

Heterogeneity of Phosphatidic Acid Levels and Distribution at the Plasma Membrane in Living Cells as Visualized by a Förster Resonance Energy Transfer (FRET) Biosensor^{*[5]}

Received for publication, June 11, 2010, and in revised form, September 1, 2010. Published, JBC Papers in Press, September 8, 2010, DOI 10.1074/jbc.M110.153007

Teruko Nishioka^{‡1}, Michael A. Frohman[§], Michiyuki Matsuda^{‡¶}, and Etsuko Kiyokawa^{‡2}

From the [‡]Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan, the [§]Program in Molecular and Cellular Pharmacology, the Department of Pharmacology, and the Center for Developmental Genetics, Stony Brook University, Stony Brook, New York 11794-5140, and the [¶]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

Phosphatidic acid (PA) is one of the major phospholipids in the plasma membrane. Although it has been reported that PA plays key roles in cell survival and morphology, it remains unknown when and where PA is produced in the living cell. Based on the principle of Förster resonance energy transfer (FRET), we generated PA biosensor, and named Pii (phosphatidic acid indicator). In these biosensors, the lipid-binding domain of DOCK2 is sandwiched with the cyan fluorescent protein and yellow fluorescent protein and is tagged with the plasma membrane-targeting sequence of K-Ras. The addition of synthetic PA, or the activation of phospholipase D or diacylglycerol kinase at the plasma membrane, changed the level of FRET in Pii-expressing cells, demonstrating the response of Pii to PA. The biosensor also detected divergent PA content among various cell lines as well as within one cell line. Interestingly, the growth factor-induced increment in PA content correlated negatively with the basal PA content before stimulation, suggesting the presence of an upper threshold in the PA concentration at the plasma membrane. The biosensor also revealed uneven PA distribution within the cell, *i.e.* the basal level and growth factor-induced accumulation of PA was higher at the cell-free edges than at the cell-cell contact region. An insufficient increase in PA may account for ineffective Ras activation at areas of cell-cell contact. In conclusion, the PA biosensor Pii is a versatile tool for examining heterogeneity in the content and distribution of PA in single cells as well as among different cells.

Like that of other phospholipids, the regulation of phosphatidic acid (PA)³ homeostasis is not simple, in part because PA

can be a precursor or product of a number of metabolic pathways, and in part because several enzymes are involved in the production of PA. Two major precursors of PA are phosphatidylcholine and diacylglycerol (DAG), which are the substrates of phospholipase D (PLD) and DAG kinases, respectively (1). The produced PA is then dephosphorylated by PA phosphatases to yield DAG, or hydrolyzed by phospholipase A to yield lyso-PA.

In mammalian cells, there are two major PLDs, PLD1 and PLD2, which are distinct from each other in terms of both overexpression and knock-down phenotype (2). In addition, PLD1 and PLD2 differ strikingly in subcellular localization (3). Such observations have suggested that the PA generation in different subcellular membrane compartments is regulated by different PLDs and that the PA in different subcellular compartment regulates different cellular functions. Similarly, mammalian DAG kinases comprise an extended family with 10 members classified into five different subtypes with different regulatory domains (4).

Biochemical methods such as the radioisotopic labeling of phosphate or fatty acids have been used to measure cellular PA content. However, spatial information cannot be obtained through this approach. To overcome this problem, the yeast t-SNARE Spo20 (5), mammalian Raf1 kinase (6), the Rac1 exchange factor DOCK2 (7), and the Ras exchange factor SOS1, all of which bind specifically to PA using relatively compact PA binding domains, have been developed as biosensors (8–10). For example, upon growth factor stimulation, the GFP-fused, PA-binding SOS1 PH domain translocates from the cytoplasm to bind to PA at the plasma membrane (11). The possible drawback of this method is its dependence on the intracellular concentration of the biosensors; either an excess or a shortfall may hamper the detection of translocation from the cytoplasm to PA-containing membrane surfaces. It is also suggested that a location with a high surface to volume ratio, such as the membrane ruffles, provides pseudo-positive signals even when the

^{*} This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was also supported in part by National Institute of Health Awards GM0171520 and GM084251 (to M. A. F.).

^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3.

¹ Supported by research fellowships from the Japan Society for the Promotion of Science for Young Scientists.

² To whom correspondence should be addressed. Tel.: 81-75-753-9450; Fax: 81-75-753-4698; E-mail: kiyokawa@lif.kyoto-u.ac.jp.

³ The abbreviations used are: PA, phosphatidic acid; BtOH, butyl alcohol; *n*-BtOH, normal BtOH; *t*-BtOH, tertial BtOH; CFP, cyan fluorescent protein; DAG, diacylglycerol; DGK γ , diacylglycerol kinase γ ; rDGK γ , rat DGK γ ; DIGDA, DAG indicator; FIPI, 5-fluoro-2-indolyl des-chlorohalopemide;

FKBP, FK506-binding protein; FRB, FKBP12-rapamycin binding domain of mTOR (mammalian target of rapamycin); MDCK, Madin-Darby canine kidney; PH, pleckstrin homology; PI(4)P, phosphatidylinositol (4)-monophosphate; PI(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate; Pii, phosphatidic acid indicator; Pippi, phosphatidylinositol phosphate indicator; PLD, phospholipase D; TGD2, trigalactosyldiacylglycerol; SS, SOS (Son of Sevenless); DK, DOCK (Downstream of Crk).

lipid distribution is uniform (12). Therefore, GFP-tagged probes require appropriate negative controls and quantitative analysis (13).

Based on the principle of Förster (or Fluorescence) resonance energy transfer (FRET), we and others have developed biosensors for various types of phosphoinositides and DAG to visualize their spatio-temporal regulation (13–16). Here, we have expanded this technique to develop a FRET biosensor for PA. Using this biosensor, we found that Ras activation in response to the presence of growth factor correlates very well with the PA response. Importantly, the response of PA to growth factor is inversely correlated with the basal PA content, which is divergent among different cell types.

EXPERIMENTAL PROCEDURES

Reagents

The following reagents were purchased for use in this study: synthetic PA, 1,2-dioctanoyl-*sn*-glycero-3-phosphate (Avanti Polar Lipids, Inc., Alabaster, AL), normal butyl alcohol (*n*-BtOH), and tertial-BtOH (*t*-BtOH) (Sigma-Aldrich), 5-fluoro-2-indolyl des-chlorohalopemide (FIPI; Sigma-Aldrich), epidermal growth factor (EGF; Sigma-Aldrich), platelet-derived growth factor-BB (PDGF; BD Biosciences), and rapamycin (LC Laboratories, Woburn, MA).

FRET Biosensors and Plasmids

Plasmids for the FRET-based monitors were constructed essentially as described previously (14, 17). The FRET biosensor for PA, Pii, consisted of CFP (amino acids 1–237), a spacer (Glu-Ala-Ala-Ala-Arg)₆, *Arabidopsis thaliana* TGD2 (amino acids 119–381) (18), the PH domain of mouse SOS1 (amino acids 423–551) or the C-terminal region of human DOCK2 (amino acids 1614–1830), a spacer (Glu-Ala-Ala-Ala-Arg)₃, Gly-Gly-(Glu-Ala-Ala-Ala-Arg)₃, YFP (amino acids 1–237), a spacer (Glu-Ala-Ala-Ala-Arg)₇, and the C-terminal region of K_i-Ras4B (amino acids 169–188). The remaining FRET biosensors used in this study were DIGDA, Raichu-Ras (Raichu-146X), and Picchu-X, all of which have been described previously (16, 17, 19). The plasmid coding rat diacylglycerol kinase γ (rDGK γ) was a gift from Dr. Naoaki Saito (20), and pERed-NLS-LDR (Lyn₁₁-targeted FRB) was described previously (21). Either the full-length rDGK γ or the kinase domain (amino acids 423–788) of rDGK γ was cloned to pCX4bsr-3HA-FKBP. To manipulate PLD activity, pERedMito-3HA-FKBP-mPLD2-dN-stop (deletion of N-terminal amino acids 1–308) (22), as well as its catalytically inactive mutant, pERedMito-3HA-FKBP-mPLD2-dN-K758R-stop, were constructed (23).

