

PtdIns(5)P activates the host cell PI3-kinase/Akt pathway during *Shigella flexneri* infection

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The virulence factor IpgD, delivered into nonphagocytic cells by the type III secretion system of the pathogen *Shigella flexneri*, is a phosphoinositide 4-phosphatase generating phosphatidylinositol 5 monophosphate (PtdIns(5)P). We show that PtdIns(5)P is rapidly produced and concentrated at the entry foci of the bacteria, where it colocalises with phosphorylated Akt during the first steps of infection. Moreover, *S. flexneri*-induced phosphorylation of host cell Akt and its targets specifically requires IpgD. Ectopic expression of IpgD in various cell types, but not of its inactive mutant, or addition of short-chain penetrating PtdIns(5)P is sufficient to induce Akt phosphorylation. Conversely, sequestration of PtdIns(5)P or reduction of its level strongly decreases Akt phosphorylation in infected cells or in IpgD-expressing cells. Accordingly, IpgD and PtdIns(5)P production specifically activates a class IA PI 3-kinase via a mechanism involving tyrosine phosphorylations. Thus, *S. flexneri* parasitism is shedding light onto a new mechanism of PI 3-kinase/Akt activation via PtdIns(5)P production that plays an important role in host cell responses such as survival.

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Introduction

Shigella flexneri, a facultative intracellular pathogen, is the causative agent of bacillary dysentery in humans. Like *Salmonella*, *Shigella* uses a type III secretion system to inject effector proteins into the host cell and induce their uptake via

a process resembling macropinocytosis (Galan and Bliska, 1996; Cossart and Sansonetti, 2004). *S. flexneri* induces a localised but massive rearrangement of the host cell surface resulting from cytoskeleton reorganisation. The formation of these entry structures comes from the concerted action of injected bacterial proteins and components of the host cell (Cossart, 1997; Sansonetti and Egile, 1998). There is now significant evidence that several microbial pathogens exploit the phosphoinositide metabolism of target cells to promote their entry and develop their virulence (Pizarro-Cerda and Cossart, 2004). Phosphoinositides are minor components of membranes but play a key role in many biological processes, including cell growth or apoptosis, motility, vesicular trafficking and glucose metabolism (Payrastra *et al*, 2001; Cantley, 2002). The *myo*-inositol head group of phosphoinositides contains five free hydroxyl groups and three of them (positions D-3, D-4 or D-5) are phosphorylated by specific kinases, which, together with phosphatases and phospholipases, accurately and locally control the level of these versatile lipids. Several phosphoinositide-binding domains (PH, FYVE, PX, ENTH, etc) have been identified, pointing to the importance of these lipids as scaffolds recruiting specific effectors and orchestrating the spatiotemporal organisation of key signalling pathways. One of the best characterised signalling pathways initiated via synthesis of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P₂) by phosphoinositide 3-kinase (PI 3-kinase) is the activation of the serine-threonine kinase Akt, a key regulator of cell survival (Song *et al*, 2005). Evidence is now accumulating that disruption of the phosphoinositide metabolism is linked to the development of different human diseases (Pendaries *et al*, 2003).

Recently, we found that the *S. flexneri* effector IpgD is injected into the host cell, where it acts as a phosphoinositide phosphatase specifically transforming PtdIns(4,5)P₂ into PtdIns(5)P (Niebuhr *et al*, 2002). IpgD is involved in the formation of fully structured entry sites (Niebuhr *et al*, 2000) but, at first glance, it is not directly involved in invasion, as the *IpgD*-deficient mutant shows the same invasive phenotype as the wild-type (WT) strain (Allaoui *et al*, 1993). The role of this *S. flexneri* effector is therefore under question. Expression of IpgD in mammalian cells leads to a reduction in the membrane/cytoskeleton adhesion energy and eventually causes membrane blebbing (Niebuhr *et al*, 2002). The role of PtdIns(4,5)P₂ in actin cytoskeleton remodelling and in plasma membrane–cytoskeleton interaction is well described (Janmey and Lindberg, 2004) and a drop in its level can explain some of the effects of IpgD. A similar decrease in plasma membrane PtdIns(4,5)P₂ was described during *Salmonella* infection and involves the IpgD homologue SigD (Terebiznik *et al*, 2002). The product generated by SigD, the substrate specificity of which is still a matter of debate (Norris *et al*, 1998; Zhou *et al*, 2001; Terebiznik *et al*, 2002), is not identified, but it is proposed that the drop in

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PtdIns(4,5)P₂ is implicated in membrane fission during the entry process (Terebiznik *et al*, 2002).

