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Cutting Edge: Dok-1 and Dok-2 Adaptor Molecules Are Regulated by Phosphatidylinositol 5-Phosphate Production in T Cells¹

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Downstream of tyrosine kinase (Dok) proteins Dok-1 and Dok-2 are involved in T cell homeostasis maintenance. Dok protein tyrosine phosphorylation plays a key role in establishing negative feedback loops of T cell signaling. These structurally related adapter molecules contain a pleckstrin homology (PH) domain generally acting as a lipid/protein-interacting module. We show that the presence of this PH domain is necessary for the tyrosine phosphorylation of Dok proteins and their negative functions in T cells. We find that Dok-1/Dok-2 PH domains bind *in vitro* to the rare phosphoinositide species, phosphatidylinositol 5-phosphate (PtdIns5P). Dok tyrosine phosphorylation correlates with PtdIns5P production in T cells upon TCR triggering. Furthermore, we demonstrate that PtdIns5P increase regulates Dok tyrosine phosphorylation *in vivo*. Together, our data identify a novel lipid mediator in T cell signaling and suggest that PH-PtdIns5P interactions regulate T cell responses. *The Journal of Immunology*, 2009, 182: 3974–3978.

Stimulation of membrane receptors such as the TCR on T cells induces the activation of protein tyrosine kinases (PTKs)⁴ and subsequently the phosphorylation of substrates, which contributes to the formation of an intracytoplasmic multiprotein network. The downstream of tyrosine kinase (Dok) proteins Dok-1 and Dok-2 are involved in negative T cell activation control (1). During T cell activation, Dok pro-

teins are tyrosine phosphorylated (2, 3). Dok-1 and Dok-2 overexpression in T cell lines decreases IL-2 production in activated cells (4, 5), and loss or reduction of Dok-1 and Dok-2 expression in primary T cells enhances TCR-mediated functions and signaling (3, 6). Moreover, mice lacking both Dok-1 and Dok-2 develop T cell-dependent Ab responses and a spontaneous autoimmunity disease (6).

Interestingly, Dok-1 and Dok-2 contain an N-terminal pleckstrin homology (PH) domain that has been poorly studied. PH domains are one of the largest and most intensively investigated families of lipid binding domains; the majority bind weakly to phosphoinositides (PIs) with little or no selectivity (7). PIs are constituents of cell membranes and play a critical role in cell signaling pathways. Their metabolism is tightly regulated by several lipid kinases/phosphatases and its disruption gives rise to several pathologies (8). These PI species mediate signals through their binding to proteins containing specific interaction domains, including PH, PX, FYVE, and ENTH domains (9).

We now report that in T cell lines or primary T cells, the PH domain of Dok proteins is necessary for their phosphorylation and functions. Interestingly, we show that the phosphatidylinositol 5-phosphate (PtdIns5P) is the most relevant ligand for the Dok-1 and Dok-2 PH domains.

PtdIns5P is a rare PI species and is starting to emerge as a potential second messenger (10, 11). Bacterial infection by *Shigella flexneri* via the virulence factor IpgD generates PtdIns5P in the host cells and induces Akt activation (12, 13). Recent evidence indicates that enhanced tyrosine phosphorylation increases cellular PtdIns5P levels (14, 15). Altogether, these data

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⁴ Abbreviations used in this paper: PTK protein tyrosine kinase; Dok, downstream of tyrosine kinase; HA, hemagglutinin; PH, pleckstrin homology; PHD, plant homeodomain; PI, phosphoinositide; PtdIns3P, phosphatidylinositol 3-phosphate; PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns5P, phosphatidylinositol 5-phosphate; SPR, surface plasmon resonance.

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argue for an important role of PtdIns5P in cell signaling. In this study, we show that PtdIns5P is generated upon TCR triggering and regulates the tyrosine phosphorylation of negative adaptor proteins of T cell activation such as Dok-1 and Dok-2.

Materials and Methods

Culture cells and transfection

Jurkat JA16 and Hut-78 T cells were grown in RPMI 1640 medium, and HeLa cells were cultured in DMEM. Both media were supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 1 mM sodium-pyruvate. PBMC from healthy donors (Etablissement Français du Sang, Marseille, France) were isolated on Ficoll-Hypaque (Pharmacia) gradients before purification of the CD4⁺ T cell subset using the CD4⁺ T cell isolation kit (Miltenyi Biotec).

