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Dok-1 and Dok-2 adaptor molecules are regulated by PdtIns5P production in T cells[1].

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Running title: Dok-1 and Dok-2 PH domains / PtdIns5P interactions

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Summary

Downstream of tyrosine kinases (Dok) proteins, Dok-1 and Dok-2 are involved in the T cell homeostasis maintenance. Dok proteins tyrosine 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 phosphoinositides species, phosphatidylinositol-5-phosphate (PtdIns5P). Dok tyrosine phosphorylation correlates with a PtdIns5P production in T cells upon TCR triggering. Further, 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.

Introduction

Stimulation of membrane receptors such as the TCR on T cells induces the activation of protein tyrosine kinases (PTKs), and subsequently the phosphorylation of substrates, which contribute to the formation of intracytoplasmic multiprotein network. The downstream of tyrosine kinases (Dok) proteins, Dok-1 and Dok-2 are involved in negative T cell activation control (1). During T cell activation, Dok proteins 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 antibody responses and a spontaneous autoimmunity disease (6).

Interestingly, Dok-1 and Dok-2 contain 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 playing 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 PIs species mediate signals through their binding to proteins containing specific interaction domains including PH, PX, FYVE or 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 Dok-1 and Dok-2 PH domains.

PtdIns5P is a rare PIs 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 evidences report that enhanced

tyrosine phosphorylation increases cellular PtdIns5P levels (14, 15). Altogether, these data argue for an important role of PtdIns5P in cell signaling. Here, 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, Hela cells were cultured in DMEM. Both medium 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 µF and 250 V using a Bio-Rad Gene Pulser or nucleofected according to the manufacturer's instructions (http://www.amaxa.com/). PBMC were transfected using Amaxa Nucleofector technology. Hela cells were transfected using Lipofectamin 2000 ™ (Invitrogen).

Plasmid constructs

The HA-Dok1 and HA-Dok2 constructs were described previously (4). All the constructs encoding the mutants lacking the PH domain: HA-ΔPHDok1, HA-ΔPHDok2, ΔPHDok1-GFP and ΔPHDok2-GFP, GFP-Dok1 and GFP-Dok2, chimeric PH domain constructs: PHDok1-GFP, PHDok2-GFP, GST-PHDok1 and GST-PHDok2, and finally several other vectors described previously are detailed in supplemental method.

PtdIns binding assays

Protein lipid-blot assays were carried out as described (16). “PIP array” and “PIP strip” were purchased from Echelon biosciences Inc. To reveal binding, 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 µM protein to lipid layers containing 90% DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine (SIGMA) and 10% PtdIns, PtdIns3P, PtdIns4P or PtdIns5P (Echelon biosciences Inc.) was measured. Protein binding for the 4 lanes were measured at the same time and all proteins were tested on the same fresh lipid layers (at least 6000 RU was coupled). Proteins were removed from the layers by addition of 20µl 25 µM NaOH at a flow rate of 100 µl/min.

Phospholipid extraction and analysis

Hut-78 (10^7) were stimulated with CD3 antibody (clone 289) (18) during indicated time. PtdIns5P was quantified by mass assay as previously described (12). The PtdIns(4,5)P_2 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 the Amaxa Nucleofector technology, 16 hrs after transfection (transfection efficiency: 40 % GFP^+ cells), mixed $CD4^+$ T cell population

(10^5) was plated on 96-wells plate and left non stimulated or stimulated with OKT3 + CD28.2 (0.5µg/ml each), or 1ng/ml PMA + 1µg/ml Ionomycin. Cells were cultured for 4 days and plates were pulsed with 1 µCi [^{3}H]-thymidine/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 also detectable in Jurkat T cells upon an optimal T cell activation via TCR plus CD28 co-stimulation (Fig. 1A). To investigate the role of the PH domain, we first compared Jurkat cells overexpressing GFP-tagged Dok-1 or Dok-2 proteins full length or deleted for their PH domain. Dok-1 and Dok-2 proteins full length were tyrosine phosphorylated in stimulated cells (Fig. 1B). Interestingly, Dok proteins tyrosine phosphorylation was undetectable when PH domain was deleted. Therefore, 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 full length or deleted of their PH domain and stimulation was carried out with CD3 and CD28 antibodies for 16 hrs. Luciferase assays revealed that IL-2 promoter activity was inhibited by Dok-1 or Dok-2 overexpression proteins full length (wild type, WT) (Fig. 1C). However, expression of PH-deleted Dok-1 or Dok-2 proteins (ΔPH) 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 full length (WT) or deleted for their PH domain (ΔPH) were transfected in human primary T cells. The cells were then stimulated by CD3 and CD28 mAbs and proliferation assays were performed (Fig. 1D). 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.