In the case of *S. flexneri*, the generation of PtdIns(5)P, a lipid present in low amount in resting cells (Rameh *et al*, 1997; Morris *et al*, 2000), could also contribute to the host cell response during infection. Little is known about the role of this least-characterised phosphoinositide, but it can be produced upon cell stimulation or stress situations with levels comparable to other phosphoinositide second messengers, like PtdIns(3,4,5)P₃ (Morris *et al*, 2000; Sbrissa *et al*, 2002; Tronchere *et al*, 2004). Recent evidence suggest that PtdIns(5)P can interact with some plant homeodomains (PHD) and may be a signalling molecule on its own (Gozani *et al*, 2003; Sbrissa *et al*, 2004; Di Lello *et al*, 2005).

Phosphatidylinositol monophosphates have long been considered as intermediate metabolites of the synthesis pathways of phosphatidylinositol bis- and tris-phosphates. However, it is now becoming clear that they can act directly as signalling molecules (Clarke, 2003; Pendaries *et al*, 2005). PtdIns(3)P regulates vesicular trafficking through interaction with a set of FYVE and PX domain-containing proteins involved in vacuolar sorting in yeast and in the endosomal pathway in mammals (Wurmser *et al*, 1999). Recently, PtdIns(4)P was shown to interact and recruit proteins such as FAPPs or the clathrin adaptor AP-1 to the *trans*-Golgi network (De Matteis and Godi, 2004).

Here, we imaged the production of PtdIns(5)P during *S. flexneri* infection or upon ectopic expression of IpgD and, through a combination of biochemical and genetic approaches, investigated its role as a signalling molecule. This bacterial intracellular parasitism model revealed a novel mechanism of PI 3-kinase/Akt activation through PtdIns(5)P production via a process involving tyrosine phosphorylations. This pathway is likely to play a critical role in direct and transcriptional control of host cell survival that would benefit the bacterium.

Results

Host-cell Akt, GSK3, FKHR and p70S6K phosphorylations specifically require IpgD

Previous studies (Steele-Mortimer *et al*, 2000; Marcus *et al*, 2001; Knodler *et al*, 2005) have shown that Akt is activated in the host cell during infection with *Salmonella*, leading to increased survival of infected epithelial cells. Interestingly, the phosphatase activity of the IpgD homologue, SigD, is required for this effect and IpgD can replace SigD for *Salmonella*-mediated Akt activation (Steele-Mortimer *et al*, 2000). Here we show that infection of HeLa cells with *S. flexneri* can also induce the phosphorylation of Akt at serine 473 and threonine 308 (Figure 1). This phosphorylation was maximal after 15 min and decreased thereafter, but was still visible 1 h after infection, while the total amount of Akt remained constant. Inhibition of PI 3-kinase by LY294002 prevented Akt phosphorylation (not shown). Accordingly, Akt substrates such as GSK3 (α and β) and FKHR were phosphorylated after 15 min of infection. Phosphorylation of the forkhead family member FKHR increased for about 1 h, whereas GSK3 phosphorylation was transient. P70S6 kinase (p70S6K), involved in cap-dependent protein translation and ribosome biogenesis, is a target of Target Of Rapamycin (TOR) downstream of Akt signalling

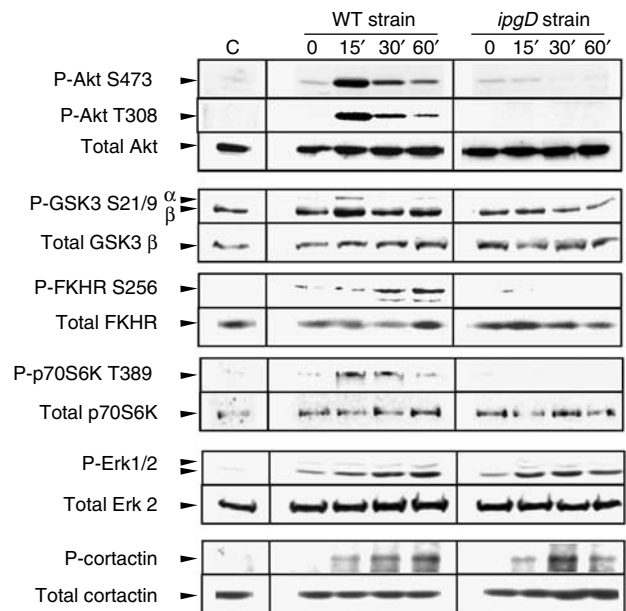


Figure 1 IpgD is required for the specific activation of the Akt pathway during infection of HeLa cells by *S. flexneri*. HeLa cells were noninfected (C) or submitted to a time course of infection (0, 15, 30, 60 min of infection) with the WT M90T or the *IpgD*-deficient (*IpgD*) strains. The phosphorylation of Akt (S473 and T308), GSK3 (S21 and S9 for α and β , respectively), FKHR (S256), p70S6K (T389), MAPK and cortactin was analysed with specific antibodies. The amount of each protein is also shown. Data are representative of three independent experiments.

