shown in blue; $n = 3$). Bar, SD. The asterisk indicates the significance of t test analysis: $*p < 0.05$ (vs. cells expressing the mutant biosensor stimulated for the same period). (D) An NIH3T3 cell expressing Digda were stimulated with PDGF (50 ng/ml). FRET images were processed as in Figure 1B. (E) The net intensities of CFP and FRET in each cell were measured and the averaged emission ratio (FRET/CFP) was calculated as in Figure 1C. Results were obtained from the wild type (shown in red; $n = 17$) and a mutant (C67/132S, shown in blue; $n = 5$). Digda-expressing cells were stimulated with PDGF. At the time point 0 min, PDGF was added to the cell as indicated by the arrow. Bar, SD. The asterisk indicates the significance of t test analysis: $*p < 0.001$ (vs. cells

and perinuclear organelles. The level of PI(4,5)P₂ was significantly higher at the plasma membrane in the front than in the rear of the cell (Figure 3A; Supplemental Movie 1). The FRET/CFP values ranged from 1.2 to 1.6 in most of the observed cells, which indicated that the concentration of PI(4,5)P₂ ranged from 4 to 8 μ M, as discussed in Supplemental Material 1. Such a gradient in the level of FRET/CFP was not observed in cells expressing the mutant Pippi-PI(4,5)P₂-R37D/R38E (Supplemental Figure S3A). Similarly to PI(4,5)P₂, DAG also exhibited a polarized distribution, with a higher concentration in the front, particularly at lamellipod, than in the back (Figure 3B). Again, such a gradient was not observed in cells expressing the Digda-C67/132S mutant (Supplemental Figure S3B). For the comparison, we also examined the distribution of products of phosphatidylinositol 3-kinase (PI3-kinase), PIP₃, and PI(3,4)P₂, in migrating MDCK cells by using the Pippi-type FRET biosensors Pippi-PIP₃ and -PI(3,4)P₂, having the PH domains of GRP and TAPP1, respectively (Aoki *et al.*, 2007; Yoshizaki *et al.*, 2007). As reported previously (Parent *et al.*, 1998; Servant *et al.*, 2000), both PtdInss were enriched in the front with an increasing gradient toward the leading edge (Figure 3, C and D). The specificity was confirmed by FRET imaging of cells expressing the Pippi-PIP₃-R284C mutant (Supplemental Figure S3C) and by the fluorescence spectrography of cells expressing Pippi-PIP₃ in the presence or absence of a hydrolysis-resistant analogue of PIP₃ (Supplemental Figure S3D). Finally, we examined the level of PI(4)P with Pippi-PI(4)P. In contrast to the other PtdInss examined in this study, PI(4)P was distributed uniformly at the entire plasma membrane (Figure 3E).

Untangling the PtdInss Pathway by Using Inhibitors of PtdInss Metabolism

To understand how such gradients of PtdInss concentration are generated, we examined the effect of inhibitors for enzymes that metabolize PtdInss. First, to examine the role of PI(4)P as a precursor of PI(4,5)P₂, MDCK cells expressing Pippi-PI(4,5)P₂ were treated with PAO. As shown in Figure 4A, PAO treatment rapidly and strongly decreased the FRET level in Pippi-PI(4,5)P₂-expressing MDCK cells, suggesting that PI(4)P is the primary source for PI(4,5)P₂ at the plasma membrane. The half-life of PI(4,5)P₂ was estimated to be $\approx$ 2 min (Figure 4, right). Next, to examine the catabolism of PI(4,5)P₂, MDCK cells were treated with LY294002 or