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 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 Dok-1 and Dok-2 PH domains have a phospholipid-binding activity, we tested 15 lipids including phosphoinositides in a protein lipid-blot assay using "PIP-Strip" membranes.

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. 2A). By SPR analysis, GST Dok-1 and Dok-2 PH domains interacted mostly with PtdIns4P and PtdIns5P but not with PtdIns3P (Fig. 2B). To quantify the relative affinity of PH Dok-1 and Dok-2 domain 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) (Fig. 2C). GST immunoblot analysis revealed that Dok-1 and Dok-2 PH domain bound most tightly to PtdIns5P. Although there is a low binding to other phosphoinositides species, PtdIns5P appears clearly as the preferential ligand for Dok-1 or Dok-2 PH domain.

Dok-1 PH domain has been previously described as a potential PtdIns(3,4,5)P_3 binding module (19), however the binding activity to phosphatidylinositol monophosphates was not addressed in this study. Here we observe a slight binding to PtdIns(3,4,5)P_3 (Fig. 2C), but the

binding of the PH domains of Dok proteins are stronger to PtdIns5P .Moreover, the PH domain of Dok proteins has not been identified as a PtdIns(3,4,5)P_3 binding module in a recent published large scale screening for PtdIns(3,4,5)P_3 partners (20). We are showing by independent in vitro assays that Dok-1 and Dok-2 PH domain 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 a PtdIns5P production in T cells

The difficulty to detect PtdIns5P explains why it has only been quite recently biochemically characterized (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. Hut-78 T cell line is not mutated in phosphoinositide biosynthetic enzymes, such as phosphatase and tensin homologue deleted in chromosome 10 (PTEN) or SH2 domain-containing inositol polyphosphate 5'-phosphatase (SHIP) (3). PtdIns5P levels were measured in T cells upon TCR triggering (Fig. 2D). Compared to unstimulated cells, a four time increase in PtdIns5P levels was detected after 2 min of TCR/CD3 stimulation. 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. 2D; upper panel). Dok-2 phosphorylation followed the same kinetic as

PtdIns5P increase, suggesting a possible link between Dok-2 tyrosine phosphorylation and PtdIns5P production.

PtdIns5P increase induces Dok-1 and Dok-2 proteins 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 *Shigella flexneri* transforming PtdIns(4,5)P_2 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 of 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 (CS) construct. Interestingly, IpgD expression but not its phosphatase-dead version leads to Dok-1 and Dok-2 tyrosine phosphorylation (Fig. 3A). These data further indicate that PtdIns5P increase induces Dok-1 and Dok-2 phosphorylation, and strongly suggest that 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 the overexpression of Dok-1 PH domain could inhibit Dok phosphorylation (competitive effect). Jurkat T cells containing detectable levels of 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). The Dok-1 PH domain expression blocks Dok-1 tyrosine phosphorylation induced by IpgD (Fig. 3B). Similar results were reproduced in Hela cells (Fig. 3C) and extended to another PtdIns5P-binding module, the GFP-trimer of PHD (Plant Homeo Domain) motif of ING2 (3X PHD ING2 GFP) (16). Both PtdIns5P partners (PH-Dok-1 or 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 co-transfected with IpgD plus GFP-tagged Inp54 or the type II PtdIns5P 4-kinaseβ (PIP4KIIβ) (Fig. 3D). Inp54 is an inositol-5-phosphatase known to transform PtdIns(4,5)P_2 into PtdIns4P and PIP4KIIβ is a kinase that transforms PtdIns5P into PtdIns(4,5)P_2 (12). Inp54 ectopic expression increasing PtdIns4P was unable to induce the tyrosine phosphorylation of Dok-1 (Fig. 3D, HA-Dok-1 plus INP54 GFP conditions). Using an *in vitro* SPR binding approach both PtdIns4P and PtdIns5P are recognized by Dok PH domains (Fig. 2B), however only PtdIns5P, but not PtdIns4P, is a regulator module for Dok-1 protein (Fig. 3D). When HA-Dok-1 plus IpgD were co-expressed in presence of PIP4KIIβ, Dok-1 phosphorylation was lost. In control, co-expression 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 proteins 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 *Shigella flexneri* probably via a PTK activation leading to the stimulation of phosphoinositide 3'-kinase (PI3K) (13). As Dok-1 and Dok-2 PH domains are able to bind PtdIns5P and PtdIns5P production induces PTK activation, the 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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Figure legends

