Biochemical and Biophysical Research Communications 387 (2009) 127–131

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

PtdIns5P protects Akt from dephosphorylation through PP2A inhibition

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ARTICLE INFO

Article history:
Received 25 June 2009
Available online 1 July 2009

Keywords:
PtdIns5P
Phosphoinositide
Akt
PP2A
PTEN
IpgD
Shigella flexneri

ABSTRACT

Phosphatidylinositol 5-phosphate (PtdIns5P), the most recently discovered phosphoinositide, has been proposed to play a role as a lipid mediator of intracellular signaling. We have previously shown that PtdIns5P generated by IpgD, an effector of the causative agent of dysentery *Shigella flexneri*, activates the PI 3-kinase/Akt pathway. Here, we demonstrate that PtdIns5P is able to protect Akt from dephosphorylation. This effect is not due to inhibition of the phosphoinositide phosphatase regulating PtdIns(3,4,5)P_3 levels PTEN but rather to PtdIns5P-induced phosphorylation and subsequent inhibition of the catalytic subunit of PP2A phosphatases. These data shed light on a new mechanism used by *S. flexneri* bacteria to sustain Akt activation to increase survival of the host cells during bacterial replication.

Introduction

Phosphoinositides play critical roles in the control of many cellular processes including cytoskeleton remodelling, cellular proliferation, survival and vesicular trafficking [1]. Most phosphoinositides have the capacity to bind proteins through specific domains like PH, PX, or FYVE domains [2]. Until recently only PtdIns(4,5)P_2 and the PI 3-kinase product PtdIns(3,4,5)P_3 were known for their role as second messengers. However, other less studied phosphoinositides, long regarded as intermediates in the metabolic routes, have emerged as important regulators of cell functions [3,4]. Among them is PtdIns5P, one of the latest phosphoinositide discovered in mammalian cells, whose cellular roles are still not entirely characterized [5]. Several pieces of evidence point out a role for PtdIns5P as a second messenger in cellular signaling. PtdIns5P level increases upon diverse external stimuli such as agonists, stress or during cell cycle progression [6–8]. PtdIns5P was detected in the nucleus where it binds the PHD (Plant Homeodomain) domain of the ING2 protein and regulates the p53-dependent apoptosis [9]. This PtdIns5P pool increases under UV and genotoxic stress and is modulated by the PIP4KIIβ and the type I 4-phosphatase, that respectively, convert PtdIns5P into PtdIns(4,5)P_2 and *vice versa* [10,11]. Besides this nuclear function, evidences indicate that PtdIns5P exists in other cell compartments. PtdIns5P can be produced by the myotubularin family of 3-phosphatases by hydrolysis of PtdIns(3,5)P_2, a phosphoinositide present in the late endosomes/lysosomes [12]. Moreover, a PtdIns5P pool was shown to be dependent on the kinase PIKfyve able to produce PtdIns(3,5)P_2 from PtdIns3P and possibly PtdIns5P from PtdIns. This kinase is known for its regulatory role in vesicular trafficking [13]. These data, together with a study on the evolution of the enzymes involved in PtdIns5P metabolism [14] strongly suggest a role for PtdIns5P in membrane trafficking. However, so far, the strongest production of PtdIns5P at the plasma membrane was measured in cells infected with the pathogen *Shigella flexneri*, by the action of the bacterial PtdIns(4,5)P_2 4-phosphatase IpgD [15]. We showed that this PtdIns5P pool activates the PI 3-kinase/Akt survival pathway of the host cell for the benefits of the bacteria [16]. Moreover, other evidences suggest that PtdIns5P may act on the regulation of Akt phosphorylation. Indeed, an inhibitory role of PtdIns5P on PtdIns(3,4,5)P_3 specific phosphatases at the plasma membrane has been proposed in cells deficient for the PIP4KIIβ. This inhibition could impact the accumulation of PtdIns(3,4,5)P_3, leading to activation of Akt and hypersensitivity to insulin [17]. Altogether, these data indicate a role for PtdIns5P in the regulation of Akt activity but the mechanism is still unclear. Here, we show that once activated *via* PtdIns5P production, Akt is protected from dephosphorylation by inhibition of the PP2A phosphatases.