(Bjornsti and Houghton, 2004). P70S6K was phosphorylated up to 30 min after infection. Phosphorylations of Akt, GSK3, FKHR and p70S6K were abolished with the *IpgD*-deficient mutant strain, demonstrating the requirement of the phosphatase for this process. Conversely, both WT *S. flexneri* and the *IpgD*-deficient mutant induced Erk1/2 and cortactin (Nhieu *et al*, 2005) phosphorylations with similar ranges and time courses. Thus, IpgD is required for the initiation of specific signalling networks during bacterial infection.

PtdIns(5)P is produced at the entry foci of *S. flexneri*, where it colocalises with phosphorylated Akt

To image PtdIns(5)P during *S. flexneri* infection of HeLa cells, we first used a GFP protein fused to three repeats of the PHD domain of the ING2 protein (GFP-PHDx3) known to interact with PtdIns(5)P (Gozani *et al*, 2003). Despite a strong nuclear signal, the GFP-PHDx3 probe clearly decorated the entry foci of WT bacteria (Figure 2A, upper panel), which are characterised by a typical actin rearrangement (Adam *et al*, 1995). The *IpgD*-deficient mutant entry sites were never labelled (Figure 2A, lower panel). The GFP-PHDx3 mutated on the zinc co-ordinating residues that interacts only very weakly with PtdIns(5)P did not label the entry foci of *S. flexneri* (data not shown). To avoid the nuclear signal generated by the GFP-PHDx3 probe that could mask a discrete membrane labelling, we developed a biotinylated GST protein fused to a tandem of PHD (GST-PHDx2) to probe PtdIns(5)P on fixed cells as described previously for the localisation of PtdIns(3)P using FYVE domains (Gillooly *et al*, 2000; Tronchère *et al*, 2004). This GST-PHDx2 probe did not decorate the nucleus, confirmed the low level of PtdIns(5)P in control HeLa cells