Figure 1. The PH domain of Dok1 and Dok2 proteins are required for their tyrosine phosphorylation and for T cell signaling down-regulation.

(A) Jurkat T cells were left unstimulated (NS) or were stimulated with CD3 and CD28 mAbs for 5 min. Immunoprecipitation (IP) with anti-Dok-1 mAbs then anti-phosphotyrosine (PY) or anti-Dok-1 (Dok-1) Western Blot 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 (ΔPHDok-1 GFP) (left panel), or the corresponding Dok-2 constructs (right panel). Cells were stimulated (+) or not (-) with CD3 plus CD28 antibodies for 5 min. Dok-1 and Dok-2 tyrosine phosphorylation was observed after anti-GFP IP. 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 HA-Dok-1 (left diagram) or HA-Dok-2 (right diagram) wild-type (WT) or deleted for the PH domain (ΔPH). Two hours after transfection, the cells were stimulated with CD3 and CD28 mAbs for 16 hours and luciferase activity was measured. Data shown are the average of three independent experiments ± s.d.

(D) Human primary T cells were transfected with an empty vector (pEGFP-N1) or vectors for the expression of GFP-tagged Dok-1 (left diagram) or Dok-2 (right diagram) wild-type (WT), or missing the PH domain (ΔPH). Twenty-four hours after transfection, cells were stimulated (+) or not (-) with CD3 and CD28 mAbs. Data shown are the average of three independent experiments ± s.d.

Figure 2. The PH domains of Dok-1 and Dok-2 bind PtdIns5P and transient increase in PtdIns5P cellular levels correlate with Dok-2 phosphorylation.

(A) Dok-1/Dok-2 PH domains bind phosphatidylinositol monophosphates. The PH domain of Dok-1 (upper panel) and Dok-2 (lower panel) fused to GST were purified and incubated at 0,5µg/ml with PIP strips. 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 part) and GST-PH Dok-2 (lower part) to reconstituted indicated liposomes. PtdIns binding values were substracted to PtdInsP binding data.

(C) GST-PH Dok-1 and GST-PH Dok-2 bind preferentially to PtIns5P. The GST-PH fusion proteins were incubated with "PIP arrays" containing serial dilutions of the indicated PI derivatives (100, 50, 25, 12.5, 6.3, 3.2 and 1.6 picomoles).