Abbreviations: PtdIns, phosphatidylinositol; PH, Pleckstrin Homology; PX, Phox Homology; FYVE, Fab1p, YOTB, Vps27 and EEA1; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SHIP, SH2 containing inositol lipid phosphatase

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doi:10.1016/j.bbrc.2009.06.139

Material and methods

Cell culture and stimulation

HeLa cells from ATCC were grown in DMEM (Invitrogen) supplemented with 10% FBS. For all experiments, cells were serum starved for 12 h after transfections. For EGF experiments, cells were stimulated with 200 ng/ml EGF (Invitrogen) and 25 μM LY 2900042 (Sigma–Aldrich) was added after 5 min. Reaction was stopped at different time of the 120 min EGF time course.

Transfections

DNA transfections were performed using Effectene (Qiagen) according to the manufacturer's instructions. The following plasmids were used: pRK5-Myc-IpgD WT and pRK5-Myc-IpgD-C438S mutant [15]; pCDNA3-GFP-Lyn-Inp54p vector (a kind gift from T. Meyer). The PIP4KIIβ was cloned in the peGFP-C1 vector (Clontech) by PCR using the pcDNA-PIP4KIIβ vector (kind gift of K. Lamia) as a template.

PP2A assay

Cells were lysed with 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and PP2A activity was measured using a PP2A immunoprecipitation phosphate assay kit (Millipore). Briefly, protein lysates were incubated 2 h at 4 °C with 2 μg of PP2Ac antibody and protein A agarose. After washes, immunoprecipitates were assessed for phosphatase activity according to manufacturer's instructions.

Protein extraction and analysis

For Western blotting, cells were scraped in Laemmli sample buffer and proteins were analysed by SDS–PAGE followed by transfert on Immobilon-P membranes (Millipore). Immunoreactive bands were detected by chemiluminescence with the SuperSignal detection system (Thermo Scientific Pierce) and quantified by genetools software. The antibodies used were anti-phospho-Akt1/2/3 (Ser473 and Thr308), anti-phospho PTEN (Ser380/Thr382/Thr383), anti-PTEN (Cell Signaling), anti-Akt1/2, anti-GFP, (Santa Cruz Biotechnology), anti-PP2Ac (Millipore), anti-phospho-PP2A (Tyr 307) (Epitomics).

PtdIns5P mass assay

PtdIns5P was quantified by mass assay as described [6] and quantified by HPLC analysis. Briefly, total lipids were extracted by the Blight and Dyer method in presence of 0.4 N HCl and separated on Silica gel G60 plate in the $CH_3CL/CH_3COOH/NH_3$ (9/7/2) solvant. Monophosphorylated PIs were scraped, eluted from silica and assessed for PtdIns(4,5)P_2 formation *in vitro* using the recombinant-specific PIP4KIIα and [γ-^{32}P]ATP (Perkin–Elmer). The radiolabelled PtdIns(4,5)P_2 was scraped, deacylated and analysed by HPLC on a Partisphere 5 SAX column (Whatman) coupled to a continuous flow in-line scintillation detector (Beckman Instruments).