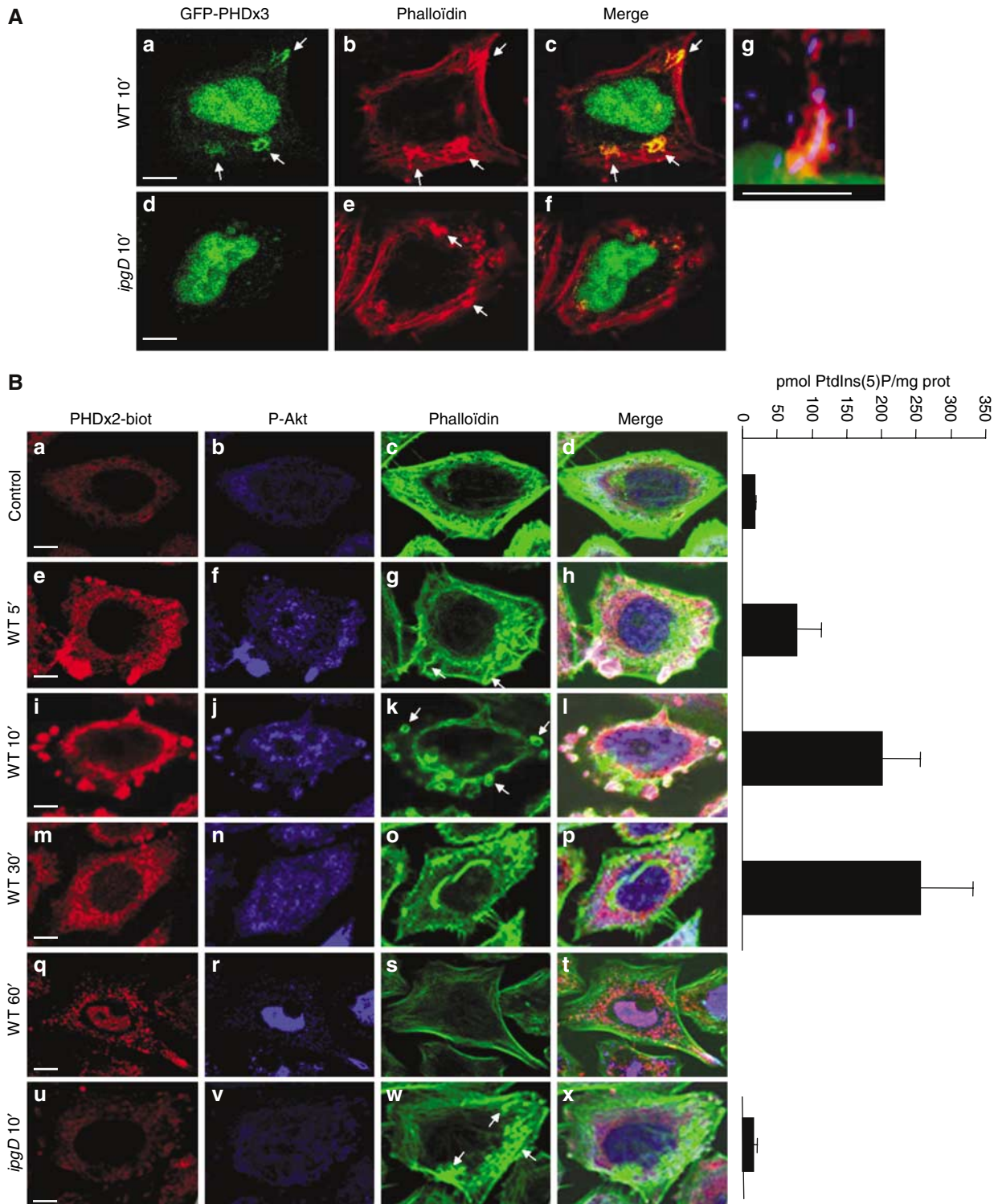


Figure 2 Colocalisation of PtdIns(5)P and phosphorylated Akt at the entry sites during early time points of HeLa cells infection by *S. flexneri*. (A) HeLa cells were transfected with the GFP-PHDx3 encoding vector and expression allowed for 24 h. Cells were fixed 10 min after infection with either the WT M90T (a–c) or the *IpgD*-deficient (*IpgD*) (d–f) strains. PtdIns(5)P was visualised with the GFP-PHDx3 probe (a, d) and actin cytoskeleton was stained with phalloidin-Alexa Fluor594 (b, e). Panel g shows, by fluorescence microscopy, the colocalisation (yellow colour) of the bacteria (visualised with an anti-LPS antibody and a secondary antibody labelled with Alexa Fluor 350 in blue) with the GFP-PHDx3 probe and the actin structure (phalloidin-Alexa Fluor594) at the entry site. (B) HeLa cells were fixed before (control, a–d) or 5, 10, 30 or 60 min after infection with the WT M90T (e–t) or the *IpgD*-deficient (*IpgD*) (u–x) strains. PtdIns(5)P was localised with the biotinylated GST-PHDx2 recombinant probe and stained with streptavidin-Alexa Fluor594 (red) (a, e, i, m, q, u). Akt phosphorylation was detected with the anti-phospho-T308 antibody and a secondary anti-IgG-Cy5 antibody (blue) (b, f, j, n, r, v). Actin cytoskeleton was stained with phalloidin-Alexa Fluor488 (green) (c, g, k, o, s, w). In both panels the preparations were analysed by confocal laser scanning using a Zeiss LSM510 equipped with a 63 × objective. White arrows show entry foci, calibration bar = 15 μm. Data are representative of three independent experiments. The amount of PtdIns(5)P (pmol/mg of proteins) in control cells or in cells infected for 5, 10 and 30 min with the WT M90T strain or, for 10 min, with the *IpgD*-deficient (*IpgD*) mutant is shown (right panel). Results are mean ± s.e.m. of three independent experiments.

(Niebuhr *et al*, 2002), and still strongly labelled *S. flexneri* entry sites visible at 5 and 15 min of infection (Figure 2B). After 30 min, the labelling of PtdIns(5)P was more diffuse, and after 1 h a punctuate pattern resembling that of internal vesicles or membranes was observed. It is also noteworthy that 1 h after the beginning of infection, a significant fraction of this lipid was detected in the nucleus. The time course of PtdIns(5)P production imaged with the GST-PHDx2 biotinylated probe correlated with the increase in PtdIns(5)P level quantified by mass assay (Figure 2, right panel). The entry sites of the *IpgD*-deficient mutant were not decorated (Figure 2B, lower panel).

Interestingly, phosphorylated Akt colocalised with PtdIns(5)P at the entry foci during the first 10 min of infection (Figure 2). Phosphorylated Akt was also detected in the nucleus as soon as 5 min after the beginning of infection and this nuclear signal increased thereafter. Consistent with the data shown in Figure 1, the *IpgD*-deficient mutant did not induce Akt phosphorylation (Figure 2B, lower panel).

The colocalisation of PtdIns(5)P and phosphorylated Akt at the entry sites of bacteria was unambiguously demonstrated by imaging bacteria using an antilipopolysaccharide antibody (Supplementary Figure 1A and B).

Ectopic expression of *IpgD* in HeLa cells and cell-permeant PtdIns(5)P cause Akt phosphorylation

To discriminate between the contribution of *IpgD* and the contribution of other bacterial factors, we transiently expressed myc-tagged *IpgD* in HeLa cells. As a control, another heterologous phosphatase, *Inp54*, known to transform PtdIns(4,5)P₂ into PtdIns(4)P (Raucher *et al*, 2000) was also used. Similar amounts of PtdIns(4,5)P₂ (nearly 25%) were hydrolysed by *Inp54* and *IpgD* after 12 h of transfection (Figure 3A). As expected, *IpgD* produced PtdIns(5)P, whereas *Inp54* produced PtdIns(4)P (Figure 3A).