Figure 2 (cont). expressing the mutant biosensor stimulated for the same period). (F) Schematic representation of the FRET biosensor for PI(4)P. The PH domain of Pippi-PI(4,5)P₂ (shown in Figure 1A) was replaced by that of FAPP1. On binding to PI(4)P, CFP in the biosensor came into proximity to YFP, thereby causing FRET. The YFP (excitation 433 nm, emission 530 nm)/CFP (excitation 433 nm, emission 475 nm) ratio of Pippi-PI(4)P was used as a representation of the FRET efficiency. (G) MDCK cells were transfected with pPippi-PI(4)P, treated with the inhibitor of PAO at the concentration of 0.1 μ M and processed as described in Figure 1B, except that phase images were obtained instead of DIC images. The numbers above are time points (in minutes). (H) MDCK cells were transfected with expression plasmids for Pippi-PI(4)P, mRFP-FKBP-5-ptase-dom, and ERed-NLS-LDR. Forty hours after transfection, cells were treated with 50 nM rapamycin; phase, RFP, CFP, and FRET images were obtained; and FRET/CFP images were processed as described in Figure 2G. The numbers above are time points after rapamycin treatment (in minutes). Bar, 20 μ m. (I) MDCK cells were transfected with pPippi-PI(4,5)P₂- (top) and -PI(4)P (bottom)-HRas-CT, which carry the C-terminal region of H-Ras. Images were processed in a manner similar to that shown in Figure 2G. Bar, 20 μ m.

Figure 3. Distribution of PI(4,5)P₂, DAG, PIP₃, PI(3,4)P₂, and PI(4)P in migrating cells. MDCK cells were transfected with FRET probes for the lipids indicated at the left. Twenty-four hours after transfection, cells were observed by the fluorescent microscopy, and the FRET ratio images were processed in a manner similar to that described in Figure 2G. Results are shown for Pippi-PI(4,5)P₂, (available as a Supplemental Video) (A), Digda (B), Pippi-PI(3,4,5)P₃ (C), Pippi-PI(3,4)P₂ (D), and Pippi-PI(4)P (E).

Figure 4. Effect of inhibitors of PtdIns metabolism on the spatial distribution of PI(4,5)P₂ and PIP₃. (A–D) MDCK cells expressing the biosensors were treated with the inhibitors as indicated. Representative FRET/CFP ratio images are shown at the indicated time points (in minutes). The normalized FRET/CFP values from three cells are expressed in the graph (A, right). The bars indicate the SD (E and F). The boxed region in D was processed for kymographic analysis (E) and line scan analysis (F). The FRET/CFP value is represented in the pseudocolor mode with the range shown at the bottom of the figure (E) and in the line scan plots at different time points (F). Bars, 20 μ m.

U73122, which inhibit PI3-kinase and PLC, respectively. As shown in Figure 4, B and D, treatment with U73122, but not LY294002, induced a rapid increase in the FRET efficiency of Pippi-PI(4,5)P₂ in the front half of the cell, indicating that PI(4,5)P₂ was primarily catabolized by PLC in migrating MDCK cells. LY294002 was found to decrease the level of FRET in Pippi-PIP₃-expressing cells (Figure 4C); therefore, the failure to detect an increase in PI(4,5)P₂ after treatment with LY294002 indicates that PI3-kinase does not contribute to the catabolism of PI(4,5)P₂ to a detectable level. Intriguingly, we noticed that the U73122-induced increase in PI(4,5)P₂ was most prominent at the lamellipod (Figure 4, E and F), suggesting the presence of an increasing gradient of PLC activity in the direction of the leading edge.

Prediction of the Activity Maps of PtdIns-metabolizing Enzymes with a One-dimensional Reaction-Diffusion Model

The finding that the concentrations of PI(4,5)P₂, PI(3,4)P₂, PIP₃, and DAG were higher in the front than in the rear of the cell could be explained simply by the high activity of type-III α PI4-kinase in the front. However, the difference in the gradients of these PtdIns and DAG should provide a clue to help in deciphering the distribution of the other PtdIns-metabolizing enzymes. To decode such activity maps

from the concentration maps of PtdIns and DAG, we developed a one-dimensional reaction-diffusion model of PtdIns metabolism at the plasma membrane. To constrain the kinetic parameters, we first examined the gradients of PtdIns and DAG that were plotted along the direction of migration (Figure 5). As already described, each of PI(4,5)P₂, DAG, PIP₃, and PI(3,4)P₂ was higher in the front than in the rear of the cell. As described in Supplemental Material 1, the difference in the concentrations between the front and back edges was estimated to be 33, 34, 57, and 39% for PI(4,5)P₂, DAG, PIP₃, and PI(3,4)P₂, respectively. Of note, PI(4,5)P₂ was distributed uniformly within the lamellipod, whereas the other lipids showed an increasing gradient toward the leading edge.