Results and discussion

We have previously shown that PtdIns5P is produced at the plasma membrane from PtdIns(4,5)P_2 4-dephosphorylation by IpgD in mammalian cells infected by *S. flexneri* and linked this PtdIns5P production to host cell survival through activation of the PI 3-kinase/Akt pathway [15,16]. One remarkable feature of Akt activation in cells transfected with IpgD is the sustained level of activation of Akt along time. To investigate this observation in more details, we have analysed the impact of IpgD on a classical activation of Akt through agonist stimulation of a tyrosine kinase receptor, the EGF receptor. In agreement with the literature, in HeLa cells, EGF stimulation leads to a rapid and transient Akt activation peaking at 5–10 min of agonist stimulation (data not shown). However, in cells transfected with IpgD, Akt is already activated in the absence of growth factor and we could not observe any additive effect of EGF even after 120 min of stimulation (data not shown). Cells transfected or not with IpgD were stimulated with EGF and the PI 3-kinase inhibitor LY294002 was added after 5 min of stimulation. Phosphorylation of Akt on serine 473 was monitored for the next 120 min (Fig. 1A). In control cells, we measured a very rapid inhibition of Akt phosphorylation upon LY294002, consistent with the well known role of PI 3-kinase to transmit signal from the EGF receptor to Akt. In cells transfected with IpgD, we also observed an inhibition of Akt phosphorylation but with a delayed onset compared to control cells, reaching the non-phosphorylated state of control cells around 120 min of treatment (Fig. 1A and B). This time course of Akt dephosphorylation in the presence of IpgD and EGF was identical to the one obtained in the absence of EGF stimulation (data not shown). It is interesting to note that the level of phosphorylation of Akt residue 473 in IpgD transfected cells is identical to the one observed after 5 min EGF stimulation. In cells, IpgD hydrolyses PtdIns(4,5)P_2 to produce PtdIns5P and it is therefore important to discriminate between the effects of a diminution of PtdIns(4,5)P_2 or an increase of PtdIns5P. For that purpose, we used Inp54p, a yeast phosphatase that hydrolyses comparable amounts of PtdIns(4,5)P_2 into PtdIns4P [16]. In that case, we did not observe the sustained activation of Akt under EGF stimulation in the presence of LY294002, demonstrating that PtdIns5P is responsible for the prolonged phosphorylation of Akt (Fig. 1A and B). From these results, we propose that IpgD, through PtdIns5P production, sustains the activation of Akt by EGF independently of PI 3-kinase, suggesting the existence of an alternative mechanism of control of Akt phosphorylation. We know that Akt activation by IpgD is PI 3-kinase dependent [16]. PI 3-kinase products PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 bind to the PH domain of Akt and drive its relocalization to the plasma membrane where it is phosphorylated on residue threonine 308 of the activation loop by the kinase PDK1. Full activation of Akt results from the phosphorylation of hydrophobic motif on serine 473 by the TORC2 complex. Once phosphorylated, Akt is released from the membrane and relocates to the cytosol or the nucleus to meet its different targets.

We next assessed how IpgD and PtdIns5P could maintain Akt activation. A first indirect way could be through the inhibition of the lipid phosphatases SHIP or PTEN that both dephosphorylate PtdIns(3,4,5)P_3. It has been proposed, based on genetic models, a role of PtdIns5P on PtdIns(3,4,5)P_3 phosphatases. Indeed, mice lacking the PIP4KIIβ, present an hypersensitivity to insulin and an overactivation of Akt, that could be explained by increased stability of the insulin-dependent PI 3-kinase signal [17]. Mice heterozygous for SHIP2 present a quite similar phenotype [18], suggesting an inhibitory role of PtdIns5P on a PtdIns(3,4,5)P_3 5-phosphatase. Consistent with these observations, overexpression of PIP4KIIβ negatively regulates insulin signaling by decreasing the cellular levels of PtdIns(3,4,5)P_3 and thus abbreviates the PI 3-kinase signal. Accordingly, we have previously observed that PtdIns5P is able to weakly inhibit SHIP1 activity *in vitro* [16]. However, in cells transfected with IpgD, we observe a concomitant increase of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which is not in favor of an inhibition of SHIP that would be associated with an increase of only PtdIns(3,4,5)P_3 [16]. We therefore looked at PTEN status in IpgD transfected cells. Six phosphorylation sites are present at the C-terminus of PTEN and impact on PTEN activity and

