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Evidence for a positive role of PtdIns5P in T-cell signal transduction pathways

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ABSTRACT

Phosphatidylinositol 5-phosphate (PtdIns5P) is emerging as a potential lipid messenger involved in several cell types, from plants to mammals. Expression of IpgD, a PtdIns(4,5)P_2 4-phosphatase induces Src kinase and Akt, but not ERK activation and enhances interleukin II promoter activity in T-cells. Expression of a new PtdIns5P interacting domain blocks IpgD-induced T-cell activation and selective signaling molecules downstream of TCR triggering. Altogether, these data suggest that PtdIns5P may play a sensor function in setting the threshold of T-cell activation and contributing to maintain T-cell homeostasis.

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)P_3 has been

Abbreviations: PIs, phosphoinositides; PtdIns5P, phosphatidylinositol 5-phosphate; IL-2, interleukin II; TCR, T-cell receptor

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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 sequestrate 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 μF and 250 V using the GenePulser Xcell™ (Bio-Rad).

2.2. Plasmid constructs

The construct βDNA4HADok-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 βDNA4 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 βDNA4 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′-ggcgc<u>ctcgag</u>atggcttccaattttaatgacatagtgaagcaaggg-3′ (XhoI site added) and antisense 5′-gcggc<u>gaattc</u>ccttggatccgtgttcctacacactccatctg-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 CD3ε 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 U872, 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 (10×10^6) were stimulated at 37 °C in RPMI 50 mM HEPES. Stimulations were carried out for the indicated times using CD3 mAb 289 at 10 μg/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 μg/ml protease inhibitors (protease inhibitors cocktail, Sigma, St. Louis, MO) and 1 mM Na_3VO_4 for 10 min at 4 °C, then centrifugated at 13 000 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 μM 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 interleukin II (IL-2) promoter, fused to firefly luciferase reporter gene and pβ-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.

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 signaling, 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 homolog deleted in chromosome 10 (PTEN) or the SH2 domain-containing inositol polyphosphate 5′-phosphatase (SHIP) as observed in Jurkat