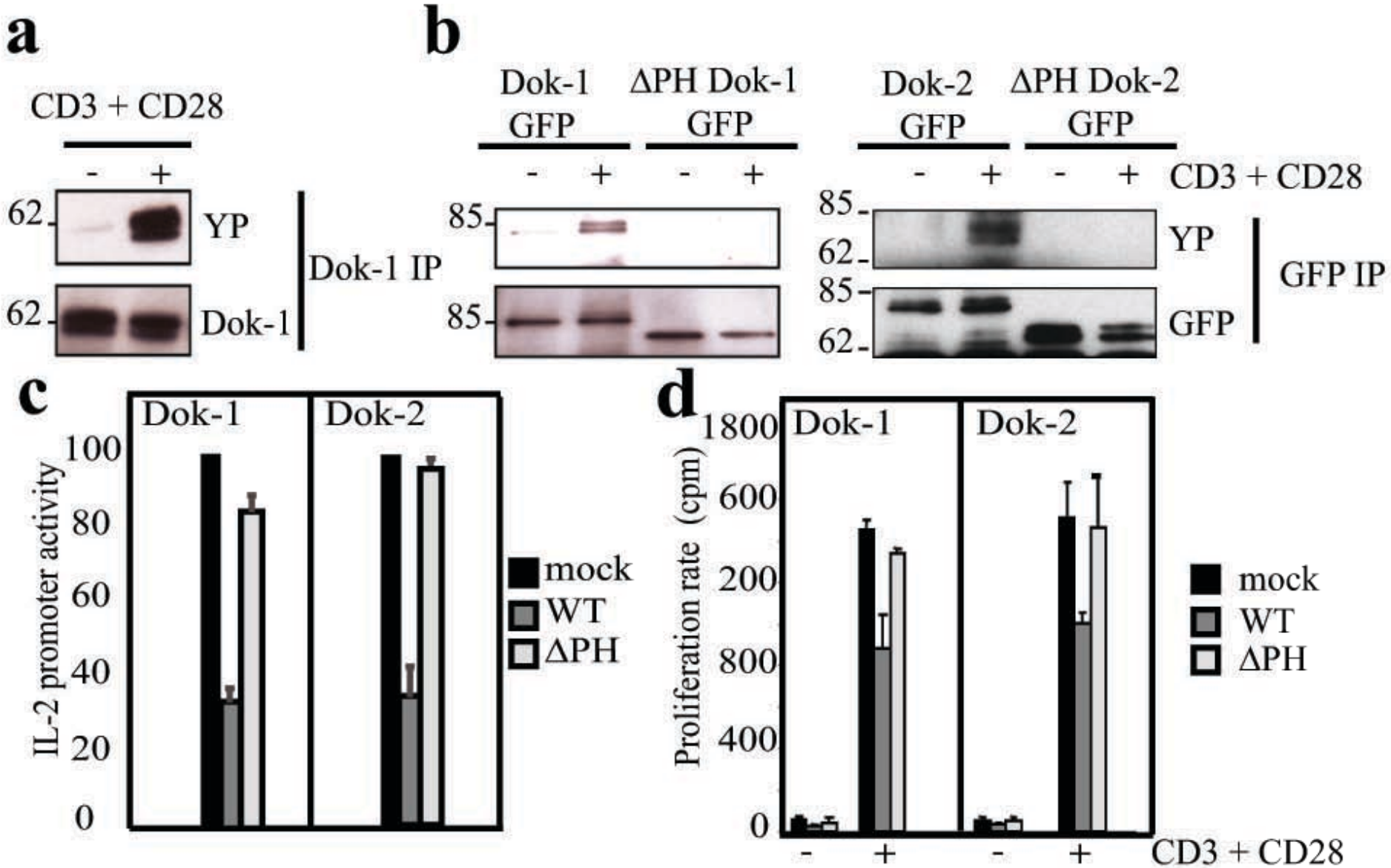
(D) In vivo PtdIns5P level increases concomitant to Dok-2 phosphorylation under TCR triggering. Hut-78 T cells were stimulated or not (NS) with CD3 mAb for the indicated periods of time. PtdIns5P amounts were measured by mass assay and the relative increase in PtdIns5P levels were plotted in the histogram. Data are expressed as means ± SEM, * $p<0.05$ (student test t-test, n=3). Cell lysates from Hut-78 cells were examined in parallel for Dok-2 and Dok-2 phosphorylation (PY351) by Western Blot.