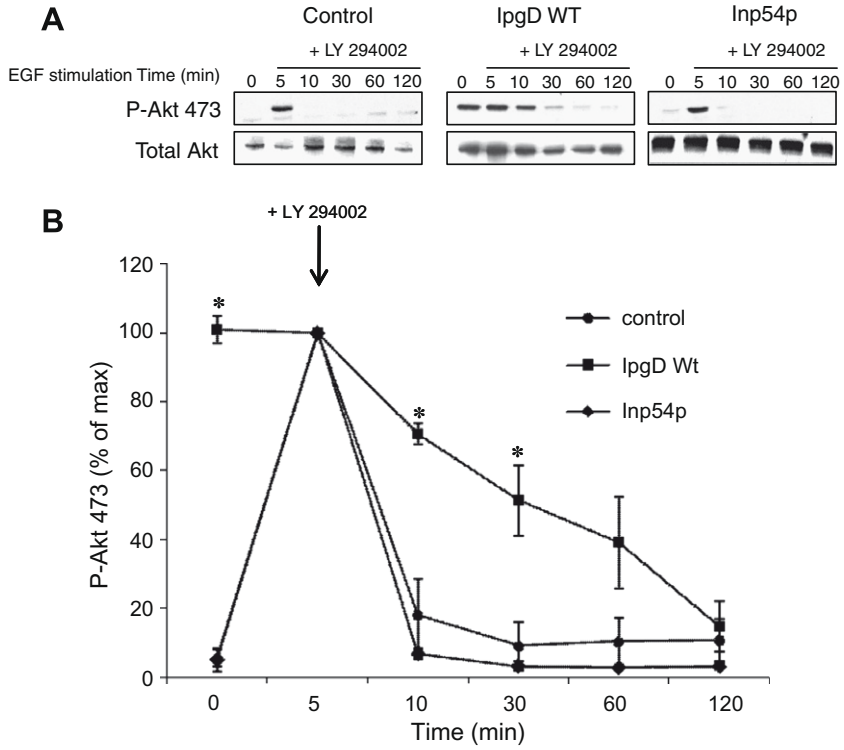

Fig. 1. Substained Akt phosphorylation in IpgD expressing cells. (A) Lysates from control, IpgD or Inp54p transfected cells treated with EGF (200 ng/ml) and with LY294002 (25 μM), added 5 min after stimulation were resolved by Western blot and analysed for phosphorylation on S473 of Akt. Loading control was determined with anti-Akt antibody. (B) Western blots presented in (A) were quantified using genetools software. Data are representative of three independent experiments. Values are presented as means ± SEM. $p < 0.05$ (Student's *t*-test).

stability. Specifically, phosphorylation of the cluster of serine 380, threonines 381 and 382 keeps PTEN in an inactive state, and activation of PTEN requires the dephosphorylation of the cluster. In control cells, we observe the phosphorylation of PTEN on those residues, reflecting low levels of activity (Fig. 2A). However, we did not see any modification of the phosphorylation of PTEN on those residues in IpgD or Inp54p transfected cells, suggesting that IpgD and PtdIns5P do not change PTEN activity *in vivo* by this mechanism and that an inhibition of the lipid phosphatase does not explain the sustained activation of Akt. Interestingly, data from an *in vitro* study proposed that PtdIns5P and PtdIns(4,5)P_2 do not inhibit, but on the contrary, activate PTEN by binding to PTEN and inducing an allosteric conformational change [19]. A role for PtdIns5P also as activator of myotubularin, a PTEN-related lipid 3-phosphatase acting on different substrates, (i.e PtdIns(3,5)P_2 and PtdIns3P), has also been described [20]. Several studies have reported the role of PtdIns(4,5)P_2 on PTEN phosphatase activity but the mechanisms involved have been a matter of debate. More recently a study has confirmed the preferential binding of PtdIns(4,5)P_2, but not PtdIns5P, to the N-terminal domain of PTEN and demonstrated that PtdIns(4,5)P_2 induces a conformational change in PTEN that results in an enhancement of its activity [21]. The decrease of PtdIns(4,5)P_2 level by IpgD could have explained a diminution of PTEN activity, however the use of Inp54p invalidates this hypothesis. Altogether, our results and these different observations suggest that PTEN is not responsible for the sustained activation of Akt observed in IpgD transfected cells. We next analysed the effect of overexpression of PTEN in IpgD expressing cells. (Fig. 2B). Cotransfection of wild type PTEN with IpgD induces the complete loss of Akt phosphorylation on threonine 308. However, when the PTEN^{C124S} mutant, that is unable to dephosphorylates PtdIns(3,4,5)P_3 was used, the level of phosphorylation of Akt was identical to the one measured for IpgD alone. These results confirm that the activation of Akt by IpgD is PI 3-kinase dependent and that PTEN is not inhibited by IpgD.