Cell Culture and Transfection

NIH3T3 and MDCK cells were purchased from the RIKEN Gene Bank (Wako-shi, Japan). NIH3T3, HeLa (a gift from Dr. Giorgio Scita), and COS7-E3 (denoted hereafter as COS7) cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio Inc., Kerrville, TX), 100 units/ml penicillin, and 100 μ g/ml streptomycin in 5% CO₂ at 37 °C. MDCK cells were cultured in minimal essential medium (MEM) containing

Earle's balanced salt solution (Invitrogen) supplemented with 10% FBS, 3% L-glutamate, 1 mM pyruvate, 100 unit/ml penicillin, and 100 μ g/ml streptomycin in 5% CO₂ at 37 °C. For the transient expression studies, cells were transfected using Polyfect (Qiagen, Hilden, Germany) or 293Fectin (Invitrogen). Cells were analyzed at 24–48 h after transfection.

FRET Imaging

FRET imaging was performed essentially as described previously (24). Briefly, cells plated on a collagen-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 auto-focusing 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 the filter sets described below and obtained images for CFP and FRET. After the background subtraction had been carried out, the FRET/CFP or CFP/FRET ratio was depicted using MetaMorph software (Molecular Devices, Sunnyvale, CA), and the resulting image was used to represent FRET efficiency. Cells were illuminated with a 75-W xenon lamp through a 6–25% ND filter (Olympus) and a 60 $\times$ oil immersion objective lens (PlanApo 60 $\times$ /1.4). The exposure time was 0.2–0.5 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 images of FRET/CFP or CFP/FRET were created with MetaMorph software and were used to represent the efficiency of the FRET.

Filters and Mirror Settings

ZDC1 Microscopy—For CFP, we used as exciter, 440AF21 (Omega Optical, Brattleboro, VT); dichroic mirror, 86006bs (Chroma, Bellows Falls, VT); and emitter, XF3075 480AF30 (Omega Optical). For FRET, we used as exciter, 440AF21; dichroic mirror, XF2034 (Omega Optical); and emitter, XF1019 535AF26 (Omega Optical).

ZDC2 Microscopy—For CFP, we used as exciter, XF1071 440AF21 (Omega Optical); dichroic mirror, FF444/521/608 (Semrock, Rochester, NY); and emitter, XF3075 480AF30 (Omega Optical). For FRET, we used as exciter, XF1071 440AF21; dichroic mirror, XF2034; and emitter, XF3079 535AF26 (Omega Optical). For RFP, we used as exciter, FF01-589/15 (Semrock); dichroic mirror, XF2052 (Omega Optical); and emitter, FF01-632/22 (Semrock).

RESULTS

Development of a PA Indicator, Pii—To investigate the spatio-temporal dynamics of PA, we developed a FRET-based monitor that we refer to as Pii, based on previously described Pippi and DIGDA biosensors, for phosphoinositides and DAG, respectively (14, 25, 16). The Pii biosensors consist of CFP, a PA binding domain, a glycine linker, YFP, and the farnesylation signal of K-Ras4B (Fig. 1A). PA has been shown to bind to a number of structurally unrelated, basic amino acid-rich domains (26). From these, we selected the PA binding domain of TGD2, the PH domain of SOS1, and the C-terminal PA bind-

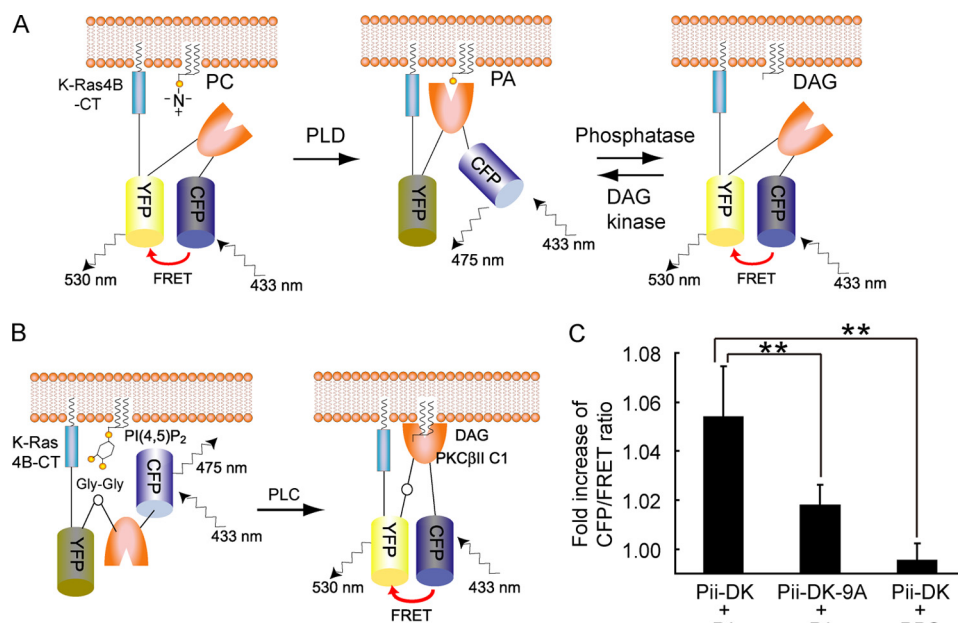


FIGURE 1. Development of FRET biosensors of PA. *A*, schematic representation of a monitor of phosphatidic acid (Pii). YFP and CFP denote a yellow-emitting mutant of GFP and cyan-emitting mutant of GFP, respectively. *K-Ras4B-CT* indicates the C-terminal region of K-Ras4B. PA is generated from phosphatidylcholine by PLC or from DAG by DAG kinase. By a reversal of the process, phosphatidic acid phosphohydrolase (phosphatase) generates DAG from PA. *B*, schematic representation of the monitor for DAG (DIGDA). *Gly-Gly* indicates glycine-glycine linker. The C1 domain of PKC β II was utilized as the DAG binding domain. In this biosensor design, the FRET level will increase when the biosensor recognizes DAG. *C*, COS7 cells transfected with pPii-DK. Twenty-four hours later, the cells were serum-starved, stimulated with synthetic PA:1,2-dioctanoyl-*sn*-glycero-3-phosphate (200 nM), and observed by fluorescent microscopy. CFP (ex 440 nm/em 480 nm) and FRET (ex 440 nm/em 530 nm) images were obtained every 1 min with a time-lapse epifluorescent microscope. After recordings were made, the ratio of CFP to FRET was calculated by MetaMorph software. At the same time, phase images were obtained. The net intensities of CFP and FRET in each cell were measured to calculate the averaged emission ratio (CFP/FRET). The CFP/FRET ratio was normalized by the average value at 0 min. The bar graph shows the averaged highest CFP/FRET values within 15 min after the addition of PA. Pii-DK-9A contains a 9 alanine-substituted DOCK2-C-terminal region, and was used as a negative control for Pii-DK. Pii-DK was incubated with PBS, the solvent for PA. Error bars indicate the S.D. $n = 15$ (Pii-DK+PA), 6 (Pii-DK-9A+PA), and 5 (Pii-DK+PBS). The asterisk indicates statistical significance, determined by *t* test analysis: $p < 0.01$ (versus cells expressing the mutant biosensors treated with PA, or versus cells expressing WT biosensor treated with PBS).

ing domain of DOCK2 (18, 11, 7) to construct Pii-TGD, Pii-SS, and Pii-DK, respectively. In the early course of the experiments, we discarded Pii-TGD because Pii-SS and Pii-DK performed better than Pii-TGD in terms of the response to PA.