A block diagram of the kinetic simulation model of PtdIns metabolism is shown in Figure 6A. PI(4)P, the concentration of which was assumed to be constant, is the inflow, and phosphatidic acid (PA) and IP₃ are the outflows of this network. Three kinases, phosphatidylinositol (4)-monophosphate 5-kinase [PI(4)P 5-kinase], PI3-kinase, and DAG-kinase; three phosphatases, PIP₂ 5-phosphatase (PIP₂ 5-Pase), phosphatase and tensin homologue (PTEN), and PIP₃ 5-phosphatase (PIP₃ 5-Pase); and one phospholipase, PLC, were integrated into this model. In the one-dimensional reaction-diffusion model along the direction of migration,

Figure 5. Quantitative analysis of the distribution of PtdInss and DAG in migrating MDCK cells. MDCK cells were imaged as in Figure 3 (A, C, E, and G). Shown are representative phase contrast images (left) and FRET/CFP ratio images in pseudocolor mode (right). The rainbow bars show ratio ranges. White bars, 20 μm . Red and blue arrows indicate the front and back of migrating cells. The origin of the cell is set on the boundary between the cell body and the front of the cell (white circle). The ordinates of the front and back ends are set to 1.0 and -1.0 in the standardized cell size, respectively. FRET/CFP values are also normalized to that at the center and plotted along the arrows (B, D, F, and H). Each panel contains data for five different time points. The slope of the gradient in the front was calculated by the least-squares method.

we set the origin of the abscissa at the border between the front and back halves and normalized the lengths of these halves to 1.0, respectively (Figure 6B). A detailed kinetic description of this model is given in the supplemental materials part 2. With this information in hand, we used the data shown in Figure 4 to constrain the distribution of enzymes that regulate the metabolism of PtdInss and DAG at the plasma membrane. Because we cannot decisively determine the distribution of the enzymes based on the present data, we will present the most likely distribution of the enzymes (Figure 6, C and D). The rationale behind this distribution is also described in the supplemental materials part 2. First, phosphatidylinositol monophosphate (PIP) 5-kinase activity is slightly higher in the front than in the back half of the cell. Second, PLC activity is set to zero in the back half of the cell, with an increasing gradient toward the leading edge. This observation is based on the data obtained in U73122-treated cells (Figure 4, D–F). Third, PI 3-kinase activity is concentrated at the area close to the leading edge. Fourth, PIP_3 5-Pase activity is slightly higher in the front than in the back half of the cell. This is predicted because the slope of PIP_3 is steeper than that of $\text{PI}(3,4)\text{P}_2$ in the front half of the cell (Figure 5, F and H). Finally, DAG-kinase PTEN and PIP_2 5-Pase were set to be constant, simply

because we do not have data to support the uneven distribution of these enzymes.

The local concentrations of PtdInss and DAG calculated by the simulation model and those estimated by the FRET images are overlaid in Figure 6, E–H, showing a reasonable correlation. Finally, the distribution and time-course of the level of $\text{PI}(4,5)\text{P}_2$ were simulated and shown using a kymograph and a line plot (Figure 6, I and J), which were very similar to those shown in Figure 4, E and F, demonstrating that the present model could reliably recapitulate the kinetics of PtdInss in migrating MDCK cells.

DISCUSSION

Uneven or polarized localization of PtdInss serves as a compass for various migrating cells, such as *D. discoideum* and neutrophils (reviewed in Bourne and Weiner, 2002). In the gradient sensing *D. discoideum*, PI 3-kinase and PTEN are accumulated in the leading edge and uropod, respectively (Funamoto *et al.*, 2002; Iijima and Devreotes, 2002). Recently, it has been reported that SHIP1 (SH2-containing inositol phosphatase 1), rather than PTEN, plays an important role in dephosphorylation of PIP_3 in chemotactic neutrophils (Nishio *et al.*, 2007). All these reports demonstrate that the