Jurkat cells were electroporated at 960 microfarads and 250 volts using a Bio-Rad Gene Pulser or nucleofected according to the manufacturer's instructions (Amaxa). PBMC were transfected using Amaxa Nucleofector technology. HeLa cells were transfected using Lipofectamine 2000 (Invitrogen).

Plasmid constructs

The HA-Dok1 and HA-Dok2 constructs (where HA is hemagglutinin) were described previously (4). The constructs encoding the mutants lacking the PH domain were HA- Δ PHDok1, HA- Δ PHDok2, Δ PHDok1-GFP, Δ PHDok2-GFP, GFP-Dok1, and GFP-Dok2, and the chimeric PH domain constructs were PHDok1-GFP, PHDok2-GFP, GST-PHDok1, and GST-PHDok2; several other vectors described previously are detailed in the online supplemental "Methods" section.⁵

PtdIns binding assays

Protein lipid blot assays were conducted as described (16). "PIP Array" and "PIP Strip" were purchased from Echelon Biosciences. To reveal binding, a GST mAb was used in immunoblotting.

Surface plasmon resonance (SPR) experiments

Detailed protocols for SPR experiments were described previously (17). Briefly, the binding of 1 μ M protein to lipid layers containing 90% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Sigma-Aldrich) and 10% phosphatidylinositol phosphate, phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol 4-phosphate (PtdIns4P), or PtdIns5P (Echelon Biosciences) was measured. Protein binding for the four lanes was measured at the same time, and all proteins were tested on the same fresh lipid layers (at least 6000 resonance units were coupled). Proteins were removed from the layers by addition of 20 μ l of 25 μ M NaOH at a flow rate of 100 μ l/min.

Phospholipid extraction and analysis

Hut-78 (10⁷) were stimulated with CD3 Ab (clone 289) (18) during the indicated time periods. PtdIns5P was quantified by mass assay as previously described (12). The phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) generated after lipid kinase assay was extracted and analyzed by HPLC for quantification.

Luciferase assay

Luciferase assay was performed as previously described (4).

T cell proliferation assay

Primary human T cells were transfected with Amaxa Nucleofector technology, and 16 h after transfection (transfection efficiency: 40% GFP⁺ cells) a mixed CD4⁺ T cell population (10⁵) was plated on 96-well plates and left nonstimulated or stimulated with OKT3 plus CD28.2 (0.5 μ g/ml each) or 1 ng/ml PMA plus 1 μ g/ml ionomycin. Cells were cultured for 4 days and plates were pulsed with 1 μ Ci of [³H]thymidine per well for the last day of culturing.

Results and Discussion

The loss of the PH domain blocks Dok-1 and Dok-2 tyrosine phosphorylation and their inhibitory properties

Tyrosine phosphorylation of Dok proteins is detectable in Jurkat T cells upon optimal T cell activation via TCR plus CD28 costimulation (Fig. 1*a*). To investigate the role of the PH do-