To begin characterizing the sensors, COS7 cells expressing Pii-SS and Pii-DK were incubated with 200 nM synthetic PA (1,2-dioctanoyl-*sn*-glycero-3-phosphate). In the previously developed DIGDA, when the PH domain binds to the lipid of interest on the cell membrane, the CFP is brought in close proximity to the YFP, increasing the level of FRET (Fig. 1*B*) (16). Unlike the FRET efficiency of DIGDA, that of Pii-SS and Pii-DK (as represented by FRET/CFP) unexpectedly decreased upon the addition of PA (Fig. 1*C* and supplemental Fig. S1*A*). In other words, the FRET efficiency of Pii showed an inverse correlation with the amount of PA at the plasma membrane. Therefore, we will hereafter express the amount of PA by the CFP (475-nm fluorescence)/FRET (530-nm fluorescence) ratio. To examine the specificity of the response, we prepared two negative controls: a truncated SOS-PH domain (amino acids 445–544) was used in Pii-SS-tr. In Pii-DK-9A, the 9 amino acids in the critical lipid binding motif were replaced with alanine (7). As expected, the FRET level of cells expressing these two mutants did not change as efficiently as that of cells expressing Pii-SS or Pii-DK

(Fig. 1*C* and supplemental Fig. S1*A*). These mutant experiments deny the possibility that these FRET probes were sensitive to the intracellular pH change, which may affect protein-lipid interaction or fluorescence characteristics. Moreover, the addition of phosphate-buffered saline (PBS) alone, a solvent for PA, was associated with only a negligible increase in Pii-SS and Pii-DK.

The structural changes in FRET biosensors upon binding to the cognate ligand are not easily predictable. We therefore do not have a clear understanding of why the FRET efficiency of Pii decreases upon binding to PA. To corroborate that Pii was detecting the level of PA at the plasma membrane, we next examined the response of Pii upon an acute increase in endogenous PA. For this purpose, we utilized a rapamycin-inducible membrane translocation system (21, 27). We sought to increase the level of PA through activation of either PLD or DGK, the roles of which in PA metabolism are depicted in Fig. 1*A*. FKBP-PLD2 and FKBP-DGK were constructed as fusion proteins containing the mouse N terminus-truncated PLD2 (22) or full-length rDGK γ 1 (20), respectively, and the FKBP. HeLa cells were co-trans-

fected with FKBP-PLD2 or FKBP-DGK together with FRB fused to a plasma membrane-targeting signal. Upon the addition of rapamycin (*i.e.* the heterodimerizer of FKBP and FRB), FKBP-PLD2 and FKBP-DGK were recruited to the plasma membrane, increasing the level of production of PA. As shown in Fig. 2*A*, wild-type PLD2, but not a catalytically inactive mutant allele (K758R), increased the CFP/FRET ratio of Pii-DK. Similarly, wild-type DGK, but not an inactive truncated mutant, increased CFP/FRET in Pii-DK-expressing cells (Fig. 2*B*), suggesting that Pii-DK can detect changes in endogenous PA in real time at the plasma membrane. Similarly, cells expressing Pii-SS showed an increase in the CFP/FRET ratio upon endogenous PA production (supplemental Fig. S1, *B* and *C*). To validate this approach further, HeLa cells co-expressing FKBP-DGK, PM-FRB, and DIGDA, a FRET probe for DAG, were stimulated similarly with rapamycin. As shown in Fig. 2*C*, the FRET/CFP level representing the amount of DAG decreased upon rapamycin treatment. The time course of the decrease in DAG correlated inversely with the time course of the increase in PA, indicating that rapamycin-induced translocation of DGK to the plasma membrane resulted in phosphorylation of DAG to produce PA there. To exclude the possibility that the probe is recognizing PI(4,5)P₂, COS cells expressing

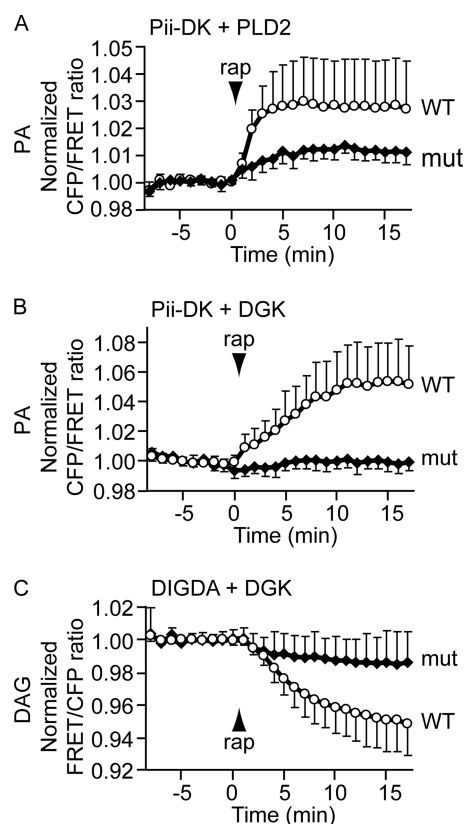


FIGURE 2. Characterization of the FRET biosensor for PA. A and B, HeLa cells were transfected with expression plasmids for Pii-DK, FKBP-truncated PLD2 (A), or FKBP-DGK γ (B), and membrane-targeted FRB. Forty hours after transfection, cells were treated with a heterodimerizer (50 nM rapamycin, denoted as rap) at 0 min (closed arrowheads) and observed using epifluorescent microscopy. The net intensities of CFP and FRET in each cell were measured to calculate the averaged emission ratio (CFP/FRET). The CFP/FRET ratio was normalized by the average value before stimulation. Results were obtained with the wild-type PLD2 (denoted as WT, open circles, $n = 9$) and a catalytically inactive (K758R) PLD2 mutant allele (mut, $n = 6$, filled diamonds). B, results for wild-type DGK γ (open circles, $n = 10$) and a truncated (amino acids 423–788) DGK γ mutant allele (mut, $n = 12$) are shown. Error bars indicate S.D. C, experiments were performed as in B, with the exception that DIGDA, a FRET biosensor for DAG, was used instead of Pii-DK. The same number of samples of full-length (open circles, $n = 9$) or short (filled diamonds, $n = 9$) rat DGK γ and ERed-NLS-LDR were processed as described in B. Error bars indicate S.D.

Pii-DK or Pippi-PI(4,5) P_2 were treated with phenylarsine oxide, which inhibits conversion of phosphatidylinositol to PI(4)P (16). Although PI(4,5) P_2 was decreased within 1 min upon phenylarsine oxide treatment, the CFP/FRET ratio for Pii-DK was not detectably affected by the phenylarsine oxide treatment (supplemental Fig. S2B). Propranolol, which inhibits type I PA phosphohydrolase activity (15), rapidly increased the CFP/FRET of Pii-DK, confirming that Pii-DK recognizes endogenous PA in the living cells (supplemental Fig. S2C).