Figure 6. In silico analysis of the metabolism of PtdInss and DAG at the plasma membrane of migrating MDCK cells. (A) The metabolic pathway of PtdInss and DAG used in the model. DAG-K, DAG kinase. All reactions and parameters are listed in the supplemental information. (B) Schematic view of the one-dimensional model of PtdInss and DAG metabolism in a migrating cell. There are 256 elements along the axis of the standardized cell described in Figure 5. In each element the reactions were calculated while taking the lateral diffusion of lipids into consideration. (C and D) A predicted distribution of kinases, PLC, and lipid phosphatases. The concentrations of their molecules are normalized to the highest region. (E–H) Distribution of PtdInss and DAG in vivo (black plots) and in silico (red lines) in a migrating cell. (I and J) In silico simulation of the U73122-induced increase in PI(4,5)P₂ in a migrating cell. The time course of the PI(4,5)P₂ increase is shown in a kymograph (I) and a line scan plot (J).

asymmetric distribution of PIP₃ and its metabolic enzymes plays critical roles in cell migration. Importantly, however, these studies do not necessarily negate the possibility that the PI(4,5)P₂ gradient shown in our study also contributes to establishment of the cell polarity directly by regulating the cytoskeleton and indirectly by increasing the concentration of the substrate for PI 3-kinase. Meanwhile, our simulation analysis implied that a simple increase in the most upstream component of this cascade, PI(4,5)P₂, cannot explain the elevation of the other components at the lamellipod, i.e., not only PIP 5-kinase, but also PI 3-kinase, PLC, and PI 5-Pase are up-regulated in the front of the migrating cells (Figure 6). Thus, we need to take not only the amount but also the

turnover rate of PtdInss into consideration of the role of PtdInss in cell migration. In support this, it has been reported that both PLCγ1 and PIPKIγ661, a variant of type I γ PIP 5-kinase, form a complex and both are localized at the leading edge of migrating cells (Piccolo *et al.*, 2002; Sun *et al.*, 2007).

PI(4)P and PI(4,5)P₂ are the two major PtdInss, although they constitute only 0.5% of the total lipids in the eukaryotic cell membrane. It is classically admitted that the concentrations of PtdInss, PI(4)P and PI(4,5)P₂ remain at the steady-state levels in the inner leaflet of the plasma membrane, due to the continuous phosphorylation/dephosphorylation reactions by specific kinases and phosphatases (Michell, 1975;

Payraastre *et al.*, 2001). This so-called “futile cycle” accounts for ~7% of the basal ATP consumption in human blood platelets (Verhoeven *et al.*, 1987; Payraastre *et al.*, 2001). In this study, we showed that the PtdIns turnover rate in the front is faster than that of the rear of the migrating MDCK cells. Then, for what purpose do cells further accelerate the “futile cycle” at the front of the cells? We speculate that such local high turnover rate increases the sensitivity to the external cues. To address this question, we need to integrate external cues into the kinetic simulation model of the PtdIns. Further on this line, this kinetic simulation model will predict the effect of drugs targeting phosphoinositide-metabolizing enzymes on cell migration.

As a possible precursor of PI(4,5)P₂, we focused on PI(4)P in the plasma membrane because the quantity of PI(5)P was much lower than the level of PI(4)P in eukaryotes (see above) (Rameh *et al.*, 1997). Although the majority of PI(4)P and its precursor PtdIns localized to the Golgi apparatus, and although it is known that type II (wortmannin-insensitive) and type III (wortmannin-sensitive) PI 4-kinases (α and β) are primarily localized to endomembranes such as the Golgi apparatus and endoplasmic reticulum, a small amount of PI(4)P also locates to the plasma membrane, due to the activity of type-III α PI 4-kinase (Balla *et al.*, 2005). By using a biosensor localized at the plasma membrane, we found that the concentration of PI(4)P was uniform over the entire plasma membrane. Meanwhile, the concentrations of its derivative, PI(4,5)P₂, and other PtdIns were higher in the plasma membrane of the front than that of the back half of the cell. This observation suggested two possibilities: first, production of PI(4,5)P₂ from PI(4)P is higher at the front than at the back of the cell; or second, PI(4,5)P₂ accumulates preferentially at the front of the cell. Although the second possibility is not negated by any data, there are many studies supporting the former possibility, as are discussed in the next paragraph.

