Non-invasive visualization of the lipid product of class I PI3K in transgenic mouse models

T. Sasaki†‡, J. Sasaki*, K. Watanabe* and A. Suzuki‡

Department of Pathology and Immunology, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
†PRESTO (Precursory Research for Embryonic Science and Technology), Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan
‡Department of Molecular Biology, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

Abstract
PI3Ks (phosphoinositide 3-kinases) regulate many critical cellular responses by producing PI(3,4,5)P₃ (phosphatidylinositol 3,4,5-trisphosphate). To facilitate the spatio-temporal characterization of PI(3,4,5)P₃ in living primary cells, we generated a novel strain of transgenic mice [AktPH (Akt pleckstrin homology domain)–GFP (green fluorescent protein)] Tg (transgenic) mice that express a fluorescent bioprobe for PI(3,4,5)P₃/PI(3,4)P₂ (phosphatidylinositol 3,4-bisphosphate). By crossing AktPH–GFP Tg mice with strains of gene-targeted ‘knockout’ mice lacking a particular phosphoinositide-metabolizing enzyme, we have been able to evaluate the contribution of each enzyme to PI(3,4,5)P₃ localization in migrating neutrophils. Our results indicate that PI3Kγ and the PI(3,4,5)P₃ phosphatase SHIP1 [SH2 (Src homology 2)-containing inositol phosphatase-1] are the key regulators of PI(3,4,5)P₃ dynamics during fMet-Leu-Phe (N-formylmethionyl-leucylphenylalanine; ‘chemotactic peptide’)–stimulated neutrophil migration. Our study has also validated the fluorescent transgenic strategy for studying PI(3,4,5)P₃ metabolism in physiological and pathological situations.

Introduction
The PI3Ks (phosphoinositide 3-kinases) constitute a family of evolutionarily conserved lipid kinases that phosphorylate the 3-hydroxy group of the inositol ring of phosphoinositides [1,2]. PI3Ks are divided into three groups on the basis of the phosphoinositides that they preferentially utilize as substrates. In vitro, all class I PI3Ks can phosphorylate PI (phosphatidylinositol), PI(4)P (phosphatidylinositol 4-phosphate) and PI(4,5)P₂ (phosphatidylinositol 4,5-bisphosphate). However, in vivo, PI(4,5)P₂ is the predominant substrate and its phosphorylation by class I PI3Ks leads mainly to the production of PI(3,4,5)P₃ (phosphatidylinositol 3,4,5-trisphosphate) [3].

A wide range of cell-surface receptors that utilize tyrosine kinase or heterotrimeric G-proteins for signal transduction can induce PI3K activation and thus stimulate PI(3,4,5)P₃ accumulation within the cell. Intense research over the last decade has unequivocally demonstrated that class I PI3Ks and PI(3,4,5)P₃ regulate a vast array of fundamental cellular responses, including proliferation, suppression of apoptosis, glucose metabolism, protein synthesis, polarization and motility [4,5]. The generation of PI(3,4,5)P₃ within cells is balanced by phosphoinositide phosphatases that counteract PI3K action. The tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) dephosphorylates the 3-position of PI(3,4,5)P₃, whereas type II phosphoinositide 5-phosphatases such as SHIP [SH2 (Src homology 2)-containing inositol phosphatase], SKIP (skeletal muscle and kidney enriched inositol phosphatase) and synaptojanin dephosphorylate the 5-position of PI(3,4,5)P₃. Given the pleiotropic roles of the PI3Ks and their lipid products in cellular responses, it has been challenging to define the precise roles and significance of these signalling molecules in vivo. Gene-targeted mice lacking individual PI3K isoenzymes or PI(3,4,5)P₃ phosphatases have been generated and studies of these mutants have identified potential links between PI(3,4,5)P₃ metabolism and diseases such as cancer, diabetes, cardiac failure, osteoporosis, allergy, and inflammatory and autoimmune disorders [6,7]. These findings have been supported by the identification in various human cancers of amplifications and mutations of genes encoding components of the PI3K signalling pathway [8,9]. Based on these studies, PI(3,4,5)P₃-metabolizing enzymes, especially the PI3Ks, have attracted pharmaceutical company interest as potential therapeutic targets for the above disorders [10,11]. Our goal has been to elucidate the spatio-temporal regulation of PI(3,4,5)P₃ synthesis/degradation in physiological and pathological situations.