Expression of *IpgD* in various cell lines, including HEK293, Vero, Cos-7 (not shown) and HeLa cells (Figure 3B), induced the phosphorylation of Akt both at serine 473 and threonine 308, as also observed in CHO-IR cells (Carricaburu *et al*, 2003). The C438S inactive *IpgD* mutant and *Inp54* had no effect. Moreover, the pool of PtdIns(5)P produced upon ectopic expression of *IpgD* in HeLa cells colocalised with phosphorylated Akt (Figure 3C), whereas cells expressing *Inp54* gave no signal (Figure 3C, lower panel).

Expression of the type II PtdIns(5)P 4-kinase β (PI5P4KII β), known to phosphorylate PtdIns(5)P into

PtdIns(4,5)P₂ (Rameh *et al*, 1997), as verified in our model by a decrease in neo-synthesised PtdIns(5)P of about 65%, strongly reduced *IpgD*-induced Akt phosphorylation (Figure 4A). Sequestration of PtdIns(5)P by overexpression of the GFP-PHDx3 protein markedly decreased the phosphorylation of Akt, whereas the GFP-PHDx3 mutant, which binds weakly to PtdIns(5)P, had no effect (Figure 4A), despite comparable levels of overexpressed proteins. Inhibition of

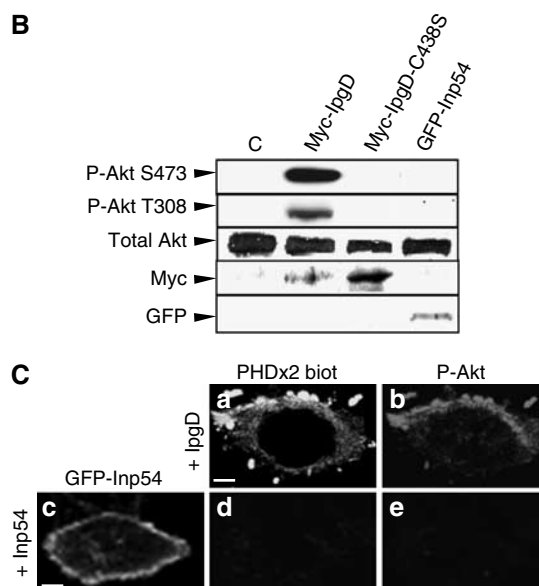
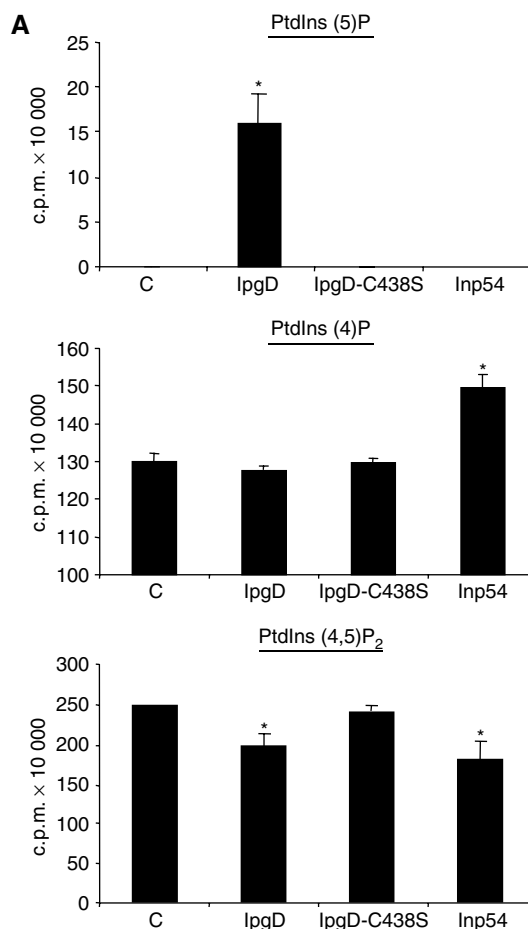


Figure 3 Effects of *IpgD* and *Inp54* phosphatase overexpressions on phosphoinositide metabolism and Akt phosphorylation. (A) HeLa cells transfected with pRK5-Myc-*IpgD*, pRK5-Myc-*IpgD*-C438S or pcDNA3-GFP-lyn-*Inp54* were labelled for 12 h with [³²P]orthophosphate. Lipids were then extracted and analysed by HPLC. The radioactivity incorporated in PtdIns(5)P, PtdIns(4)P and PtdIns(4,5)P₂ was quantified and expressed as c.p.m. Results are the mean \pm s.e.m. of three independent experiments. **P* < 0.05. (B) HeLa cells were cotransfected with pcDNA3-HA-Akt and empty vector (C), pRK5-Myc-*IpgD*, pRK5-Myc-*IpgD*-C438S or pcDNA3-GFP-lyn-*Inp54*, lysed and probed with anti-Akt and anti-phospho-Akt antibodies. (C) The localisation of PtdIns(5)P in *IpgD*- or *Inp54*-expressing HeLa cells was assessed using the biotinylated GST-PHD \times 2 recombinant probe (a, d) and phospho-Akt was followed using the anti-phospho-T308 antibody (b-e). *Inp54* was detected by GFP fluorescence (c). Calibration bar = 15 μ m.