GTPases of the Rho family and Arf6 stimulate the production of PI(4,5)P₂ via type I α PIP 5-kinase (reviewed in Ling *et al.*, 2006 and Santarius *et al.*, 2006). Using FRET-based biosensors for GTPases, we and others have found that the activities of RhoA, Rac1, and Cdc42 are increased toward the leading edge of migrating cells (Kraynov *et al.*, 2000; Itoh *et al.*, 2002; Nalbant *et al.*, 2004; Kurokawa and Matsuda, 2005). In addition to the GTPases, Ajuba, which is a component of integrin-mediated adhesive complexes and regulates localization of the p130^{Cas}/Crk/DOCK/Rac signaling cascade (Pratt *et al.*, 2005), interacts with and activates type I α PIPK (Kisseleva *et al.*, 2005). Therefore, although the mechanisms could be divergent, there is ample evidence that activation of type I α PIP 5-kinase causes accumulation of PI(4,5)P₂ in the lamellipod of the front of the cell. In agreement with these observations, when PI(4,5)P₂ was rapidly reduced by plasma membrane targeting of PI 5-Pase, the cells stopped migrating and PI(4)P increased in the peri-nuclear region (Figure 2H).

For analysis of the spatio-temporal regulation of PtdIns, three methods have been used successfully. The first method involves PtdIns-specific antibodies (Gascard *et al.*, 1991). For the immunostaining, however, cells have to be fixed. Because lipids are not fixed by paraformaldehyde, permeabilization of the plasma membrane by detergent may misconduct the distribution. The second method is to use GFP-tagged PH domains, which are translocated from the cytoplasmic pool to membranes upon the increase in PtdIns (reviewed in Balla, 2005). This method has enabled us for the first time to view the dynamics of PtdIns metabolism in living cells. However, the method has drawbacks in terms of

quantifying the membrane translocation of the probes. For example, this method is sensitive to the thickness of the cells, i.e., when cells increase their thickness during migration or upon stimulation, particularly at the membrane ruffles, accumulation of GFP-tagged proteins tends to be erroneously scored as membrane-targeting. In addition, this method usually cannot detect the membrane targeting of GFP-tagged proteins to the plasma membrane below or above the perinuclear area, due to the bright background from cytoplasmic GFP proteins. Therefore, the GFP-tagged PH domain usually cannot be used to examine the intracellular gradient of the PtdIns. The third method, which is based on FRET, was originally developed by Umezawa and colleagues to detect the level of PIP₃ (Sato *et al.*, 2003). One drawback, or maybe a merit, of this method is that the biosensor has to be anchored to the membranes of interest. Here, we used biosensors fused with the C-terminal region of K-Ras proteins, which brings the proteins preferentially to the plasma membrane. Therefore, we neglected the events in the endomembrane compartments. This problem can be overcome by using different membrane-targeting signals. For example, by using the carboxyl-terminus of the H-Ras protein, we could compare the concentration of PI(4,5)P₂ between the plasma membrane and Golgi apparatus (Figure 2I).

In summary, using FRET-based biosensors, we visualized the gradient of PtdIns and DAG in the plasma membrane of migrating MDCK cells and predicted the activity map of PtdIns-metabolizing enzymes. We concluded that, although the difference in the steady-state level of PtdIns is less than twofold, PtdIns were more rapidly turned over at the front than the rear of the migrating MDCK cells. The physiological significance of this high turnover rate should be addressed in a subsequent study.

ACKNOWLEDGMENTS

We thank Dr. Tamas Balla (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD) for the generous gift of the plasmid coding for mRFP-FKBP-5ptase-dom. We are grateful to N. Yoshida, N. Fujimoto, A. Abe, K. Fukuhara, and Y. Kasakawa for technical assistance. We also thank the staff of the Matsuda laboratory for technical advice and helpful input. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the New Energy and Industrial Technology Development Organization; the Mitsubishi Foundation; and the Japan Health Science Foundation.