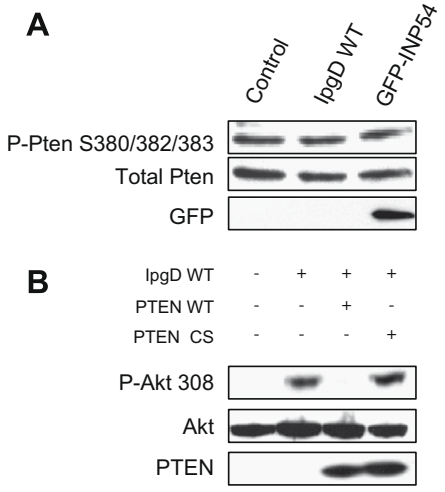

Fig. 2. PTEN is not inhibited by PtdIns5P production. (A) Lysates from control, IpgD or Inp54p transfected cells were resolved by Western blot and analysed for phosphorylation on S380/T382/T383 of PTEN. Loading control was performed with anti-PTEN antibody. GFP-Inp54p expression was followed by anti-GFP antibody. (B) Lysates from control, IpgD or IpgD cells co-transfected with a PTEN WT or a lipid phosphatase dead PTEN (PTEN CS) were resolved by Western blot and analyse for phosphorylation on S473 and T308 of Akt. Loading control was determined with anti-Akt antibody and PTEN expression by a PTEN antibody. Data are representative of three independent experiments.

Another direct way of regulating Akt activity is to modify the phosphorylation of its activating residues, serine 473 and threonine 308. Many studies have shown that the serine/threonine

protein phosphatase 2A (PP2A) mediates this dephosphorylation at both sites [22]. More recently, another phosphatase, the PH domain leucine-rich repeat phosphatase (PHLPP), a phosphatase related to the PP2C family, was proposed to contribute to the dephosphorylation of only serine 473 [23]. Although we cannot, at present, formally exclude PHLPP, we believe that PP2A has a major role in regulating Akt phosphorylation in our system. Indeed, our results point to the maintenance of Akt phosphorylation on both serine 473 and threonine 308. The predominant form of PP2A is a heterotrimeric holoenzyme, composed of a scaffolding subunit (A), a catalytic subunit (C), and a variable regulatory subunit (B). The B subunits are divided in three families, B (PR55), B′ (B56/PR61), and B″ (PR72) each with several isoforms, producing a large family of oligomeric PP2A with a common catalytic subunit. PP2A activity was measured after immunoprecipitation of the PP2A catalytic subunit in cells transfected with IpgD. Our results show a 75% inhibition of the serine/threonine phosphatase compared to control cells indicating that IpgD is able to downregulate PP2A activity (Fig. 3A). Immunoprecipitation of comparable amount of PP2Ac was verified by Western blotting. It is important to note that the catalytic subunit is common to the different PP2A enzymes and we might therefore underestimate the inhibition. The B56 and PR55 families have been implicated in controlling the phosphorylation of both Ser473 and Thr308 of Akt [24,25]. This is in agreement with our results, as we observed a sustained phosphorylation at both sites of activated Akt and it will be interesting to determine which regulatory subunit is involved in Akt regulation in our system. The role of PtdIns5P as the molecule that mediates the inhibition was confirmed by the use of the PIP4KIIβ. This lipid kinase uses PtdIns5P as a substrate to produce PtdIns(4,5)P