PA Production upon Growth Factor Stimulation—To examine the spatio-temporal regulation of PA distribution in the plasma membrane, COS7 cells were expressed with Pii-DK, stimulated with EGF, and time-lapse-imaged (Fig. 3). Upon EGF stimulation, the amount of PA increased rapidly at the plasma membrane, especially at the lamellipodia (Fig. 3A), and then reached the maximum at 5 min after stimulation, after which it decreased gradually to the basal level within 30 min (Fig. 3B). Such changes in CFP/FRET were not observed in

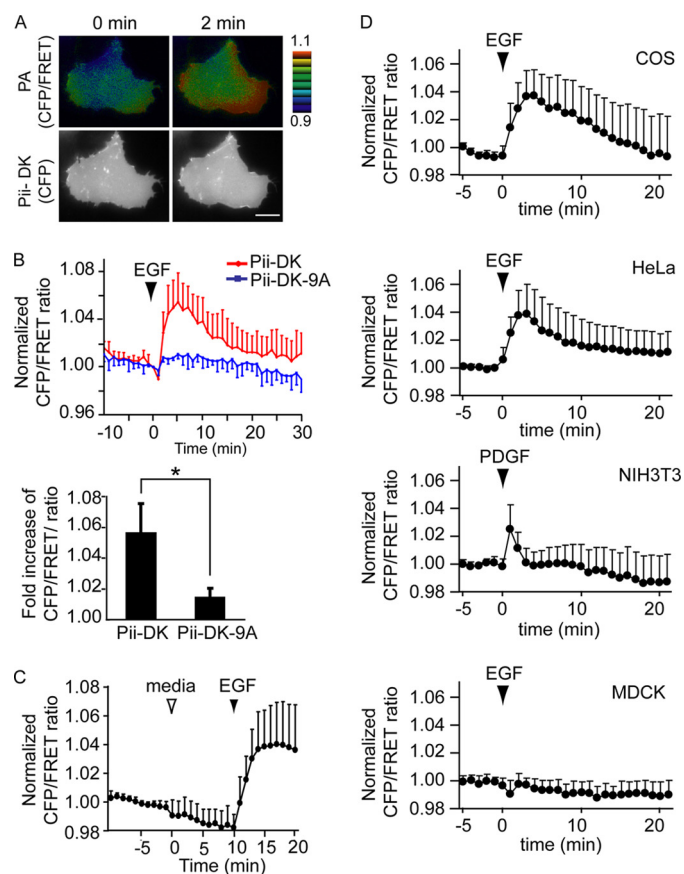


FIGURE 3. PA production upon stimulation by various growth factors. A, COS7 cells were transfected with pPii-DK. Twenty-four hours later, the cells were serum-starved, stimulated with EGF (50 ng/ml), and observed by fluorescent microscopy. CFP (ex 440 nm/em 480 nm) and FRET (ex 440 nm/em 530 nm) images were obtained every 1 min with a time-lapse epifluorescent microscope. After recordings were made, the CFP/FRET ratio was calculated using MetaMorph software. The calculated FRET efficiency is differentiated by color in intensity-modulated display (IMD) modes shown at the right. The upper and lower limits of the ratio range are also shown at the right of the panel. B, COS cells were transfected with pPii-DK or -DK-9A. Twenty-four hours later, the cells were serum-starved, stimulated with EGF (50 ng/ml), and observed by fluorescent microscopy. The net intensities of CFP and FRET in each cell were measured to calculate the averaged emission ratio (CFP/FRET). The results obtained using the wild-type DOCK2-C-terminal region are shown in red (Pii-DK, $n = 4$). The biosensor containing the 9 amino acid substitution in the DOCK2-C-terminal region was used as a negative control (Pii-DK-9A, shown in blue, $n = 3$). Error bars indicate the S.D. The bar graph (below) shows the averaged highest CFP/FRET values after EGF stimulation. The asterisk indicates statistical significance, determined by t test analysis. *, $p < 0.05$ (versus cells expressing the mutant biosensor). C, COS7 cells expressing Pii-DK were stimulated with medium at 0 min (white arrowhead) and EGF (50 ng/ml) at 10 min (black arrowhead). The normalized CFP/FRET ratio was calculated as in (Fig. 2). $n = 8$. The bar graph below shows the averaged highest CFP/FRET values after the addition of PA. Error bars indicate the S.D. The asterisk indicates statistical significance, determined by t test analysis. $p < 0.05$ (versus cells expressing the mutant biosensors treated with PA). D, cells expressing Pii-DK were stimulated with growth factors at 0 min (closed arrowheads). The following pairs of cells and growth factors were used: COS7 cells and 50 ng/ml EGF ($n = 15$), HeLa cells and 50 ng/ml EGF ($n = 15$), NIH3T3 cells and 50 ng/ml PDGF ($n = 11$), and MDCK cells and 50 ng/ml EGF ($n = 18$). Error bars indicate the S.D.

EGF-stimulated cells expressing Pii-DK-9A (Fig. 3B) or, in the mock-stimulated Pii-DK-expressing cells (Fig. 3C), which demonstrated that the change in CFP/FRET indeed depended on the increase in PA. Cells expressing Pii-SS also showed similar changes in CFP/FRET upon EGF stimulation, but the dynamic range of CFP/FRET was smaller than

that of Pii-DK (supplemental Fig. S1, D–F), suggesting the superiority of Pii-DK in the live-cell imaging of PA. Thus, Pii-DK was used in the following experiments.

We extended our analyses to other cell types. As shown in Fig. 3D, PA was increased most prominently in COS7 cells and HeLa cells, less in NIH3T3 cells, but not in MDCK cells. Notably, prominent membrane ruffles were evident in the COS7, HeLa, NIH3T3, and MDCK cells upon stimulation (data not shown), indicating activation of the receptors in all of the cell types. When COS7 and MDCK cells were stimulated with ATP, which stimulates both P2X and P2Y receptors (28), PA was transiently produced both in COS7 cells and MDCK cells, albeit the increase was less in MDCK cells (supplemental Fig. S3). Thus, the lack of PA increase in EGF-stimulated MDCK cells suggests differences in MDCK cells in the signaling pathways that couple EGF to PA production in COS7 and HeLa cells.

Divergence in Phosphatidic Acid Content among Various Cell Types—During the course of the experiments, we noticed that the amount of PA as represented by the CFP/FRET ratio was significantly divergent among individual cells and among different cell lines. As shown in Fig. 4A, COS7, NIH3T3, and HeLa cells showed lower basal PA levels than did MDCK cells. In contrast, MDCK cells contained lower DAG levels. This observation suggested that the lack of increase in PA in EGF-stimulated MDCK cells might reflect high PA levels in the basal state. To corroborate this hypothesis, we examined the correlation between the basal level and the increase in PA upon growth factor stimulation. As shown in Fig. 4B, there was a clear inverse correlation between the basal level and the growth factor-induced increase in PA levels in COS7 cells. A similar correlation between the basal level and the growth factor-induced increase in PA was observed in HeLa cells stimulated with EGF (Fig. 4C) and in NIH3T3 cells stimulated with PDGF (Fig. 4D). In contrast, the CFP/FRET ratio in MDCK cells did not change upon EGF stimulation, regardless of the basal PA content (Fig. 4B). Because Pii-DK in MDCK cells stimulated with ATP enabled the detection of an increase in PA (supplemental Fig. S3), this inert response to EGF was not due to limitation in the dynamic range of Pii-DK. Thus, we concluded that the lower the basal PA content at PM, the higher the increase in PA after EGF stimulation, suggesting the presence of a negative feedback loop maintaining the level of PA within a predetermined range. Although serum starvation increases negative regulation of PLD2 and thereby decreases PA levels in cancer cells (29), we did not observe any reduction in PA in the MDCK cells after even 18 h of serum starvation (data not shown).