REFERENCES

- Aoki, K., Nakamura, T., Fujikawa, K., and Matsuda, M. (2005). Local phosphatidylinositol 3,4,5-trisphosphate accumulation recruits Vav2 and Vav3 to activate Rac1/Cdc42 and initiate neurite outgrowth in nerve growth factor-stimulated PC12 cells. *Mol. Biol. Cell* 16, 2207–2217.
- Aoki, K., Nakamura, T., Inoue, T., Meyer, T., and Matsuda, M. (2007). An essential role for the SHIP2-dependent negative feedback loop in neuritogenesis of nerve growth factor-stimulated PC12 cells. *J. Cell Biol.* 177, 817–827.
- Apolloni, A., Prior, I. A., Lindsay, M., Parton, R. G., and Hancock, J. F. (2000). H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. *Mol. Cell Biol.* 20, 2475–2487.
- Balla, A., Tuymetova, G., Tsiomenko, A., Varnai, P., and Balla, T. (2005). A plasma membrane pool of phosphatidylinositol 4-phosphate is generated by phosphatidylinositol 4-kinase type-III α : studies with the PH domains of the qxysterol binding protein and FAPP1. *Mol. Biol. Cell* 16, 1282–1295.
- Balla, T. (2005). Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. *J. Cell Sci.* 118, 2093–2104.
- Berridge, M. J., Heslop, J. P., Irvine, R. F., and Brown, K. D. (1984). Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. *Biochem. J.* 222, 195–201.
- Bourne, H. R., and Weiner, O. (2002). Cell polarity: a chemical compass. *Nature* 419, 21.