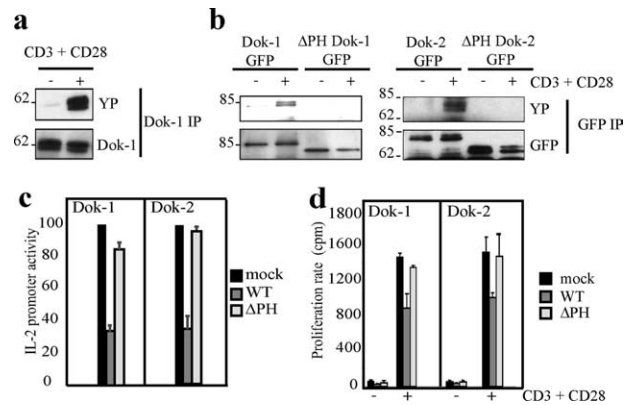


FIGURE 1. The PH domain of the Dok1 and Dok2 proteins are required for their tyrosine phosphorylation and for T cell signaling down-regulation. *a*, Jurkat T cells were left unstimulated or were stimulated with CD3 and CD28 mAbs for 5 min. Immunoprecipitation (IP) with anti-Dok-1 mAbs and then anti-phosphotyrosine (YP) or anti-Dok-1 (Dok-1) Western blotting was performed. Similar results were obtained from three independent experiments. *b*, Jurkat cells expressing GFP-tagged Dok-1 (Dok-1 GFP) or GFP-tagged Dok-1 missing the PH domain (Δ PH Dok-1 GFP) (left panel) or the corresponding Dok-2 constructs (right panel). Cells were stimulated (+) or not (-) with CD3 plus CD28 Abs for 5 min. Dok-1 and Dok-2 tyrosine phosphorylation was observed after anti-GFP immunoprecipitation. Similar results were obtained from two independent experiments. *c*, Jurkat T cells were transfected with a luciferase reporter construct for IL-2 promoter activity plus an empty vector (mock) or vectors for the expression of HA-Dok-1 (left) or HA-Dok-2 (right), WT or deleted for the PH domain (Δ PH). Two hours after transfection, the cells were stimulated with CD3 and CD28 mAbs for 16 h and luciferase activity was measured. Data shown are the average of three independent experiments \pm SD. *d*, Human primary T cells were transfected with an empty vector (pEGFP-N1) or vectors for the expression of GFP-tagged Dok-1 (left) or Dok-2 (right), WT or missing the PH domain (Δ PH). Twenty-four hours after transfection, cells were stimulated (+) or not (-) with CD3 and CD28 mAbs. Data shown are the average of three independent experiments \pm SD.

main, we first compared Jurkat cells overexpressing GFP-tagged Dok-1 or Dok-2 proteins at full length or deleted for their PH domain. Dok-1 and Dok-2 proteins at full length were tyrosine phosphorylated in stimulated cells (Fig. 1*b*). Interestingly, Dok protein tyrosine phosphorylation was undetectable when the PH domain was deleted. Therefore, the PH domain is important for Dok-1 and Dok-2 phosphorylation.

Overexpression of Dok-1 or Dok-2 inhibits IL-2 promoter activity in TCR plus CD28-activated Jurkat cells (4). We used this assay to address the importance of the PH domain for the function of Dok proteins. Cells were transfected with HA-tagged Dok-1 and Dok-2 at full length or deleted of their PH domain and stimulation was conducted with CD3 and CD28 Abs for 16 h. Luciferase assays revealed that IL-2 promoter activity was inhibited by Dok-1 or Dok-2 overexpression proteins at full length (wild type (WT); Fig. 1*c*). However, expression of PH-deleted Dok-1 or Dok-2 proteins (Δ PH) resulted in a loss of inhibitory effects on IL-2 promoter activity. To confirm the importance of the PH domain in the regulatory function of Dok proteins, constructs encoding GFP-tagged Dok-1 or Dok-2 at full length (WT) or deleted for their PH domain (Δ PH) were transfected in human primary T cells. The cells were then stimulated by CD3 and CD28 mAbs and proliferation assays were performed (Fig. 1*d*). We observed an inhibition of T cell proliferation upon overexpression of Dok-1 or Dok-2, but not upon expression of their PH-deleted versions.

⁵ The online version of this article contains supplemental material.