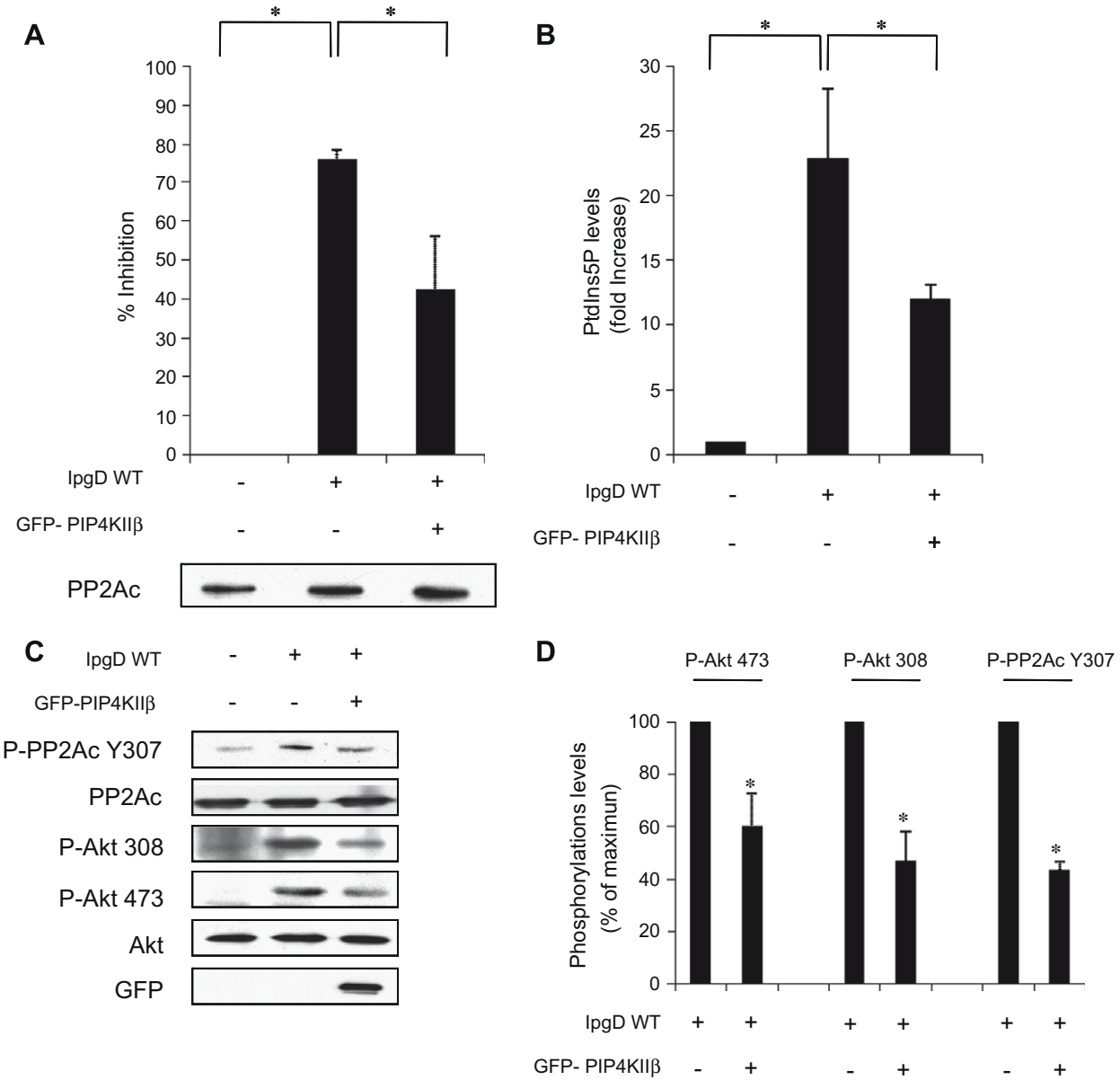

Fig. 3. PP2A phosphatases are inhibited by PtdIns5P. (A) PP2Ac was immunoprecipitated from lysates of control, IpgD or IpgD co-transfected with GFP-PIP4KIIβ cells. PP2A activity was quantified as described in Materials and methods. The amount of PP2Ac immunoprecipitated was determined by Western blot analysis using anti-PP2Ac antibody. (B) Quantification of PtdIns5P levels in control, IpgD or IpgD co-expressing the GFP-PIP4KIIβ cells was performed by mass assay. (C) Lysates from control, IpgD or IpgD co-transfected with GFP-PIP4K IIβ cells were resolved by Western blot and analyse for phosphorylation of Y307 of PP2Ac, and S473 and T308 of Akt. Loading control was performed by using PP2Ac and Akt antibodies. GFP-PIP4KIIβ expression was followed by anti-GFP antibody. Western blot quantification was performed as in Fig. 1 and are represented in (D). Data are representative of three independent experiments. Values are presented as means ± SEM. $p < 0.05$ (Student's t-test).

and has been used to modulate PtdIns5P levels in cells. When co-transfected with IpgD, PIP4KIIβ induces a 50% decrease in the inhibitory effect of IpgD on PP2A activity (Fig. 3A). This partial effect of PIP4KIIβ is in agreement with the fact that the PIP4KIIβ does not fully abolish the level of PtdIns5P produced by IpgD (Fig. 3B). The status of the phosphorylation of tyrosine 307 of the PP2A catalytic subunit was also determined in cells transfected with IpgD. This residue, localized in the well conserved C-terminus domain of the PP2A catalytic subunit, was shown to be phosphorylated by non-receptor and receptor tyrosine kinases [26]. Phosphorylation of Y307 leads to the inactivation of the PP2A enzyme, probably by inducing the dissociation of the catalytic subunit from the regulatory B subunit [27]. In IpgD transfected cells, we observe an increase in the phosphorylation of Y307 compared to control cells, that decreased in cells co-transfected with IpgD and the PIP4KIIβ (Fig. 3C). These results demonstrate that