Current methods for analysing PI(3,4,5)P₃ metabolism and localization
The standard biochemical procedure for determining intracellular phosphoinositide levels is to label cells with myo-[3H]inositol or [32P]phosphorous in vitro, extract and...
deacylate the labelled lipids, and fractionate them by TLC or HPLC [12]. This approach allows the determination of the total cellular synthesis and mass of PI(3,4,5)P₃, and has revealed that PI(3,4,5)P₃ is barely detectable in quiescent cells but accumulates rapidly in cells stimulated by engagement of a wide range of cell-surface receptors. However, PI(3,4,5)P₃ is only a minor constituent of total cellular phospholipids, so that the incorporation of high levels of radioisotope into millions of cells is required to detect PI(3,4,5)P₃ in this way. This method is therefore often limited or not even feasible for many cell types. A more sensitive method of detecting phosphoinositides consists of monitoring the distribution of fluorescently tagged phosphoinositide-binding domains in living cells [13]. This microscopic method has substantiated the notion that rapid and localized production of specific phosphoinositides can provide a discriminating signal for the selective recruitment and activation of signalling molecules in particular membrane compartments. For example, the serine/threonine protein kinase Akt (also known as protein kinase B) is the major downstream target of PI3K. Upon PI3K activation, PI(3,4,5)P₃ is generated that can bind to Akt and induce its translocation from the cytosol to the membrane [14,15]. PI(3,4)P₃ that is formed by 5-dephosphorylation of PI(3,4,5)P₃ is also reported to bind to Akt [16]. This recruitment brings Akt into close proximity to the regulatory kinase PDK1 (phosphoinositide-dependent protein kinase 1), which is also capable of binding to PI(3,4,5)P₃ [17,18]. The phosphorylation of Akt by PDK1 then activates the enzymatic function of Akt. The binding of Akt to PI(3,4,5)P₃/PI(3,4)P₃ is mediated by the AktPH (Akt pleckstrin homology) domain, and when this domain is fused to EGFP (enhanced GFP, green fluorescent protein), an AktPH–GFP bioprobe is created that can be used to track the localization of PI(3,4,5)P₃/PI(3,4)P₃ in living cells [19]. The AktPH–GFP bioprobe has been widely used to detect PI(3,4,5)P₃/PI(3,4)P₃ in cell lines but cannot be applied to primary cell cultures due to the difficulties in achieving efficient and non-invasive transfection of the bioprobe into such cells.

Generation and characterization of AktPH–GFP Tg (transgenic) mice

To monitor the dynamics and spatial organization of PI(3,4,5)P₃ in living primary cells, a transgenic mouse strain expressing the AktPH–GFP bioprobe was developed by Cantrell and co-workers [20]. These researchers generated transgenic mice expressing AktPH–GFP in a T-cell-lineage-restricted manner by placing AktPH–GFP expression under the control of the CD2 promoter. These mutant animals have served as an excellent tool for the analysis of the immunological synapse [20]. We have made use of the β-actin promoter to generate AktPH–GFP Tg mice that express AktPH–GFP in all tissues [21]. Because AktPH binds to PI(3,4,5)P₃ and sequesters it, and because mice lacking PI(3,4,5)P₃ due to gene targeting of PI3Kα or PI3Kβ are embryonic lethal [22,23], we anticipated that AktPH–GFP Tg mice might also show some embryonic lethality. Indeed, our yield of AktPH–GFP Tg mice was significantly lower than that of GFP Tg mice generated using the same promoter construct. In addition, we noted that viable AktPH–GFP Tg mice never expressed the transgene product at the high levels seen in EGFP Tg mice. However, the lethality of high levels of AktPH–GFP also acted as a screening tool in our experiments and prevented the survival of mutant mice in which excessive production of the bioprobe in vivo might have undesirable effects. When we examined chemoattractant-induced Akt activation in neutrophils of AktPH–GFP Tg mice, we noted that expression of AktPH–GFP did not have any deleterious effects compared with controls. In addition, cell numbers and development of both T- and B-lymphocytes were normal in AktPH–GFP Tg mice.