Effect of PLD Inhibition on Ras Activation—Because one of the major targets of the PA produced by PLD2 is SOS, a guanine nucleotide exchange factor of Ras (30), we next examined the relationship of PA and Ras activation. It has been reported that upon EGF stimulation, PA is produced by PLD2 at the plasma membrane and recruits SOS to activate Ras (11). Cells expressing Raichu-Ras were treated with the PLD inhibitor *n*-BtOH and its negative control, *t*-BtOH, for 5 min prior to EGF stimulation (Fig. 5). As reported previously, pretreatment with *n*-BtOH, but not with *t*-BtOH, affected the membrane ruffling induced by EGF treatment (31) (data not shown). Ras activation and membrane ruffling were delayed in the *n*-BtOH-treated

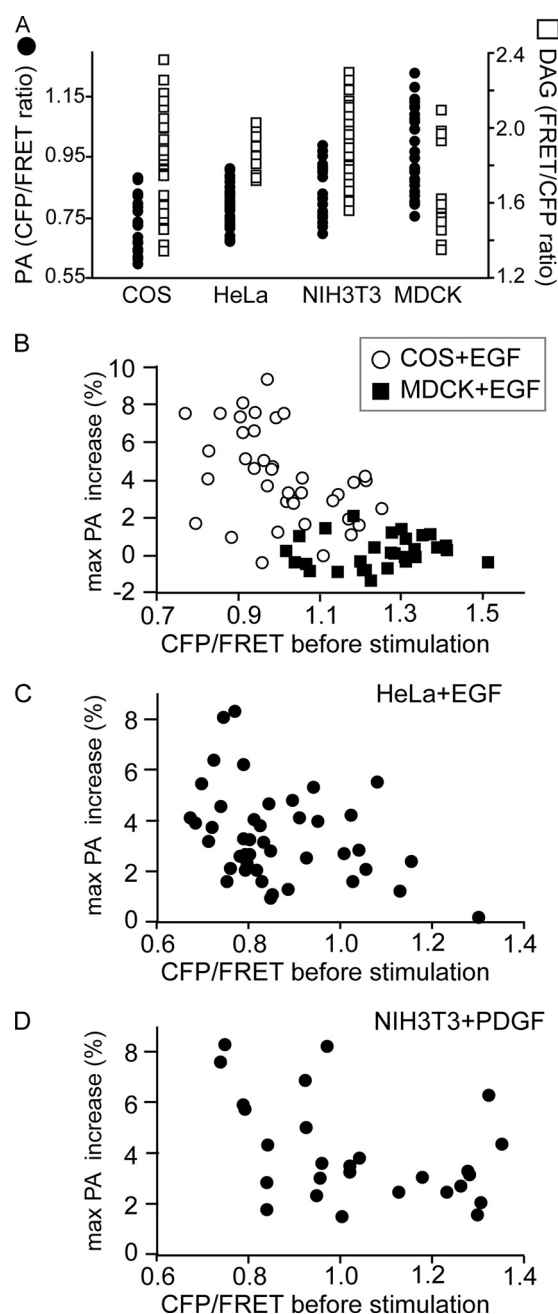


FIGURE 4. Measurement of PA in quiescent and stimulated cells. A, COS7 (noted as COS), HeLa, NIH3T3, and MDCK cells were transfected with pPii-DK or pDIGDA and observed by fluorescent microscopy (ZDC2) for 8 min. Images were obtained every minute, and the net intensities of CFP and FRET were obtained from a single cell. The averages from nine images were used to determine basal FRET efficiency using the CFP/FRET ratio for PA (left axis, filled circles) or by FRET/CFP for DAG (right axis, open boxes). The number of cells examined in each case was as follows: 24 (COS), 25 (HeLa), 26 (NIH3T3), and 39 (MDCK) for PA; and 38 (COS), 12 (HeLa), 28 (NIH3T3), and 14 (MDCK) for DAG. B–D, various cells expressing Pii-DK were stimulated with the indicated growth factors at a concentration of 50 ng/ml. The basal FRET efficiency was obtained as described in A. The maximum poststimulation increase in CFP/FRET obtained from each cell was determined by imaging analysis, as described in Fig. 3D. Microscopies ZDC1 (B) and ZDC2 (C and D) were utilized to obtain images.

cells compared with the *t*-BtOH treated cells. Intriguingly, the PA level was decreased by *n*-BtOH treatment prior to stimulation, but then increased upon EGF stimulation. This finding is consistent with a prior report that found that the amount of *n*-BtOH used here (0.5%) decreases but does not eliminate PA

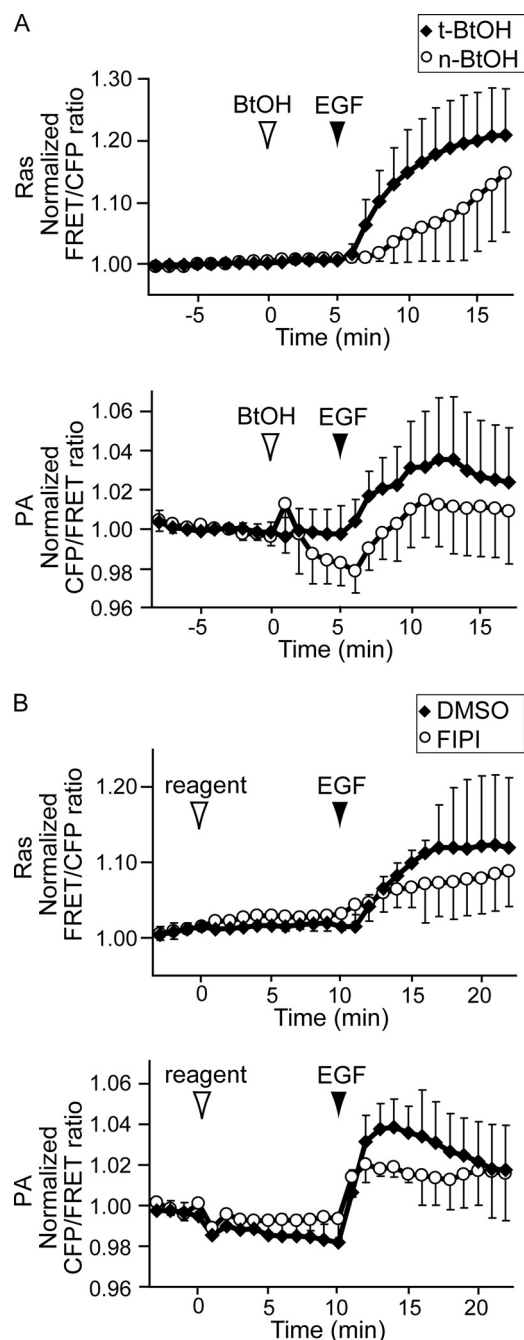


FIGURE 5. PLD inhibition and Ras activation upon stimulation by EGF. A, COS7 cells expressing Raichu-Ras and Pii-DK were treated with 0.5% t-BtOH or n-BtOH at 0 min (open arrowheads) followed by the addition of 25 ng/ml EGF at 5 min (filled arrowheads). Time-lapse imaging was processed as in Fig. 2B. Error bars indicate the S.D. $n = 11$ (Raichu-Ras) and 13 (Pii-DK). B, COS7 cells expressing Raichu-Ras and Pii-DK were treated with 750 nM FIPI or dimethyl sulfoxide (DMSO) at 0 min (open arrowheads), followed by the addition of 25 ng/ml EGF at 10 min (filled arrowheads). Time-lapse imaging was processed as in Fig. 2B. Bars indicate the S.D. $n = 5$ (Raichu-Ras+DMSO), 9 (Raichu-Ras+FIPI), 8 (Pii-DK+DMSO), and 4 (Pii-DK+FIPI).

production (32). Because concerns have been raised about potential off-target effects, cells were treated with PLD-specific inhibitor FIPI prior to EGF stimulation (9). As shown in Fig. 5B, both Ras activation and PA production were partially suppressed by FIPI treatment.