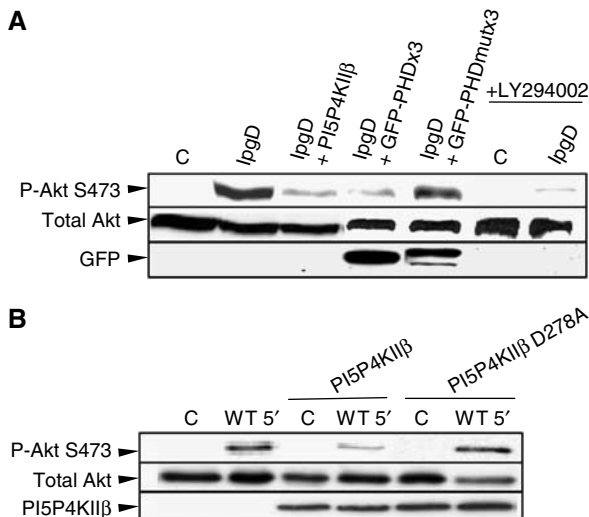


Figure 4 IpgD-mediated production of PtdIns(5)P induces Akt phosphorylation in a PI 3-kinase-dependent manner. (A) HeLa cells were cotransfected with pcDNA3-HA-Akt and pRK5-Myc-IpgD plus empty vector (C), pcDNA3-PI5P4KII β , pEGFP-PHDx3 (GFP-PHDx3) or pEGFP-PHDx3 mutated on the zinc co-ordinating residues (GFP-PHDmutx3) and expression allowed for 12 h. The PI 3-kinase inhibitor LY294002 (50 μ M) was added 4 h before lysis and Akt phosphorylation status was analysed by Western blotting. (B) HeLa cells were transfected with pcDNA3-PI5P4KII β or its inactive form, pcDNA3-PI5P4KII β D278A, and infected with the WT M90T strain. C: non infected cells, WT5': HeLa cells infected 5 min with the WT M90T strain. Data are from one experiment representative of six (A) and three (B).

PI 3-kinase by LY294002 almost totally inhibited IpgD-induced Akt phosphorylation (Figure 4A).

Importantly, expression of PI5P4KII β in HeLa cells also prevented Akt phosphorylation induced by infection with *S. flexneri* (Figure 4B), whereas the inactive kinase had no effect, again pointing to a role for PtdIns(5)P in this process. Incubation of HeLa cells with cell-permeant, short chains, C4-PtdIns(5)P, led to a transient phosphorylation of Akt (Figure 5A), whereas C4-PtdIns(3)P and C4-PtdIns(4)P had only marginal effects (Figure 5B). As shown by the GST-PHDx2 biotinylated probe, after 10 min of incubation, a significant fraction of the exogenous di-C4-PtdIns(5)P incorporated in cell membranes and a colocalisation with phosphorylated Akt was observed (Figure 5C, upper panel). As a control, cells incubated with di-C4-PtdIns(3)P were negative for both signals (Figure 5C, lower panel), despite the fact that a fluorescent di-C8-NBD6-PtdIns(3)P efficiently incorporated in cell membranes under similar conditions (not shown). Together, these results indicate that PtdIns(5)P acts as a signalling molecule to induce Akt phosphorylation.

The phosphoinositide phosphatase activity of IpgD is sufficient to induce class IA PI 3-kinase activation

A time course of phosphoinositide synthesis during infection of HeLa cells with *S. flexneri* shows that PtdIns(5)P appears before PI 3-kinase products (Supplementary Figure 2). In agreement, expression of IpgD in HeLa cells increased PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ levels, whereas the inactive mutant of IpgD or Inp54 had no effect (Figure 6). To investigate the type of PI 3-kinase involved in this process, we used MEF6 cells lacking the p85 β regulatory subunit of PI

