

1. Introduction

Phosphoinositides (PIs) are constituents of cell membranes playing a critical role in cell signaling pathways. Their biosynthesis is tightly regulated by several lipid kinases and phosphatases, which disruption can give rise to several pathologies [1]. Among PIs, phosphatidylinositol 5-phosphate (PtdIns5P) is emerging as a potential lipid messenger [2-3]. Indeed, enhanced tyrosine phosphorylation induced by pervanadate treatment reveals detectable cellular PtdIns5P levels in epithelial cells [4]. Moreover, stimulation of some receptor tyrosine kinases such as the insulin receptor induces a PtdIns5P increase [5]. An increase in PtdIns5P levels has been also reported in hematopoietic cells expressing the oncogenic tyrosine kinase nucleophosmin anaplastic lymphoma kinase (NPM-ALK), a chimeric protein found in the large majority of anaplastic large cell lymphomas (ALCLs) [6]. In blood platelets, thrombin stimulation can also increase PtdIns5P production [7]. Bacterial infection by *Shigella flexnerii* generates, via the virulence factor IpgD, PtdIns5P at the plasma membrane of the host cells leading to Akt activation [8-9].

PtdIns5P has been identified in different subcellular compartments such as the plasma membrane and the nucleus [3]. These data suggest that PtdIns5P is entering the large investigation field of signaling pathways.

PIs mediate signals through their binding to proteins containing specific interaction domains [10]. Interestingly, we found that Dok-1/Dok-2 pleckstrin homology (PH) domains bind *in vitro* to PtdIns5P [11]. Previous studies have identified ATX1 and ING2 plant homeo domain (PHD) as PtdIns5P binding module [12-13]. However, it remains still essential to identify selective PtdIns5P binder modules in order to better characterize its function. Using surface plasmon resonance analysis, we identify in this study a PtdIns5P partner harboring a strong selectivity for this lipid. This new partner is the PH domain of another Dok family member, Dok-5.

T cells are a major cell type from the adaptive immune system that is involved in controlling the immune response. T cell activation is initiated by the stimulation of the T cell receptor (TCR). Production of the PIs species such as PtdIns(3,4,5)P₃ has been reported to act as a key checkpoint in T cell signaling [14]. Recently, we observed that PtdIns5P is produced in T cells upon TCR triggering. This production leads to Dok-1 and Dok-2 tyrosine phosphorylation and subsequent T cell signaling negative regulation, suggesting that PtdIns5P is a second messenger downstream of TCR stimulation [11].

Using PH-Dok-5 as a tool to sequester cognate PtdIns5P, as well as IpgD to artificially increase the pool of PtdIns5P, we found that PtdIns5P is indeed required for T-cell signaling. Altogether our data argue for an early impact of PtdIns5P during T cell activation. PtdIns5P seems to be a newly identified player in T cell signaling and is able to activate Src family tyrosine kinases and Akt pathways that are crucial in T cell activation.

2. Materials and methods

2.1. Culture cells and transfection

Hut-78 T cells were grown in RPMI 1640 medium supplemented with 10 % heat inactivated FCS, 2 mM L-glutamine, and 1 mM sodium-pyruvate. For transfection, Hut-78 T cells (10^7) were electroporated at 960 μ F and 250 V using the GenePulser XcellTM (BioRad).

2.2. Plasmid constructs

The construct β DNA4HADok-5 corresponding to the wild-type (WT) full-length human Dok-5 cDNA tagged with HA epitope at the N-terminus, was generated by subcloning of the pGEM-T easy vector containing HA tagged Dok-5, described previously [15], into the β DNA4 vector, using the restriction site NotI. The constructs encoding the Dok-4 and Dok-5 PH domains fused to the GST protein were obtained by PCR amplification of human Dok-4 [16] or human Dok-5 tagged HA epitope, both in β DNA4 expression vector. The PCR products were sequenced and cloned in pGEX-4T3 (Amersham Biosciences Limited, England, UK).

The GFP-tagged Dok-5 PH domain construct was generated by PCR amplification from pGEX-4T3 PH-Dok-5 using the following primers: sense 5'-ggcgctcgagatggcttccaattttaatgacatagtgaagcaaggg-3' (XhoI site added) and antisense 5'-gcggcgaattcccttggatccgtgttcctacacactccatctg-3' (EcoRI site added). The PCR products were sequenced and cloned in pEGFP-N1 XhoI/EcoRI (Clontech, Palo Alto, CA). Myc epitope-tagged IpgD WT and IpgD-C438S, a phosphatase-dead mutant were in the pRK5 expression vector as described previously [8].