the PtdIns5P produced by IpgD, increases the phosphorylation level of tyrosine 307 of PP2Ac, thereby leading to the inactivation of the PP2A enzyme, which correlates with the level of Akt phosphorylation on residues threonine 308 and serine 473 (Fig. 3C). Quantification of the phosphorylation of PP2A and Akt are presented in Fig. 3D. Finally, phosphorylation of Akt was followed in cell transfected with IpgD and treated with okadaic acid, a compound known to inhibit PP2A but which has no effect on PP2C family members such as PHLPP. We observed a slight increase of Akt phosphorylation on residue serine 473 with increasing concentration of okadaic acid (data not shown), suggesting not only that PHLPP was not involved in the regulation of Akt by IpgD and PtdIns5P, but also that PP2A is already fully inhibited by IpgD (Fig. 3A). As mentioned above, our results show that the inhibition of PP2A activity by PtdIns5P needs phosphorylation of the catalytic subunit. It was reported that this phosphorylation is mediated by cytosolic or receptor tyrosine kinases in order to allow efficient signaling after stimulation [26]. Interestingly, we have shown that the PI 3-kinase/Akt activation by PtdIns5P involves tyrosine kinase activity [16]. We are currently characterizing this mechanism and we hypothesize that the same tyrosine kinase activated by PtdIns5P could activate the PI 3-kinase/Akt module and also inhibit PP2A to favor the survival of the host cells during infection by *S. flexneri*.

In conclusion, this study provides the first observation on how PtdIns5P can induce a sustained Akt signaling by inhibiting PP2A phosphatases. It confirms an important regulatory role of PtdIns5P on Akt activity and signaling during infection by *S. flexneri*.

Acknowledgments

This work was supported by grants from INSERM, ARC (#4937), ANR Blanc and ANR e-rare. DR is supported by grant from Ministère de la Recherche and ARC. We thank P. Sansonetti and G. Tran Van Nhieu for helpful discussions.

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