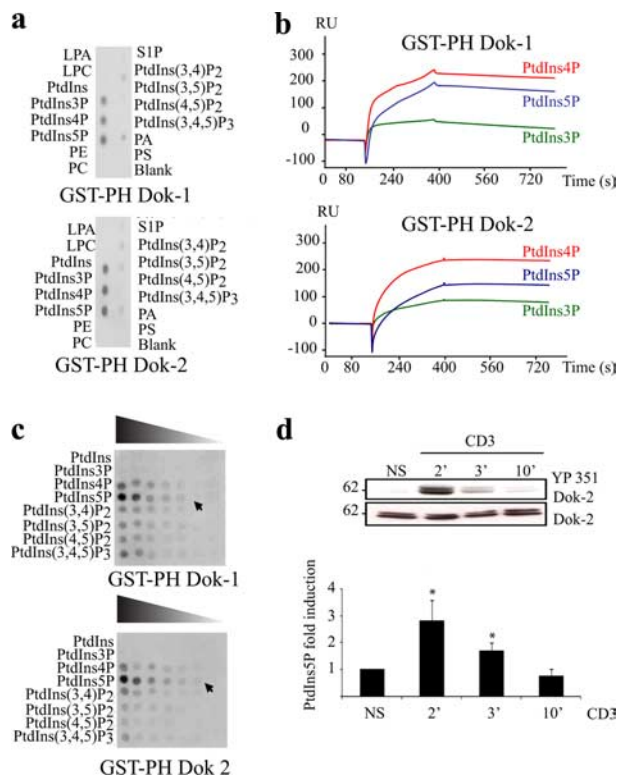


FIGURE 2. The PH domains of Dok-1 and Dok-2 bind PtdIns5P, and a transient increase in PtdIns5P cellular levels correlates with Dok-2 phosphorylation. *a*, Dok-1/Dok-2 PH domains bind phosphatidylinositol monophosphates. The PH domains of Dok-1 (*upper panel*) and Dok-2 (*lower panel*) fused to GST were purified and incubated at 0.5 $\mu\text{g/ml}$ with “PIP Strips” (Echelon Biosciences). PA, Phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; LPA, lysophosphatidic acid; LPC, lysophosphocholine; S1P, sphingosine-1-phosphate. *b*, The PH domain of Dok-1 and Dok-2 bind to PtdIns5P- and PtdIns4P-containing liposomes in SPR. Sensorgrams showing the binding of 1 μM GST-PH Dok-1 (*upper graph*) and GST-PH Dok-2 (*lower graph*) to reconstituted indicated liposomes. PtdIns binding values were subtracted from PtdInsP binding data. *c*, GST-PH Dok-1 and GST-PH Dok-2 bind preferentially to PtdIns5P. The GST-PH fusion proteins were incubated with “PIP Arrays” (Echelon Biosciences) containing serial dilutions of the indicated PI derivatives (100, 50, 25, 12.5, 6.3, 3.2, and 1.6 picomoles). *d*, In vivo PtdIns5P level increases concomitant to Dok-2 phosphorylation under TCR triggering. Hut-78 T cells were stimulated or not (NS) with CD3 mAb for the indicated periods of time. PtdIns5P amounts were measured by mass assay and the relative increases in PtdIns5P levels were plotted in the histogram. Data are expressed as means \pm SEM; *, $p < 0.05$ (Student’s *t* test; $n = 3$). Cell lysates from Hut-78 cells were examined in parallel for Dok-2 and Dok-2 phosphorylation (PY351) by Western blotting.

Taken together, these results indicate that the PH domain is necessary for Dok phosphorylation and to trigger the inhibitory effects of Dok-1 and Dok-2 in activated T cells. We then decided to characterize the ligand of these PH domains.

Dok-1 and Dok-2 PH domains bind PtdIns5P

PH domains are commonly described as phospholipid binding domains (7). Although little is known about Dok-1 and Dok-2 PH domain binding specificity, it was reported that the PH domain of Dok-1 recognizes polyphosphoinositides incorporated in multilamellar vesicles (19). To establish whether both the Dok-1 and the Dok-2 PH domains have phospholipid binding activity, we tested 15 lipids including phosphoinositides in a

protein lipid blot assay using “PIP Strip” membranes (Echelon Biosciences).

After incubation with recombinant GST-PH Dok-1 or GST-PH Dok-2, a signal was clearly detectable with phosphatidylinositol monophosphate species PtdIns3P, PtdIns4P, and PtdIns5P (Fig. 2*a*). By SPR analysis, GST Dok-1 and Dok-2 PH domains interacted mostly with PtdIns4P and PtdIns5P, but not with PtdIns3P (Fig. 2*b*). To quantify the relative affinity of the PH Dok-1 and Dok-2 domains for phosphoinositides, we probed a lipid blot with serial dilutions of eight different phosphoinositides with GST-PH Dok-1 or GST-PH Dok-2 proteins (“PIP Array” membranes; Echelon Biosciences) (Fig. 2*c*). GST immunoblot analysis revealed that the Dok-1 and Dok-2 PH domains bound most tightly to PtdIns5P. Although there is a low binding to other phosphoinositide species, PtdIns5P appears clearly as the preferential ligand for the Dok-1 or Dok-2 PH domain.