2.3. Antibodies and Western blotting

CD3 mAb (clone 289) recognizing CD3 ϵ subunit of the CD3-TCR complex was reported previously [17]. Anti-Myc epitope mAb, 9E10 and anti-phosphotyrosine mAb, 4G10 were purchased from Millipore (Billerica, MA). Anti-GFP mAbs were purchased from Roche (Meylan, France). The rabbit polyclonal antibodies recognizing Akt (# 9272), phospho-LAT (Tyr191) (# 3584), phospho-ZAP-70 (Tyr493) (# 2704), phospho-Src family kinases (Tyr416) (# 2101) and the rabbit mAbs recognizing phospho-p44/42 MAPK (ERK-1/2) (Thr202/Tyr204) (# 4376), phospho-Akt (Ser473) (# 4058) were purchased from Cell signaling technology, Inc. Anti-GST mAb ND2.1 was a kind gift of Dr. J. L. Teillaud (Inserm U 872, Centre de Recherche des Cordeliers, Paris, France).

For Western blotting, samples were resolved by standard 12% SDS-polyacrylamide gels. Membranes were blocked and probed with specific antibodies. Blots were then incubated with the appropriate secondary antibodies, anti-rabbit IgG or anti-mouse IgG, both conjugated with horseradish peroxidase (DAKO Denmark A/S). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences Limited, England, and UK).

2.4. Stimulation and cell lysates

Hut-78 T cells ($10 \cdot 10^6$) were stimulated at 37 °C in RPMI 50mM Hepes. Stimulations were carried out for the indicated times using CD3 mAb 289 at 10 μ g/ml. The cells were pelleted in a microcentrifuge and lysed in buffer containing 50 mM Hepes (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 20 mM NaF, 20 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml protease inhibitors (protease inhibitors cocktail, Sigma, St Louis, MO) and 1 mM Na₃VO₄ for 10 min at 4°C, then centrifugated at 13000 rpm for 10 min at 4°C.

2.5. Phospholipids binding assays

Protein lipid-blot assays were carried out as described [18]. “PIP strip” membranes were purchased from Echelon biosciences Inc. To reveal PIs – GST PH domains interactions, the anti-GST mAb was used in immunoblotting.

2.6. BiaCore ®/ Surface plasmon resonance (SPR) experiments

Detailed protocols for SPR experiments were described previously [19]. Briefly, the binding of 1 μ M protein was measured on reconstituted liposomes containing 90% DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine (Sigma) and 10% PtdIns or mono-PtdInsP (Echelon biosciences Inc.) immobilized on L1 chips. At least 6000 RU of liposomes were coupled. Binding to PtdIns and mono-PtdInsP were measured at the same time and all proteins were tested on the same freshly prepared liposomes.

2.7. Luciferase assays

The promoter assay plasmids pIL-2-Luc composed of IL-2 promoter, fused to firefly luciferase reporter gene and p β -actin-Rluc composed of β -actin promoter, fused with *Renilla* luciferase gene were previously reported and luciferase assay was performed as previously described [20]. PMA was purchased from Sigma (St Louis, MO).

2.8. Phospho flow by FACS Analysis

Phospho flow analysis was performed by cytometry as previously described [21]. Briefly, cells were fixed and permeabilized, incubated with anti-phospho-Src Y416 (# 2101, Cell Signaling Technology) antibodies and appropriate biotinylated secondary antibodies. Finally, revelation was performed using Streptavidin-phycoerythrin solution (#IM3325, Beckman Coulter).

3. Results

3.1. PtdIns5P increase is sufficient to trigger downstream cell signaling in T cells

Ectopic IpgD expression induces high PtdIns5P production in eukaryotic cells and activation of the PI 3-kinase /Akt pathway [8-9]. To study the impact of IpgD and thereby of PtdIns5P increase on T cell signalling, Hut-78 T cells were transfected with Myc-tagged IpgD WT or a C438S (CS) mutant as control. The Hut-78 T cell line was used because it is not mutated in key enzymes of the PIs metabolism, such as the phosphatase and tensin homologue deleted in chromosome 10 (PTEN) or the SH2 domain-containing inositol polyphosphate 5'-phosphatase (SHIP) as observed in Jurkat cells [22]. IpgD expression in epithelial cell lines induces Akt activation as detected by its phosphorylation at serine position 473 [9]. As shown in Fig1A, IpgD WT expression but not its phosphatase-dead version leads to Akt phosphorylation at serine 473 4h after transfection. Thus, IpgD expression is also able to trigger signaling events in this T cell line.