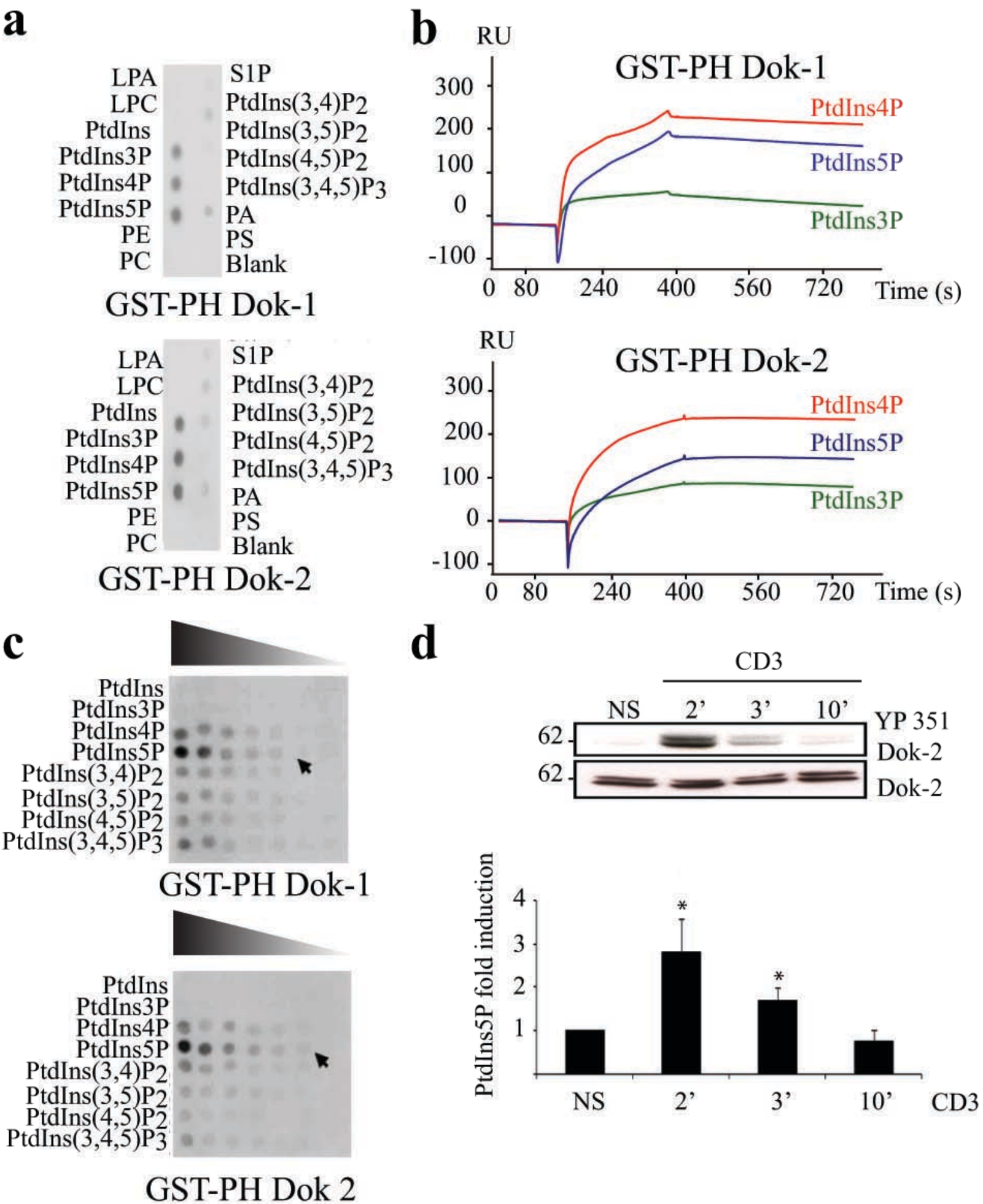
Figure 3. PtdIns5P production and availability control Dok-1 and Dok-2 phosphorylation.