The Dok-1 PH domain has been previously described as a potential phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5) P_3) binding module (19); however, the binding activity to phosphatidylinositol monophosphates was not addressed in this study. We did observe a slight binding to PtdIns(3,4,5) P_3 (Fig. 2*c*), but the binding of the PH domains of the Dok proteins are stronger to PtdIns5P. Moreover, the PH domains of Dok proteins have not been identified as a PtdIns(3,4,5) P_3 binding modules in a recently published large-scale screening for PtdIns(3,4,5) P_3 partners (20). We are showing by independent in vitro assays that the Dok-1 and Dok-2 PH domains recognize phosphatidylinositol monophosphates and preferentially PtdIns5P. PtdIns5P increase has been described in few reports, and this lipid appears as a second messenger involved in cell signaling (11). Our results raise the possibility of a yet unknown Dok-1 and Dok-2 regulation via PtdIns5P binding; moreover, they suggest that PtdIns5P could have an impact on T cell activation. Consequently, we decided to measure PtdIns5P levels under T cell activation.

TCR triggering induces PtdIns5P production in T cells

The difficulty of detecting PtdIns5P explains why it has only been biochemically characterized quite recently (10). Few studies report on an increase in PtdIns5P levels (21–23), and classically this lipid is present in low amounts in resting cells. As the method to measure PtdIns5P necessitates a large amount of cellular material, we used a T cell line, Hut-78, instead of primary T cells. The Hut-78 T cell line is not mutated in phosphoinositide biosynthetic enzymes, such as PTEN (phosphatase and tensin homologue deleted in chromosome 10) or SHIP (3). PtdIns5P levels were measured in T cells upon TCR triggering (Fig. 2*d*). Compared with unstimulated cells, a four-times increase in PtdIns5P levels was detected after 2 min of TCR/CD3 stimulation. The PtdIns5P amount then rapidly dropped to basal levels after 10 min. These data show for the first time that PtdIns5P is detectable in T cells upon TCR triggering and suggest that it might be involved in early T cell signaling. In parallel, we investigated the status of Dok-2 tyrosine phosphorylation (Fig. 2*d*, *upper panel*). Dok-2 phosphorylation followed the same kinetic as PtdIns5P increase, suggesting a possible link between Dok-2 tyrosine phosphorylation and PtdIns5P production.

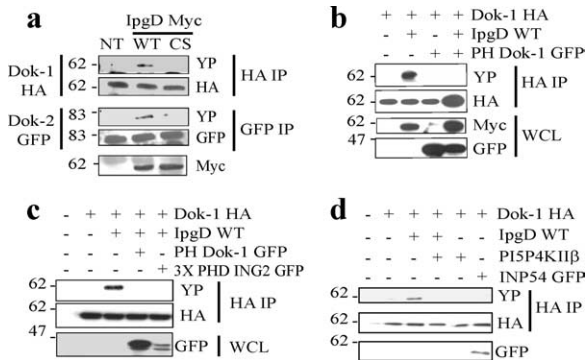


FIGURE 3. PtdIns5P production and availability control Dok-1 and Dok-2 phosphorylation. *a*, Dok-1 and Dok-2 tyrosine phosphorylation increases upon the expression of IpgD WT. Jurkat T cells expressing HA-Dok1 or Dok2 GFP alone (NT) or cotransfected with pRK5-Myc IpgD or pRK5-Myc IpgD C438S (CS) for the expression of IpgD WT or its inactive form, CS, were analyzed 12 h after transfection. Note the increase in Dok-1 and Dok-2 tyrosine phosphorylation (YP) in the presence of active IpgD. PtdIns5P sequestration prevents Dok-1 tyrosine phosphorylation. *b* and *c*, Jurkat T cells (*b*) and HeLa cells (*c*) overexpressing different combinations of proteins as indicated at the *top* were analyzed by Western blotting. Note that Dok-1-increased phosphorylation upon IpgD gain of function is abolished by the overexpression of its isolated PH domain or overexpression of a triplet of the PHD domain of ING2 (3X PHD ING2 GFP). *d*, Dok-1 phosphorylation relies on PtdIns5P but not PtdIns4P or PtdIns4,5P₂ production. HeLa cells overexpressing different combinations of enzymes (INP54 stimulates PtdIns4P production and PIP4KIIβ transforms PtdIns5P in PtdIns4,5P₂) as indicated at the *top* were analyzed by Western blotting. INP54 was overexpressed as GFP fusion and controlled by Western blotting of cell lysates with anti-GFP Abs. IP, Immunoprecipitation; WCL, whole cell lysate.