Validation of AktPH–GFP Tg mice for the study of spatio-temporal regulation of PI(3,4,5)P₃ in neutrophils

Neutrophils are a major type of blood leucocytes and are crucial for host defence against pathogens and inflammation. Neutrophils easily move up a chemoattractant gradient and efficiently migrate to a site of infection or inflammation. Many studies of neutrophil chemotaxis have been carried out using the human promyelocytic leukaemia HL60 cell line, which can be induced to differentiate in vitro into neutrophil-like cells. Landmark studies of HL60 cells stably expressing the AktPH–GFP have shown that the bioprobe is concentrated at the leading edge of the chemotaxing cells [19]. Similarly, PI(3,4,5)P₃, detected by a specific antibody applied to fixed cells, also localized at the leading edge [24], providing direct evidence for polarized distribution of PI(3,4,5)P₃ in chemoattractant-stimulated HL60 cells. These results have been corroborated in similar studies employing the amoeba Dictyostelium discoideum [25,26]. However, attempts to replicate these experiments in primary neutrophils have been laborious and difficult because neutrophils resist cDNA transfection by electroporation, lipofection, viral infection or micropinjection [27,28]. We circumvented this difficulty by isolating primary neutrophils from our AktPH–GFP Tg mice and examining their chemotaxis in vitro. We found that AktPH–GFP Tg neutrophils migrated just as well as non-transgenic neutrophils in response to the chemoattractants fMet-Leu-Phe (N-formylmethionyl-leucylphenylalanine; ‘chemotactic peptide’) and the complement factor C5a. We therefore set out to non-invasively monitor PI(3,4,5)P₃ dynamics during the polarization and chemotaxis of AktPH–GFP Tg neutrophils.

In resting AktPH–GFP Tg neutrophils, AktPH–GFP was distributed mainly in the cytoplasm (Figure 1A). Upon exposure of the cells to fMet–Leu–Phe, the AktPH–GFP bioprobe translocated uniformly to the entire circumference of the plasma membrane within 15 s (results not shown). Over the next 3 min, the neutrophils displayed periods of selective recruitment of AktPH–GFP to the leading edge during migration (Figure 1A, arrowheads). By 5 min, the response had subsided significantly (results not shown).
Figure 1 | AktPH–GFP dynamics during neutrophil migration in the absence of PI(3,4,5)P3-metabolizing enzymes

Shown are examples of AktPH–GFP localization in neutrophils prepared from AktPH–GFP Tg mice whose genetic background was either WT (A, A′), p110γ−/− (B, B′), LysMCrePtenlox−/− (C, C′) or ship1−/− (D, D′). AktPH–GFP localization before (A–D) and after stimulation with fMet-Leu-Phe for the indicated time (A′–D′) was visualized by confocal fluorescence microscopy. Arrowheads in (A′) and (C′) indicate the localization of AktPH–GFP at the neutrophil leading edge. In (B′), a p110γ-deficient cell displays polarized morphology but does not show selective membrane translocation of AktPH–GFP. Arrow in (B′) indicates pseudopod extension without localization of AktPH–GFP. Arrowheads in (D′) point to the broad distribution of AktPH–GFP in the plasma membrane of a ship1−/− neutrophil. The figure is adapted from [21] with permission.