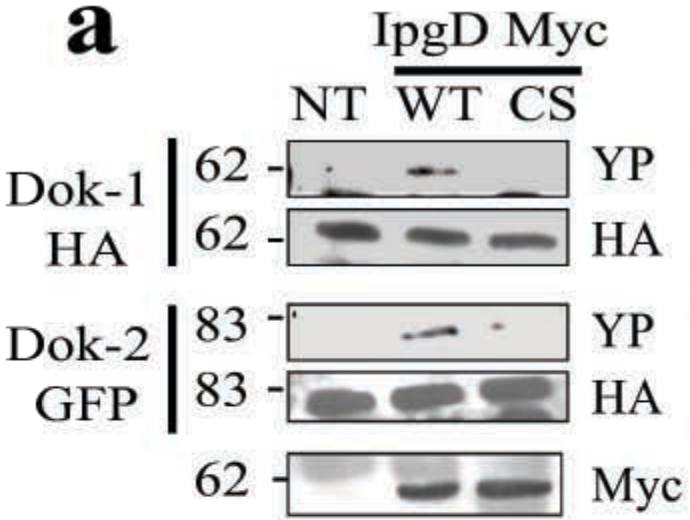
(A) Dok-1 and Dok-2 tyrosine phosphorylation increases upon the expression of IpgD WT. Jurkat T cells expressing HA-Dok1 or Dok2 GFP alone (NT), or co-tranfected respectively with pRK5-Myc IpgD or pRK5-Myc IpgD C438S for the expression of IpgD wild-type (WT) or its inactive form (CS) were analysed 12 hours after transfection. Note the increase in Dok-1 and Dok-2 tyrosine phosphorylation in the presence of active IpgD.

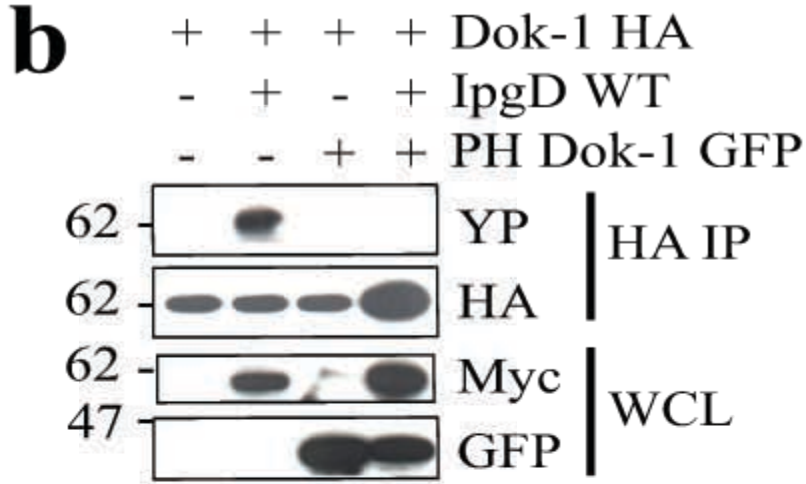
PtdIns5P sequestration prevents Dok-1 tyrosine phosphorylation. Jurkat T cells (B) and Hela cells (C) overexpressing different combinations of proteins as indicated on top were analysed in Western Blot. Note that Dok-1 increased phosphorylation upon IpgD gain of function is abolished by the overexpression of its isolated PH domain or of a triplet of the PHD domain of ING2.

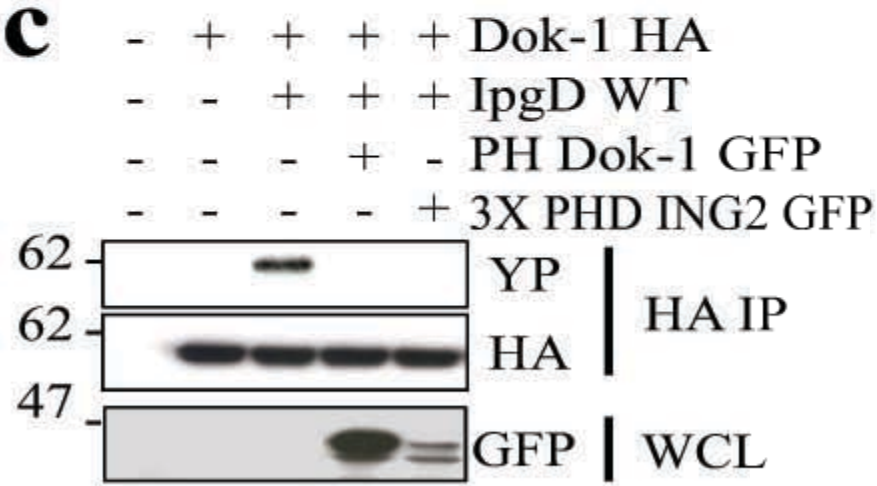
(D) Dok-1 phosphorylation relies on PtdIns5P but not PtdIns4P or PtdIns4,5P_2 production. Hela cells overexpressing different combinations of enzymes (INP54 stimulates PtdIns4P production and PI5P4KIIβ transforms PtdIns5P in PtdIns4,5P_2) as indicated on top were analysed in Western Blot. INP54 was overexpressed as GFP fusion and controlled by Western Blot of cell lysates with anti-GFP antibodies.