The importance of PLD in COS7 cells was further supported by the observation that DAG was produced only slightly in

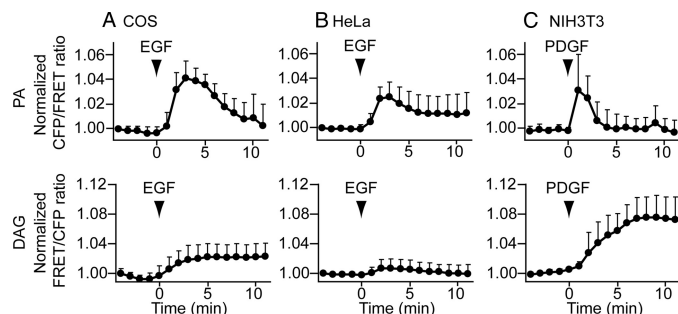


FIGURE 6. PA and DAG production in various cells. A–C, COS7 (A), HeLa (B), and NIH3T3 (C) cells were transfected with pPii-DK (upper graphs) or pDIGDA (lower graphs) and stimulated with 50 ng/ml growth factor as indicated (filled arrowheads). After imaging by fluorescent microscopy was carried out, the images were analyzed as in Fig. 2B. The number of cells examined in each case was as follows: 7 (COS7), 13 (HeLa), and 10 (NIH3T3), for PA (upper graphs); and 12 (COS7), 12 (HeLa), and 10 (NIH3T3) for DAG (lower graphs). Error bars indicate S.D.

EGF-stimulated COS7 cells, although PA was produced in parallel experiments (Fig. 6A). Similarly, PA, but not DAG, was increased significantly in EGF-stimulated HeLa cells (Fig. 6B), whereas in PDGF-stimulated NIH3T3 cells, both PA and DAG were prominently increased (Fig. 6C). These findings indicate that cells or receptors utilize different pathways to elevate PA levels and suggest that the balance between PA and DAG might be brought by different activation of enzymes such as phospholipase C, PLD, and DAG kinases. Moreover, the results suggest that in COS7 and HeLa cells, PA derived from phosphatidylcholine might be the major pathway for PA production upon EGF stimulation.

Suppression of PA Production by Cell-Cell Contact—Normal epithelial cells cannot replicate in confluent states by a process known as “contact inhibition.” One mechanism underlying this phenomenon is that growth factor-induced Ras activation is inhibited in cell-cell contact areas (17); however, the molecular mechanism remains unknown. Because EGF-induced Ras activation is dependent on PLD (Fig. 5), and PLD2 at the plasma membrane is suppressed in quiescent cells (33), we hypothesized that PA may be preferentially produced at cell-free edges, but not in cell-cell contact regions, upon stimulation by growth factor. To investigate this hypothesis, COS7 cells expressing Pii-DK were time-lapse-imaged before and after EGF stimulation (Fig. 7A). We found that PA levels in the basal state were higher in the cell-free edge areas compared with in cell-cell contact areas. After EGF stimulation, rapid PA production was observed in all regions of the cells examined. Among these regions, PA production was observed most prominently in the nascent lamellipodia, and the lowest levels of production were seen in cell-cell contact areas. This distribution is very similar to that of Ras activity before and after EGF stimulation (17) (Fig. 7B). In contrast, the activity of tyrosine kinase as visualized by a FRET biosensor, Picchu-X (19), was distributed evenly across the entire area of the cells, including both the cell-cell contact areas and cell-free edges (Fig. 7C). These observations strongly suggest that the localized PA produced in this manner determines the spatial regulation of Ras activity.

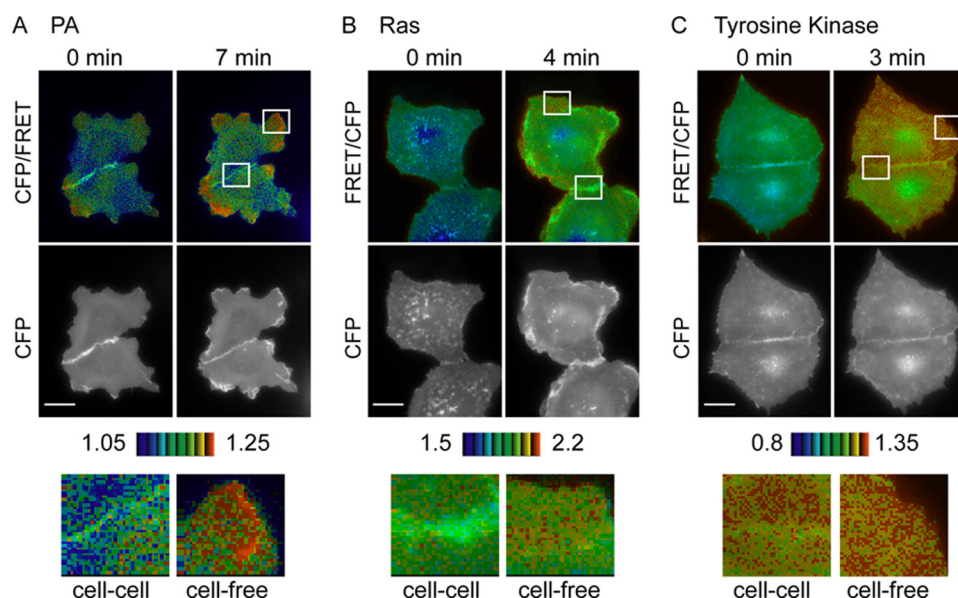


FIGURE 7. **Heterogeneous distribution of PA.** A–C, COS7 cells expressing Pii-DK, Raichu-Ras, or Picchu-X were stimulated with 25 ng/ml EGF for the indicated periods. The CFP/FRET image represents PA content (A) and the FRET/CFP images represent Ras activity and tyrosine kinase activity (B and C, respectively). Enlarged images from the top panels are shown at the bottom.

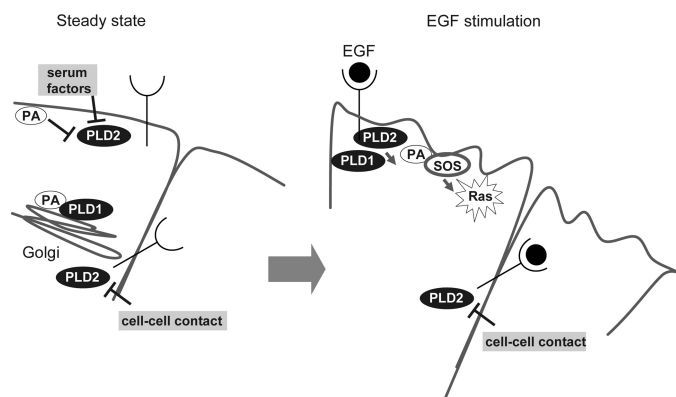


FIGURE 8. **Model for potential roles of PA in the regulation of Ras activity.** Under steady-state conditions (left), factors in the serum or cell-cell contact area suppress PLD2 activity at the plasma membrane. Upon EGF stimulation, PA is produced at the cell-free edge, either by PLD2 activation or by PLD1 translocation, followed by Ras activation by SOS. However, in the same cell in the area of cell-cell contact, PLD2 activity remains suppressed.