These dynamics are consistent with those reported in other studies using PH domain-based probes (AktPH–GFP, PhdA–GFP and CracPH–GFP) to detect PI(3,4,5)P3 in HL60 cells [19] and in D. discoideum cells [25,26].

p110γ and SHIP1 regulate PI(3,4,5)P3 accumulation at the leading edge of neutrophils

Because neutrophils are terminally differentiated cells, they are not readily amenable to genetic manipulation. In addition, the short life span of a mature differentiated neutrophil precludes practical application of the RNA interference technique. Hence, the mechanisms controlling PI(3,4,5)P3 accumulation at the leading edge of a chemotaxing primary neutrophil have remained obscure. We decided to investigate these mechanisms by crossing our AktPH–GFP Tg mice with gene-targeted mouse strains lacking a phosphoinositide-metabolizing enzyme or other signalling molecules of interest. Our goal was to analyse the functions of these molecules in the regulation of PI(3,4,5)P3 localization. One of the first molecules we analysed was p110γ, the catalytic subunit of the class IB PI3K, PI3Ky. We and others have shown that p110γ−/− neutrophils produce little or no PI(3,4,5)P3 in response to chemoattractant stimulation [29–31]. Accordingly, we could not detect AktPH–GFP translocation to the plasma membrane of fMet-Leu-Phe-stimulated p110γ−/− AktPH–GFP Tg neutrophils (compare Figure 1B and Figure 1B′) [21,32]. These results substantiate a critical role for PI3Ky in generating the PI(3,4,5)P3 that accumulates at the leading edge of migrating neutrophils.

It has been unequivocally demonstrated in D. discoideum that PTEN regulates spatially localized PI(3,4,5)P3 accumulation and thereby controls cell polarization and chemotaxis in this organism [25,26]. To determine if PTEN had the same role in mammalian cells, we crossed our AktPH–GFP Tg mice with LysMCre mice [33], Pten+/− mice [34] and Ptenlox/lox mice [35] to prepare neutrophils that lacked PTEN and expressed AktPH–GFP. Interestingly, the bioprobe accumulated just as well at the leading edge of fMet-Leu-Phe-stimulated LysMCrePtenlox/– AktPH–GFP Tg neutrophils as it did in migrating AktPH–GFP Tg neutrophils (compare Figure 1A′ and Figure 1C′). These results suggested that PTEN was not required for controlling PI(3,4,5)P3 generation in chemotaxing mammalian neutrophils. We then examined whether the 5-phosphatase SHIP1 might be assuming this role in place of PTEN. We crossed our AktPH–GFP Tg mice with ship1−/− mice [36] to generate ship1−/− AktPH–GFP Tg mice. In contrast with Pten-deficient fMet-Leu-Phe-stimulated AktPH–GFP Tg neutrophils, the bioprobe was distributed broadly around the perimeter of SHIP1-deficient fMet-Leu-Phe-stimulated AktPH–GFP Tg neutrophils and was not confined to a single membrane region (Figure 1D′). Moreover, the bioprobe exhibited a more prolonged association with the plasma membrane of ship1−/− AktPH–GFP Tg neutrophils than with the membrane of WT (wild-type) AktPH–GFP Tg neutrophils. These results indicate that it is SHIP1, and not PTEN, that is indispensable for PI(3,4,5)P3 accumulation at the leading edge of migrating neutrophils. These results are consistent with our findings on the role of each PI(3,4,5)P3 phosphatase in neutrophil chemotaxis and polarization [21].

Concluding remarks

We have developed a novel transgenic mouse model in which to analyse the spatio-temporal localization of PI(3,4,5)P3 in primary cell cultures. Importantly, we have successfully applied this system to the study of neutrophils, a cell type known to resist transfection. Our AktPH–GFP Tg mice have also proved helpful in monitoring PI3K activation in cell types present in low abundance, such as NK (natural killer) T-cells [37]. Lastly, our AktPH–GFP Tg mice have been validated as an effective tool for the in situ detection of PI3K activation. For example, administration of insulin to AktPH–GFP Tg mice causes redistribution of AktPH–GFP in the plasma membrane of hepatocytes, and this response is inhibited by prior treatment of the mice with PI3K inhibitors (R. Iizuka, M. Nishio, A. Suzuki and T. Sasaki, unpublished work). Additional studies of our AktPH–GFP Tg mice may provide useful insights into PI3K activation during pathogenesis and may offer unique opportunities for drug development.

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