TCR ligation activates protein tyrosine kinases (PTKs) like Src kinase family members and ZAP-70, which regulate several signalling events such as MAP kinases and PI3-kinase/Akt pathways [23]. To determine whether PtdIns5P production could regulate some of the TCR-mediated signalling events, activation of several PTKs was analyzed using different anti-phosphoprotein antibodies on lysates of IpgD WT or IpgD CS expressing cells (Fig. 1B). Pervanadate treatment of Hut-78 T cells was used as a positive control. The Src kinase family members, but not ZAP-70 were tyrosine phosphorylated upon IpgD expression. These results are in accordance with previous data demonstrating that IpgD enhances the tyrosine phosphorylation of Src kinase substrates in T cells such as Dok-1 and Dok-2 proteins [11,24]. Moreover, the tyrosine phosphorylation of a ZAP-70 substrate, LAT was not increased in presence of IpgD WT, confirming that PtdIns5P production does not lead to ZAP-70

activation. The phosphorylation status of other signalling proteins such as ERK-1/2, was not altered by IpgD expression. The activation of signalling pathways in T cells results in the production of several cytokines including interleukin (IL)-2, a key cytokine involved into the T cell development and proliferation during an adoptive immune response. It has been reported that Akt can positively regulate the transcriptional activity of IL-2 [25]. To characterize the role of PtdIns5P in T cell signaling, we measured IL-2 promoter activity in Hut-78 T cells (Fig. 1C). Therefore, Hut-78 T cells were co-transfected with an IL-2 promoter-dependent luciferase reporter plasmid and IpgD WT or IpgD CS. We used as a positive control, phorbol myristate acetate (PMA) plus ionomycin treatment to determine the highest induction of IL-2 promoter activity in this cell system. Interestingly, we were able to detect a great IL-2 promoter enhancement upon IpgD WT transfection as compared to IpgD CS. However, a slight effect of catalytically-dead IpgD is detectable. As IpgD is a bacterial protein, we assume that its expression in mammalian cells may induce some cellular stress response detectable both in the case of IpgD WT or CS forms that may interfere at the level of gene expression. At the molecular level, it has been reported that SigD, the ortholog of IpgD in *Salmonella typhimurium* can interfere with Cdc42 functions independently of its catalytic activity [26] suggesting that IpgD CS could have also some direct effects. However, so far no functional protein domain, apart its catalytic region, has been identified in the peptide sequence of IpgD.

Altogether, these data suggest that PtdIns5P increase induces positive activation events in T cells.

3.2. Identification of a new PtdIns5P binding module: the PH domain of Dok-5 protein

To further establish that PtdIns5P enhances some T cell signaling events, we sought to identify a selective PtdIns5P interacting domain, which could be used to sequestrate

endogenous PtdIns5P generated by TCR stimulation. We previously described Dok-1 and Dok-2 PH domain binding-specificity [11]. These PH domains have a double specificity for PtdIns4P and PtdIns5P with a preference for PtdIns5P in a protein-lipid blot [11]. Moreover, PtdIns5P enhances Dok-1 and Dok-2 phosphorylation levels. Here, we checked the lipid binding properties of the PH domains of two other Dok family members also expressed in T cells [15], Dok-4 and Dok-5 by fat blotting (Fig. 2 A) and SPR analysis (Fig.2 B).

To establish whether both Dok-4 and Dok-5 PH domains have phospholipid-binding properties, we tested 15 lipids including phosphoinositides in a protein lipid-blot (Fig. 2A). After incubation with recombinant GST-PH Dok-5, a signal was detectable with the phosphatidylinositol monophosphate species, PtdIns4P and PtdIns5P. The spectrum of binding of the GST-PH Dok-4 is a bit larger, since it recognizes also PtdIns3P. To further establish the Dok-4 and Dok-5 PH domain binding properties, we used SPR analysis, a sensitive method to analyze monophosphorylated-PIs binding. SPR biosensor analysis revealed that the Dok-5 PH domain is able to strongly bind PtdIns5P compared to the other tested monophosphorylated PIs (Fig. 2B and supplemental figure S1). Dok-4 PH domain is also able to bind PtdIns5P, but to a lesser extent and with less selectivity. As positive controls, different known PI protein interactors were tested, GST-2X-FYVE Hrs [27], GST-PH OSBP [28] and GST-2X PHD ING-2 [13] which bind respectively PtdIns3P, PtdIns4P and PtdIns5P (supplemental figure S1). In our experiments, the dimer of the PHD domain of ING-2 failed to efficiently bind PIs *in vitro*, on the contrary to the two other controls. Altogether, our *in vitro* data revealed a strong and selective interaction between Dok-5 PH domain and PtdIns5P, better than with any other yet identified PtdIns5P binding domain.