DISCUSSION

The major advantage of using a FRET probe is that it enables measurement of the lipid concentration in the steady state. Here, we found that the PA as well as the DAG concentration varies among cell lines (Fig. 4A) and that the PA content showed inter-individual heterogeneity within the same cell line as well (Fig. 4, B and C). There is evidence suggestive of aberrant PLD activity in cells transformed by v-Src (34), H-Ras (35), and v-Raf (36), as well as evidence for the overexpression of PLD in cancer tissues (37). In the presence of 0.5% serum, the aggressive breast cancer cell MDA-MB-231 shows higher PLD activity than that of another relatively benign breast cancer cell line, MCF-7 (38). In our study, the basal PA level in the plasma membrane of MDA-MB-231 cells was higher than that of MDCK cells.⁴ Therefore, this newly devel-

oped FRET probe can be applied for screening the level of PA at the plasma membrane to identify potentially highly invasive malignancies.

The negative correlation between growth factor-induced PA production and the basal PA concentration indicated that a high PA concentration negatively regulates PA production upon growth factor stimulation (Fig. 4, B–D) in COS7, HeLa, and NIH3T3 cells. Although it has not yet been clarified that PA itself interacts with and regulates its metabolic enzymes, such a negative correlation between the PA level and growth factor-induced PA production suggests the possibility that the PA level at the plasma membrane is tightly regulated in the cell and that the PA threshold is cell-type dependent. Along these lines, we had reported previously that exces-

sively produced PI(4)P at the plasma membrane increased the PI(4)P level at the endomembrane, suggesting a limit to lipid levels at the plasma membrane (16). The manner in which PA regulates cellular function has not yet been elucidated, although it has been suggested that PA function is largely dependent on cell type. For example, PLD is activated upon integrin stimulation in ovarian cancer-derived OVCAR-3 cells and the inhibition of PLD has been shown to reduce cell spreading (39). In contrast, in CHO cells, cell attachment to the substrate reduced PLD activity and cell spreading (10) and an overexpression of PLD2-suppressed cell spreading (40), both of which suggest that tight PA regulation enables cell adherence. Such PA discrepancies might be brought about by different PA-binding protein expression patterns, including those of PI(4)P 5-kinase, SOS, Raf, DOCK2, Rac, mTOR (mammalian target of rapamycin), and Fer (3, 7, 41).

Contact inhibition is a process of arresting cell growth when cells come into contact with each other (42) and is often used as a hallmark to discriminate between normal cells and cancer cells. Although the molecular mechanism of contact inhibition is not yet fully understood, it is generally thought that the molecules that accumulate in cell-cell contact areas inhibit signals for replication. For example, Ras, a critical signal transducer of the growth signal, is suppressed in cell-cell contact areas (17). Using the newly developed FRET biosensor, we for the first time demonstrated that PA production is suppressed in cell-cell contact areas in living cells (Fig. 7). Because EGF-triggered PA production was decreased in the presence of PLD inhibitors (Fig. 5, A and B) and little DAG was increased by EGF in COS7 cells (Fig. 6), it is likely PLD, rather than DGK, regulates such uneven PA distribution after EGF stimulation (Fig. 8). These observations also indicated that the DAG-PKC-PLD1 axis in the caveolin-enrichment microdomain (43) might not be the major determinant of PA heterogeneity in EGF-treated COS7 cells. It has been reported that EGFR binds to PLD2, thereby

⁴ E. Kiyokawa, unpublished observation.

suppressing PLD2 activity (44). However, in the present study, the tyrosine kinase activity pattern accessed by the FRET probe Picchu-X revealed the homogeneous activation of tyrosine kinases in areas in between the cell-free edges and cell-cell contact areas (Fig. 7C), suggesting that mechanisms other than the tyrosine phosphorylation by EGFR might regulate PLD activity in cell-free edge areas.

Even though Pii shares a backbone with other FRET probes of various Pippis and DIGDA, the change in FRET efficiency observed with Pii had a different directionality (Fig. 1 and supplemental Fig. S1). With the previous probes, FRET efficiency increased upon lipid binding, whereas that of the PA probe Pii described in this study decreased upon exogenous PA addition or endogenous PA increase (Figs. 1 and 2). The reason for this reversal of reactivity remains unknown; structural analyses of the probe together with the lipid will reveal the mechanism(s) of action. One plausible explanation is that the modes of interaction differ for the probe and the lipid. We believe that Pii recognizes the three-carbon glycerol backbone and phosphate; however, the possibility cannot be excluded that the probe also recognizes the acyl chains of the lipids. A recent report shows that DOCK2 is first recruited to the plasma membrane by phosphatidylinositol (3,4,5)-trisphosphate via the DHR1 domain and is maintained there following PA binding to the polybasic region at the C terminus (7). It is therefore possible that the FRET probe recognizes the particular PA closely associated with phosphatidylinositol (3,4,5)-trisphosphate. These factors may affect FRET efficiency, which is governed not only by the distance from the fluorophores, but also by their anisotropy (45).

In summary, we here established a novel probe for PA based on FRET principles, which enabled the detection of PA levels and distribution at the plasma membrane. One drawback of this probe is that it has to be anchored to the plasma membrane. We therefore neglected the events in the endomembrane compartments. Considering that mitochondrial PLD regulates mitochondrial fusion via PA production from cardiolipin (46), and that the kinetics of DAG upon ATP stimulation differ between the plasma membrane and endomembranes (15), the use of FRET probes with different targeting signals aimed at various organelles will shed light on PA regulation and function in living cells. We expect that there is not a single operative protein that regulates such deviations in PA; instead, such variance appears to be the result of a summation of protein expression or of the activities of multiple proteins. The computer-assisted generation of metabolic maps (16) and systematic lipid analyses (47) carried out using newly developed probes will help elucidate the contribution of lipids to various biological and pathological phenomena.

Acknowledgments—We thank Dr. Naoaki Saito (Kobe University, Japan) for the rat DGK γ plasmid; A. Abe, Y. Inaoka, K. Hirano, Y. Kasakawa, and N. Nonaka for technical assistance; and the staff at the Matsuda laboratory for technical advice and helpful input.