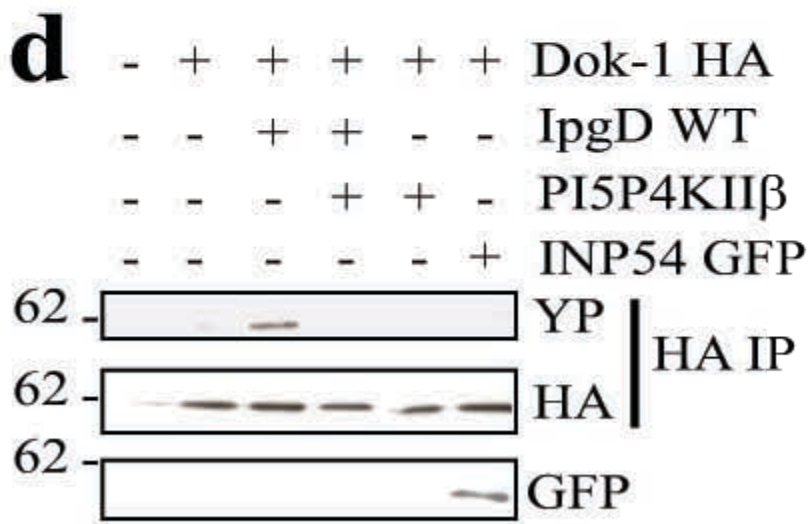

a
CD3 + CD28
- +
62
YP
62
Dok-1
Dok-1 IP
b
Dok-1 GFP
ΔPH Dok-1 GFP
Dok-2 GFP
ΔPH Dok-2 GFP
- + - +
- + - +
CD3 + CD28
85
85
85
62
85
62
YP
GFP
GFP IP
c
Dok-1
Dok-2
IL-2 promoter activity
100
80
60
40
20
0
mock
WT
ΔPH
d
Dok-1
Dok-2
Proliferation rate (cpm)
1800
600
200
800
400
0
mock
WT
ΔPH
- + - +
CD3 + CD28


a
LPA
LPC
PtdIns
PtdIns3P
PtdIns4P
PtdIns5P
PE
PC
S1P
PtdIns(3,4)P2
PtdIns(3,5)P2
PtdIns(4,5)P2
PtdIns(3,4,5)P3
PA
PS
Blank
GST-PH Dok-1
GST-PH Dok-2
b
RU
GST-PH Dok-1
PtdIns4P
PtdIns5P
PtdIns3P
300
200
100
0
-100
0 80 240 400 560 720 Time (s)
GST-PH Dok-2
c
PtdIns
PtdIns3P
PtdIns4P
PtdIns5P
PtdIns(3,4)P2
PtdIns(3,5)P2
PtdIns(4,5)P2
PtdIns(3,4,5)P3
GST-PH Dok-1
GST-PH Dok 2
d
CD3
NS 2' 3' 10'
YP 351
62
Dok-2
PtdIns5P fold induction
4
3
2
1
*
NS 2' 3' 10' CD3


a
IpgD Myc
NT WT CS
Dok-1 HA
62
62
YP
HA
HA IP
Dok-2 GFP
83
83
YP
HA
GFP IP
62
Myc


b
+ + + + Dok-1 HA
- + - + IpgD WT
- - + + PH Dok-1 GFP
62
62
YP
HA
HA IP
62
47
Myc
GFP
WCL


c
- + + + + Dok-1 HA
- - + + + IpgD WT
- - - + - PH Dok-1 GFP
- - - - + 3X PHD ING2 GFP
62
62
47
YP
HA
HA IP
GFP
WCL


d
- + + + + + Dok-1 HA
- - + + - - IpgD WT
- - - + + - PI5P4KIIβ
- - - - - + INP54 GFP
62
62
62
YP
HA
HA IP
GFP