3.3. The Dok-5 PH domain expression blocks T cell signalling events

To validate the ability of the Dok-5 PH domain to block functional events induced by PtdIns5P increase in T cells, we next investigated IL-2 promoter activity in IpgD-transfected T cells in the presence or absence of Dok-5 PH domain (Fig. 3A). To this aim, Hut-78 cells were triple-transfected with the IL-2 promoter–dependent luciferase reporter plasmid, with IpgD WT or IpgD CS and with constructs encoding the GFP alone or the Dok-5 PH domain fused with GFP. Cells transfected with GFP alone showed expected feature with enhanced IL-2 promoter activity in the presence of IpgD WT, in the same magnitude than previously observed (Fig. 1C). Importantly, cells co-transfected with IpgD WT and PH Dok-5 GFP, did not show any significant increase of the IL-2 promoter activity. This observation suggests that the PH domain of Dok-5 can sequester PtdIns5P preventing IpgD-induced IL-2 promoter activity. Thus, the Dok-5 PH domain expression blocks cellular effects induced by PtdIns5P increase, acting as a “PtdIns5P-downstream signalling inhibitor”. Endogenous TCR ligation induced by CD3 mAbs stimulates transiently a cellular PtdIns5P increase in Hut-78 T cells [11]. We investigated the impact of Dok-5 PH domain expression on TCR-induced signalling events. Hut-78 T cells were transfected or not with the GFP-tagged Dok-5 PH domain, then stimulated by CD3 mAbs. The phosphorylation status of different signalling proteins was evaluated upon TCR engagement in Western blot (Fig. 3B). As expected, TCR triggering led to the activation of PTKs such as Src family members, and activation of both ERK-1/2 and Akt. The presence of Dok-5 PH domain blocked some TCR-induced tyrosine phosphorylation events detected by anti-phosphotyrosine blots (Fig.3B). TCR-induced Src kinase family members and Akt phosphorylation, but not ERK-1/2 phosphorylation, were largely decreased by the presence of the PH domain of Dok-5. These inhibitory effects on proximal TCR-activated PTKs (Src family kinases) were confirmed by a second set of experiments using anti-phospho Src (Tyr416) antibodies in Western blot (Fig. 3C) or phosphoflow cytometry analysis (Fig. 3D). By both experimental approaches, TCR-induced

Src family kinases activation signal was reduced in presence of Dok-5 PH domain. These results correlate with data presented in Figure 1, suggesting that PdtIns5P impact on Src kinases and Akt activation processes.

4. Discussion

Altogether our findings suggest that PtdIns5P increase regulates signalling events in T cells. Some of them, such as the Akt activation, have been previously reported in epithelial cells [9]. Two kinds of regulatory mechanisms have been suggested where PtdIns5P increase prevents Akt dephosphorylation; (i) through an inhibition of the PP2A phosphatase [29] or (ii) through a PTK activation that will phosphorylate YxxM motif-containing proteins involved in the PI3-kinase class IA activation [9]. Here, we show that at least in T cells, the PtdIns5P increase induces an activation of Src family members, where two of them, Lck and Fyn, are being largely involved in T cell signalling, [23]. The activity of these Src kinases is regulated within lipid rafts structures in T cells [30]. As it has been reported that some PI signalling events occur in the raft nanodomains [31], it is tempting to investigate the role of PtdIns5P located at the plasma membrane, in the context of a lipid raft disruption by using inhibitors of the sphingolipid and cholesterol metabolism. As other intracellular PtdIns5P pools have been described in late endosomes and in the nucleus [32-33], we cannot exclude a participation of these pools in the regulation of late T cell activation events such as the cytokine promoter activities.

Concerning Dok adaptor proteins, Dok-1, Dok-2, Dok-4 and Dok-5 can be expressed in T cells [15]. We reported that these proteins can be classified in two subgroups based on bioinformatics studies (Dok-1 and Dok-2 = group A and Dok-4 and Dok-5 = group B). It has been reported that Dok family members harbor different regulatory functions [34]. Thus, the different Dok members are using different molecular mechanisms to generate negative feedback loops during T cell activation [16,20]. Instead of the three other DOK genes (DOK1, 2 and 4), DOK5 gene is only expressed in pre-activated T cells. This point will prompt us to study the PtdIns5P generation in both naïve or effector/memory T cells.