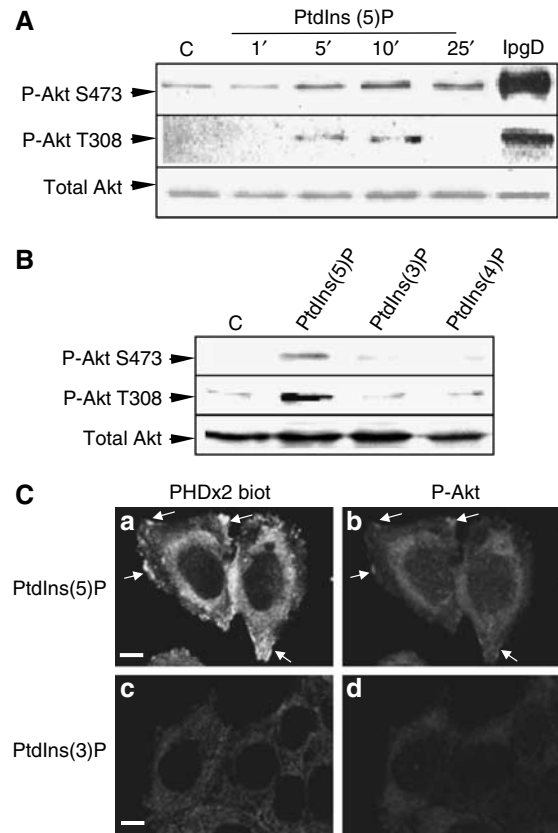


Figure 5 Intracellular delivery of short-chain PtdIns(5)P activates Akt phosphorylation. (A) HeLa cells were transfected with pcDNA3-HA-Akt and expression allowed for 12 h. Cells were lysed before (C) or after the indicated times of incubation with short acyl chains C4-PtdIns(5)P and Akt phosphorylation (S473 and T308) was assessed. (B) HeLa cells treated for 10 min with C4-PtdIns(5)P, C4-PtdIns(3)P or C4-PtdIns(4)P and probed with the anti-phospho-Akt antibodies (S473 or T308) and for total Akt. (C) The intracellular distribution of PtdIns(5)P upon 10 min treatment with C4-PtdIns(5)P (a, b) or C4-PtdIns(3)P (c, d) was followed using the biotinylated GST-PHDx2 recombinant probe (a, c). Akt phosphorylation was detected with the anti-phospho-T308 antibody (b, d). Data are representative of three independent experiments. Calibration bar = 15 μ m.

3-kinase and partly p85 α and MEF9 cells lacking p85 β , p85 α and p55 α and p50 α (Brachmann *et al*, 2005a, b). Akt phosphorylation was markedly reduced in MEF6 and no longer observed in MEF9 cells either upon expression of IpgD (Figure 7A, upper panel) or during infection with *S. flexneri* (Figure 7A, middle panel), indicating the requirement of these regulatory subunits of class IA PI 3-kinase. Moreover, the knockdown of p110 β catalytic subunit of class IA PI 3-kinase using small interfering RNA (siRNA) prevented Akt activation in infected cells (Figure 7A, lower panel).

Conversely, the knockdown of class II PI 3-kinase C2 α and C2 β by specific siRNA (Figure 7B, right panel) only abolished the weak LY294002-resistant Akt phosphorylation, indicating a modest participation of class II PI 3-kinases in IpgD-mediated Akt activation (Figure 7B, left panel).

Furthermore, *S. flexneri* infection (Figure 8A, left panel) and ectopic IpgD expression (Figure 8A, right panel) increased PI 3-kinase activity measured in anti-p85 immunoprecipitates, indicating an activation of a class IA PI 3-kinase. To gain insights into this mechanism of activation, we first

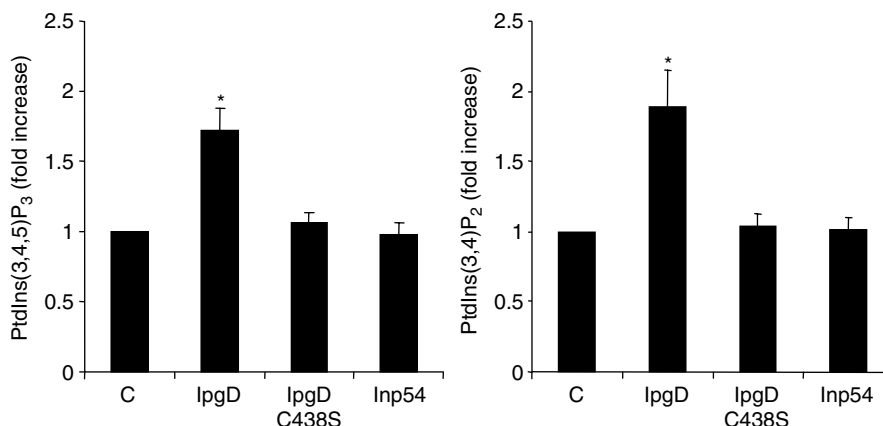


Figure 6 Expression of IpgD causes formation of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. HeLa cells were transfected with the empty vector (C), IpgD, IpgD-C438S or Inp54 encoding vectors and expression allowed for 12 h (the percentage of transfection was nearly 40%). Cells were labelled with [³²P]orthophosphate for 8 h and lipids were analysed by HPLC. Results are the mean ± s.e.m. of four independent experiments and are expressed as fold increase compared to cells transfected with the empty vector (corresponding to 38 000 c.p.m. of [³²P]PtdIns(3,4,5)P₃ and 195 000 c.p.m. of [³²P]PtdIns(3,4)P₂ in average). **P* < 0.05.