- Charest, P. G., and Firtel, R. A. (2006). Feedback signaling controls leading-edge formation during chemotaxis. *Curr. Opin. Genet. Dev.* 16, 339–347.
- Craske, M. L., Fivaz, M., Batada, N. N., and Meyer, T. (2005). Spines and neurite branches function as geometric attractors that enhance protein kinase C action. *J. Cell Biol.* 170, 1147–1158.
- Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443, 651–657.
- Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R. A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* 109, 611–623.
- Gascard, P., Tran, D., Sauvage, M., Sulpice, J. C., Fukami, K., Takenawa, T., Claret, M., and Giraud, F. (1991). Asymmetric distribution of phosphoinositides and phosphatidic acid in the human erythrocyte membrane. *Biochem. Biophys. Acta* 1069, 27–36.
- Iijima, M., and Devreotes, P. (2002). Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* 109, 599–610.
- Itoh, R. E., Kurokawa, K., Ohba, Y., Yoshizaki, H., Mochizuki, N., and Matsuda, M. (2002). Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol. Cell. Biol.* 22, 6582–6591.
- Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., and Rhee, S. G. (1991). PDGF stimulation of inositol phospholipid hydrolysis requires PLC- γ 1 phosphorylation on tyrosine residues 783 and 1254. *Cell* 65, 435–441.
- Kisseleva, M., Feng, Y., Ward, M., Song, C., Anderson, R. A., and Longmore, G. D. (2005). The LIM protein Ajuba regulates phosphatidylinositol 4,5-bisphosphate levels in migrating cells through an interaction with and activation of PIPK1 α . *Mol. Cell. Biol.* 25, 3956–3966.
- Kitano, H., Funahashi, A., Matsuoka, Y., and Oda, K. (2005). Using process diagrams for the graphical representation of biological networks. *Nat. Biotechnol.* 23, 961–966.
- Kraynov, V. S., Chamberlain, C., Bokoch, G. M., Schwartz, M. A., Slabaugh, S., and Hahn, K. M. (2000). Localized Rac activation dynamics visualized in living cells. *Science* 290, 333–337.
- Kurokawa, K., and Matsuda, M. (2005). Localized RhoA activation as a requirement for the induction of membrane ruffling. *Mol. Biol. Cell* 16, 4294–4303.
- Ling, K., Schill, N. J., Wagoner, M. P., Sun, Y., and Anderson, R. A. (2006). Movin' on up: the role of PtdIns(4,5)P₂ in cell migration. *Trends Cell Biol.* 16, 276–284.
- Luo, L. (2000). RHO GTPASES in neuronal morphogenesis. *Nat. Rev. Neurosci.* 1, 173–180.
- Michell, R. H. (1975). Inositol phospholipids and cell surface receptor function. *Biochem. Biophys. Acta* 415, 81–147.
- Miyawaki, A., Nagai, T., and Mizuno, H. (2003). Mechanisms of protein fluorophore formation and engineering. *Curr. Opin. Chem. Biol.* 7, 557–562.
- Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A., and Matsuda, M. (2001). Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. *Nature* 411, 1065–1068.
- Nalbant, P., Hodgson, L., Kraynov, V., Touthkine, A., and Hahn, K. M. (2004). Activation of endogenous Cdc42 visualized in living Cells. *Science* 305, 1615–1619.
- Nishio, M. *et al.* (2007). Control of cell polarity and motility by the PtdIns(3,4,5)P₃ phosphatase SHIP1. *Nat. Cell Biol.* 9, 36–44.
- Parent, C. A., Blacklock, B. J., Froehlich, W. M., Murphy, D. B., and Devreotes, P. N. (1998). G protein signaling events are activated at the leading edge of chemotactic cells. *Cell* 95, 81–91.
- Payraastre, B., Missy, K., Giuriato, S., Bodin, S., Plantavid, M., and Gratacap, M. (2001). Phosphoinositides: key players in cell signalling, in time and space. *Cell Signal.* 13, 377–387.
- Piccolo, E., Innominato, P. F., Mariggio, M. A., Tania, Iacobelli, S., and Falasca, M. (2002). The mechanism involved in the regulation of phospholipase C γ 1 activity in cell migration. *Oncogene* 21, 6520–6529.
- Pollok, B. A., and Heim, R. (1999). Using GFP in FRET-based applications. *Trends Cell Biol.* 9, 57–60.
- Pratt, S. J., Eppler, H., Ward, M., Feng, Y., Braga, V. M., and Longmore, G. D. (2005). The LIM protein Ajuba influences p130^{Cas} localization and Rac1 activity during cell migration. *J. Cell Biol.* 168, 813–824.
- Rameh, L. E., Tolias, K. F., Duckworth, B. C., and Cantley, L. C. (1997). A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. *Nature* 390, 192–196.
- Santarius, M., Lee, C. H., and Anderson, R. A. (2006). Supervised membrane swimming: small G-protein lifeguards regulate PIPK signalling and monitor intracellular PtdIns(4,5)P₂ pools. *Biochem. J.* 398, 1–13.
- Sato, M., Ueda, Y., Takagi, T., and Umezawa, Y. (2003). Production of PtdInsP₃ at endomembranes is triggered by receptor endocytosis. *Nat. Cell Biol.* 5, 1016–1022.
- Sato, M., Ueda, Y., and Umezawa, Y. (2006). Imaging diacylglycerol dynamics at organelle membranes. *Nat. Methods* 3, 797–799.
- Servant, G., Weiner, O. D., Herzmark, P., Balla, T., Sedat, J. W., and Bourne, H. R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 287, 1037–1040.
- Sun, Y., Ling, K., Wagoner, M. P., and Anderson, R. A. (2007). Type I γ phosphatidylinositol phosphate kinase is required for EGF-stimulated directional cell migration. *J. Cell Biol.* 178, 297–308.
- Takenawa, T., and Itoh, T. (2001). Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. *Biochem. Biophys. Acta* 1533, 190–206.
- Van Keymeulen, A., Wong, K., Knight, Z. A., Govaerts, C., Hahn, K. M., Shokat, K. M., and Bourne, H. R. (2006). To stabilize neutrophil polarity, PIP3 and Cdc42 augment RhoA activity at the back as well as signals at the front. *J. Cell Biol.* 174, 437–445.
- Varnai, P., Thyagarajan, B., Rohacs, T., and Balla, T. (2006). Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells. *J. Cell Biol.* 175, 377–382.
- Verhoeven, A. J. M., Tysnes, O. B., Aarbakke, G. M., Cook, C. A., and Holmsen, H. (1987). Turnover of the phosphomonoester groups of polyphosphoinositol lipids in unstimulated human platelets. *Eur. J. Biochem.* 166, 3–9.
- Wong, K., Pertz, O., Hahn, K., and Bourne, H. (2006). Neutrophil polarization: spatiotemporal dynamics of RhoA activity support a self-organizing mechanism. *Proc. Natl. Acad. Sci. USA* 103, 3639–3644.
- Yoshizaki, H., Mochizuki, N., Gotoh, Y., and Matsuda, M. (2007). Akt-PDK1 complex mediates epidermal growth factor-induced membrane protrusion through Ral activation. *Mol. Biol. Cell* 18, 119–128.
- Yoshizaki, H., Ohba, Y., Kurokawa, K., Itoh, R. E., Nakamura, T., Mochizuki, N., Nagashima, K., and Matsuda, M. (2003). Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. *J. Cell Biol.* 162, 223–232.