Finally, another point that will be critical to solve, is to identify enzymes that directly produce PtdIns5P in T cells. Few enzymes have been identified in the metabolism of PtdIns5P [3]. Mammalian PtdIns(4,5)P₂ 4-phosphatases have been described, which are involved in EGF-R receptor degradation [35]. The genes encoding for these two 4-phosphatases are expressed in lymphoid cells as well as the 5-kinase, PIKfyve, and the PtdIns(3,5)P₂ 3-phosphatase MTM1 which are both involved directly or indirectly in PtdIns5P synthesis [36-37]. The analysis of the functions of these enzymes in immune system, will help to understand how the lipid messenger, PtdIns5P, participates in the fine-tuning of T cell signalling.

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Figures legends

Fig. 1. T cell signalling upon PtdIns5P increase in Hut-78 T cells.

A. Cells were transfected or not with myc-tagged IpgD WT or the kinase dead construct, IpgD CS. Cell lysates were examined in Western blot after different periods of time, as indicated. Note the Akt phosphorylation (pAkt) on Ser 473 upon IpgD expression after 4 hours transfection, most probably as a consequence of transient PtdIns5P increase. *B.* Phosphorylation of different signaling molecules in cells lysates of cells transfected for 4 hours with different expression vectors, as indicated on top. As a positive control of phosphorylation pervanadate treatment was performed (pV) *C.* IpgD expression stimulates IL-2 promoter activity. Cells were transfected with a luciferase reporter construct for IL-2 promoter activity and myc-tagged IpgD WT, IpgD CS expressing constructs or an empty vector (mock). Luciferase activity was measured 6 hours after transfection. Results are expressed as the percentage obtained by dividing the luciferase activity observed by the luciferase activity measured when cells are stimulated by phorbol myristate acetate (PMA) plus ionomycin, a treatment known to induce IL-2 promoter activity. Data show the average of three independent experiments \pm s.d.

Fig. 2. Dok-4 and Dok-5 PH domain lipid binding.

A. Dok-5 PH domain preferentially binds to PtdIns4P and PtdIns5P, while Dok-4 PH binds all three PIs in a protein-lipid blot assay. The PH domain of Dok-4 or Dok-5 fused to GST were purified and tested for binding at 0.5 μ g/ml. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. *B.* The PH domains of Dok-4 and Dok-5 bind to PtdIns5P containing liposomes in SPR. Sensorgrams showing the binding of 1 μ M GST-PH Dok-4 (upper part) and GST-PH Dok-5 (lower part) to reconstituted liposomes containing 90% DOPC (1,2- dioleoyl-sn-glycero-3-phosphocholine)

and 10% PIs, as indicated, after subtraction of background signals obtained with liposomes containing PtdIns. Note the selectivity of the PH domain of Dok-5 for PtdIns5P.

Fig. 3. Dok-5 PH domain expression blocks T cell signalling events in Hut-78 T cells.

A. Dok-5 PH domain expression prevents IL-2 promoter activity induction upon IpgD expression. Cells were transfected with a luciferase reporter construct for testing IL-2 promoter activity and Dok-5 PH domain fused to GFP or GFP alone as control during 24 hours. Cells were additionally transfected for 6 hours with expression constructs for myc-tagged IpgD WT or IpgD CS before luciferase activity measurement. Results are expressed as the percentage obtained by dividing the luciferase activity observed by the luciferase activity measured when cells are stimulated by phorbol myristate acetate (PMA) plus ionomycin, a treatment known to induce IL-2 promoter activity. Data show the average of three independent experiments \pm s.d. *B.* Dok-5 PH domain expression during T cell activation prevents specific signaling events (not pErk1/2). Cells were left nonstimulated (NS) or were stimulated with CD3 mAbs for 2 min in the presence or not the Dok-5 PH domain fused to GFP. Cell lysates were probed in Western Blot with antibodies recognizing different phosphoproteins as indicated, or GFP. *C.* Cells expressing the Dok-5 PH domain fused to GFP or GFP alone were left non-stimulated (NS) or were stimulated with CD3 mAbs for the indicated periods of time before analysis of cell lysates by Western blot. *D.* Phosphoflow cytometry showing that the expression of the Dok-5 PH domain blocks Src phosphorylation. Cells expressing the Dok-5 PH domain fused to GFP (blue) or not (green) were left non-treated (NT, red) or were stimulated with CD3 mAbs for 2 min. The mean fluorescence intensity (MFI) values for the CD3-stimulated conditions correspond to MFI = 50 (green curve, without Dok-5 PH domain expression) or MFI = 13 (blue curve, with Dok-5 PH domain expression). This experiment is representative of three independent experiments.