PtdIns5P increase induces Dok-1 and Dok-2 protein tyrosine phosphorylation

To further establish a link between PtdIns5P production and Dok-1 and Dok-2 regulation, we used the bacterial inositol-4-phosphatase IpgD (12). IpgD is a virulence factor of *S. flexneri* transforming PtdIns(4,5)P₂ into PtdIns5P via its 4-phosphatase activity. IpgD ectopic expression induces high PtdIns5P production in Jurkat T cells (23). To study the impact of IpgD and thereby that of the PtdIns5P increase on Dok-1 and Dok-2 tyrosine phosphorylation, Jurkat T cells were transfected with a Myc-tagged IpgD phosphatase (WT) expression construct or its phosphatase-dead C438S mutant construct. Interestingly, IpgD expression but not its phosphatase-dead version leads to Dok-1 and Dok-2 tyrosine phosphorylation (Fig. 3*a*). These data further indicate that PtdIns5P increase induces Dok-1 and Dok-2 phosphorylation and strongly suggest that the Dok-1 and Dok-2 proteins are PTK substrates downstream of PtdIns5P production. To demonstrate a more direct effect of PtdIns5P on Dok-1 and Dok-2 phosphorylation, we tested whether overexpression of the Dok-1 PH domain could inhibit Dok phosphorylation (competitive effect). Jurkat T cells containing detectable levels of a tyrosine-phosphorylated Dok-1 protein (overexpressing IpgD together with HA-tagged Dok-1) were transfected with plasmids encoding the GFP-PH domain of Dok-1 (PH Dok-1 GFP). Dok-1 PH domain expression blocks Dok-1 tyrosine phosphorylation induced by IpgD (Fig. 3*b*). Similar results were reproduced in HeLa cells (Fig. 3*c*) and extended to another PtdIns5P binding module, the GFP trimer of the plant homeodomain (PHD) motif of ING2 (3× PHD ING2 GFP) (16). Both PtdIns5P partners (PH-Dok-1 and

PHD-ING2) were able to block Dok-1 tyrosine phosphorylation induced by PtdIns5P increase.

To test for the specific contribution of PtdIns5P increase during ectopic IpgD expression on Dok-1 and Dok-2 phosphorylation, HeLa cells expressing HA-tagged Dok-1 were cotransfected with IpgD plus GFP-tagged Inp54 or the type II PtdIns5P 4-kinaseβ (PIP4KIIβ) (Fig. 3*d*). Inp54 is an inositol 5-phosphatase known to transform PtdIns(4,5)P₂ into PtdIns4P and PIP4KIIβ is a kinase that transforms PtdIns5P into PtdIns(4,5)P₂ (12). Inp54 ectopic expression increasing PtdIns4P was unable to induce the tyrosine phosphorylation of Dok-1 (HA-Dok-1 plus INP54 GFP conditions; Fig. 3*d*). Using an *in vitro* SPR binding approach, both PtdIns4P and PtdIns5P are recognized by Dok PH domains (Fig. 2*b*); however, only PtdIns5P, but not PtdIns4P, is a regulator module for the Dok-1 protein (Fig. 3*d*). When HA-Dok-1 plus IpgD were coexpressed in the presence of PIP4KIIβ, Dok-1 phosphorylation was lost. In control, coexpression of HA Dok-1 and PIP4KIIβ without IpgD did not lead to Dok-1 phosphorylation. Taken together, our results indicate that PtdIns5P production controls Dok protein tyrosine phosphorylation in cells.

PtdIns5P could be a new, unexpected lipid second messenger in T cell activation. It has been reported that the PtdIns5P production induces Akt activation in epithelial cells infected by *S. flexneri* probably via PTK activation leading to the stimulation of PI3K (13). As the Dok-1 and Dok-2 PH domains are able to bind PtdIns5P, and PtdIns5P production induces PTK activation, PtdIns5P could bring together activated PTKs and their substrates (Dok proteins). PtdIns5P production could support positive cell signals via PI3K signaling and negative cell signals via Dok proteins. PtdIns5P might thereby function as a sensor in setting the threshold for T cell activation and in maintaining T cell homeostasis. To investigate this hypothesis, further studies are required to better understand the molecular mechanisms inducing PtdIns5P increase in eukaryotic cells.

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Disclosures

The authors have no financial conflict of interest.

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