REFERENCES

1. Wang, X., Devaiah, S. P., Zhang, W., and Welti, R. (2006) *Prog. Lipid Res.* **45**, 250–278

2. Riebeling, C., Morris, A. J., and Shields, D. (2009) *Biochim. Biophys. Acta* **1791**, 876–880
3. Jenkins, G. M., and Frohman, M. A. (2005) *Cell Mol. Life Sci.* **62**, 2305–2316
4. Cai, J., Abramovici, H., Gee, S. H., and Topham, M. K. (2009) *Biochim. Biophys. Acta* **1791**, 942–948
5. Nakanishi, H., de los, S. P., and Neiman, A. M. (2004) *Mol. Biol. Cell* **15**, 1802–1815
6. Rizzo, M. A., Shome, K., Watkins, S. C., and Romero, G. (2000) *J. Biol. Chem.* **275**, 23911–23918
7. Nishikimi, A., Fukuhara, H., Su, W., Hongu, T., Takasuga, S., Mihara, H., Cao, Q., Sanematsu, F., Kanai, M., Hasegawa, H., Tanaka, Y., Shibasaki, M., Kanaho, Y., Sasaki, T., Frohman, M. A., and Fukui, Y. (2009) *Science* **324**, 384–387
8. Corrotte, M., Chasserot-Golaz, S., Huang, P., Du, G., Ktistakis, N. T., Frohman, M. A., Vitale, N., Bader, M. F., and Grant, N. J. (2006) *Traffic* **7**, 365–377
9. Su, W., Yeku, O., Olepu, S., Genna, A., Park, J. S., Ren, H., Du, G., Gelb, M. H., Morris, A. J., and Frohman, M. A. (2009) *Mol. Pharmacol.* **75**, 437–446
10. Zeniou-Meyer, M., Zabari, N., Ashery, U., Chasserot-Golaz, S., Haeberlé, A. M., Demais, V., Bailly, Y., Gottfried, I., Nakanishi, H., Neiman, A. M., Du, G., Frohman, M. A., Bader, M. F., and Vitale, N. (2007) *J. Biol. Chem.* **282**, 21746–21757
11. Zhao, C., Du, G., Skowronek, K., Frohman, M. A., and Bar-Sagi, D. (2007) *Nat. Cell Biol.* **9**, 706–712
12. Craske, M. L., Fivaz, M., Batada, N. N., and Meyer, T. (2005) *J. Cell Biol.* **170**, 1147–1158
13. Yoshizaki, H., Mochizuki, N., Gotoh, Y., and Matsuda, M. (2007) *Mol. Biol. Cell* **18**, 119–128
14. Sato, M., Ueda, Y., Takagi, T., and Umezawa, Y. (2003) *Nat. Cell Biol.* **5**, 1016–1022
15. Sato, M., Ueda, Y., and Umezawa, Y. (2006) *Nat. Methods* **3**, 797–799
16. Nishioka, T., Aoki, K., Hikake, K., Yoshizaki, H., Kiyokawa, E., and Matsuda, M. (2008) *Mol. Biol. Cell* **19**, 4213–4223
17. Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A., and Matsuda, M. (2001) *Nature* **411**, 1065–1068
18. Awai, K., Xu, C., Tamot, B., and Benning, C. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103**, 10817–10822
19. Kurokawa, K., Mochizuki, N., Ohba, Y., Mizuno, H., Miyawaki, A., and Matsuda, M. (2001) *J. Biol. Chem.* **276**, 31305–31310
20. Yamaguchi, Y., Shirai, Y., Matsubara, T., Sanse, K., Kuriyama, M., Oshiro, N., Yoshino, K., Yonezawa, K., Ono, Y., and Saito, N. (2006) *J. Biol. Chem.* **281**, 31627–31637
21. Aoki, K., Nakamura, T., Inoue, T., Meyer, T., and Matsuda, M. (2007) *J. Cell Biol.* **177**, 817–827
22. Sung, T. C., Altshuler, Y. M., Morris, A. J., and Frohman, M. A. (1999) *J. Biol. Chem.* **274**, 494–502
23. Du, G., Huang, P., Liang, B. T., and Frohman, M. A. (2004) *Mol. Biol. Cell* **15**, 1024–1030
24. Yoshizaki, H., Ohba, Y., Kurokawa, K., Itoh, R. E., Nakamura, T., Mochizuki, N., Nagashima, K., and Matsuda, M. (2003) *J. Cell Biol.* **162**, 223–232
25. Aoki, K., Nakamura, T., Fujikawa, K., and Matsuda, M. (2005) *Mol. Biol. Cell* **16**, 2207–2217
26. Stace, C. L., and Ktistakis, N. T. (2006) *Biochim. Biophys. Acta* **1761**, 913–926
27. Varnai, P., Thyagarajan, B., Rohacs, T., and Balla, T. (2006) *J. Cell Biol.* **175**, 377–382
28. Abbracchio, M. P., Burnstock, G., Verkhratsky, A., and Zimmermann, H. (2009) *Trends Neurosci.* **32**, 19–29
29. Garcia, A., Zheng, Y., Zhao, C., Toschi, A., Fan, J., Shraibman, N., Brown, H. A., Bar-Sagi, D., Foster, D. A., and Arbiser, J. L. (2008) *Clin. Cancer Res.* **14**, 4267–4274
30. Zhang, Y., and Du, G. (2009) *Biochim. Biophys. Acta* **1791**, 850–855
31. Santy, L. C., Ravichandran, K. S., and Casanova, J. E. (2005) *Curr. Biol.* **15**, 1749–1754
32. Skippen, A., Jones, D. H., Morgan, C. P., Li, M., and Cockcroft, S. (2002) *J. Biol. Chem.* **277**, 5823–5831

33. Freyberg, Z., Siddhanta, A., and Shields, D. (2003) *Trends Cell Biol.* **13**, 540–546
34. Song, J. G., Pfeffer, L. M., and Foster, D. A. (1991) *Mol. Cell. Biol.* **11**, 4903–4908
35. Xu, L., Frankel, P., Jackson, D., Rotunda, T., Boshans, R. L., D'Souza-Schorey, C., and Foster, D. A. (2003) *Mol. Cell. Biol.* **23**, 645–654
36. Frankel, P., Ramos, M., Flom, J., Bychenok, S., Joseph, T., Kerkhoff, E., Rapp, U. R., Feig, L. A., and Foster, D. A. (1999) *Biochem. Biophys. Res. Commun.* **255**, 502–507
37. Lee, C. S., Kim, K. L., Jang, J. H., Choi, Y. S., Suh, P. G., and Ryu, S. H. (2009) *Biochim. Biophys. Acta* **1791**, 862–868
38. Zheng, Y., Rodrik, V., Toschi, A., Shi, M., Hui, L., Shen, Y., and Foster, D. A. (2006) *J. Biol. Chem.* **281**, 15862–15868
39. Chae, Y. C., Kim, J. H., Kim, K. L., Kim, H. W., Lee, H. Y., Heo, W. D., Meyer, T., Suh, P. G., and Ryu, S. H. (2008) *Mol. Biol. Cell* **19**, 3111–3123
40. Du, G., and Frohman, M. A. (2009) *Mol. Biol. Cell* **20**, 200–208
41. Itoh, T., Hasegawa, J., Tsujita, K., Kanaho, Y., and Takenawa, T. (2009) *Sci. Signal.* **2**, ra52
42. Seluanov, A., Hine, C., Azpurua, J., Feigenson, M., Bozzella, M., Mao, Z., Catania, K. C., and Gorbunova, V. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 19352–19357
43. Kim, Y., Han, J. M., Han, B. R., Lee, K. A., Kim, J. H., Lee, B. D., Jang, I. H., Suh, P. G., and Ryu, S. H. (2000) *J. Biol. Chem.* **275**, 13621–13627
44. Slaaby, R., Jensen, T., Hansen, H. S., Frohman, M. A., and Seedorf, K. (1998) *J. Biol. Chem.* **273**, 33722–33727
45. Piston, D. W., and Kremers, G. J. (2007) *Trends Biochem. Sci.* **32**, 407–414
46. Choi, S. Y., Huang, P., Jenkins, G. M., Chan, D. C., Schiller, J., and Frohman, M. A. (2006) *Nat. Cell Biol.* **8**, 1255–1262
47. Pettitt, T. R., Dove, S. K., Lubben, A., Calaminus, S. D., and Wakelam, M. J. (2006) *J. Lipid Res.* **47**, 1588–1596

Heterogeneity of Phosphatidic Acid Levels and Distribution at the Plasma Membrane in Living Cells as Visualized by a Förster Resonance Energy Transfer (FRET) Biosensor

Teruko Nishioka, Michael A. Frohman, Michiyuki Matsuda and Etsuko Kiyokawa

J. Biol. Chem. 2010, 285:35979-35987.

doi: 10.1074/jbc.M110.153007 originally published online September 8, 2010

Access the most updated version of this article at doi: [10.1074/jbc.M110.153007](https://doi.org/10.1074/jbc.M110.153007)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

Supplemental material:

<http://www.jbc.org/content/suppl/2010/09/08/M110.153007.DC1>

This article cites 47 references, 29 of which can be accessed free at <http://www.jbc.org/content/285/46/35979.full.html#ref-list-1>