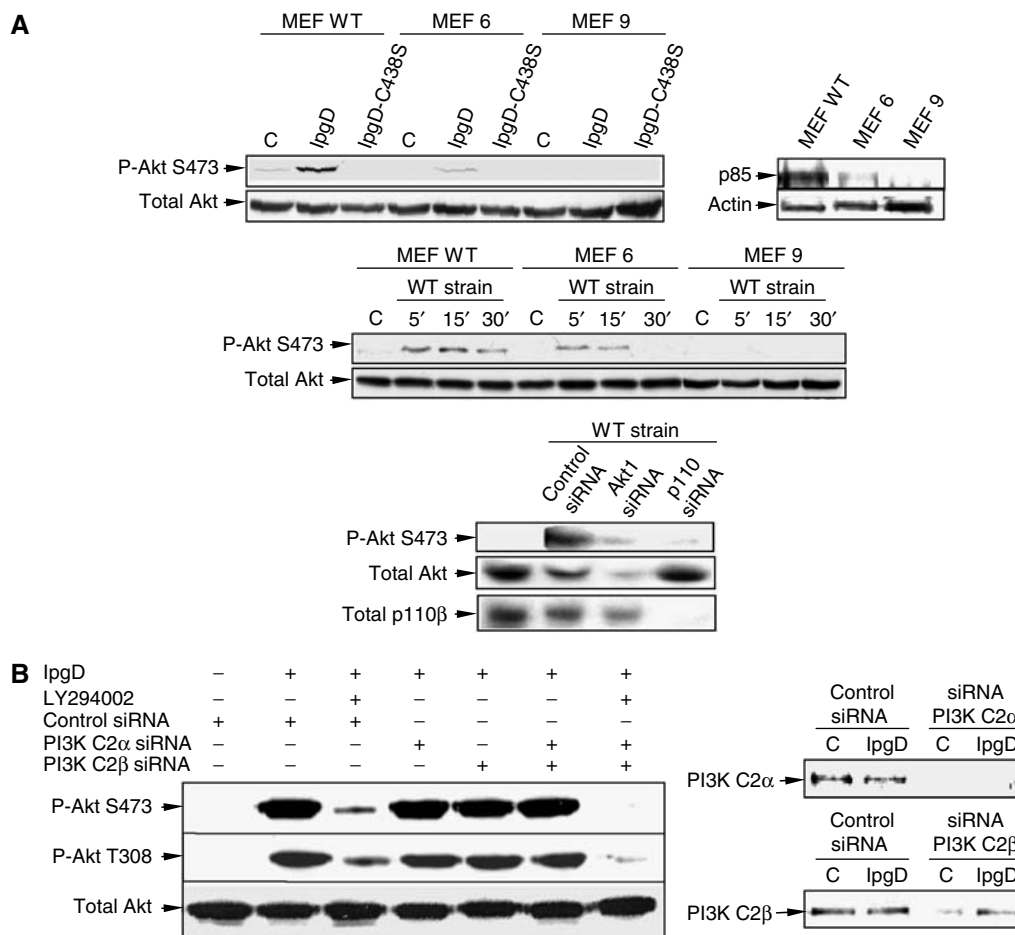


Figure 7 Class IA PI 3-kinase is essential for PtdIns(5)P-induced Akt phosphorylation. (A) Effects of PtdIns(5)P on Akt phosphorylation in MEF WT, MEF p85 α +/–, p55 α +/–, p50 α +/–, p85 β –/–, p55 γ +/+ (MEF6) or MEF p85 α –/–, p55 α –/–, p50 α –/–, p85 β –/–, p55 γ +/+ (MEF9) cells. MEF cells were cotransfected with pcDNA3-HA-Akt alone (C) or in combination with pRK5-Myc-IpgD or pRK5-Myc-IpgDC438S (upper panel, left). P85 subunits were immunodetected in MEF cells (upper panel, right). MEFs cells were non infected (C) or infected for 5, 15 and 30 min with the WT M90T strain (middle panel). HeLa cells were incubated for 48 h with siRNA against Akt1 or PI 3-kinase p110 β subunit or a control siRNA (lower panel). The decrease in p110 β and Akt1 expression reached 93 ± 2 and 90.5 ± 8% (*n* = 3), respectively. Results shown are representative of three independent experiments. (B) Western blotting of phosphorylated Akt in HeLa cells cotransfected with pcDNA3-HA-Akt and pRK5-Myc-IpgD or the empty vector and treated with siRNA against Class II PI 3-kinase C2 α , C2 β , a mix of the two siRNA or a control siRNA (left panel). The decrease in PI 3-kinase C2 α and C2 β expression (right panel) reached 91 ± 4 and 82 ± 9% (*n* = 3), respectively.

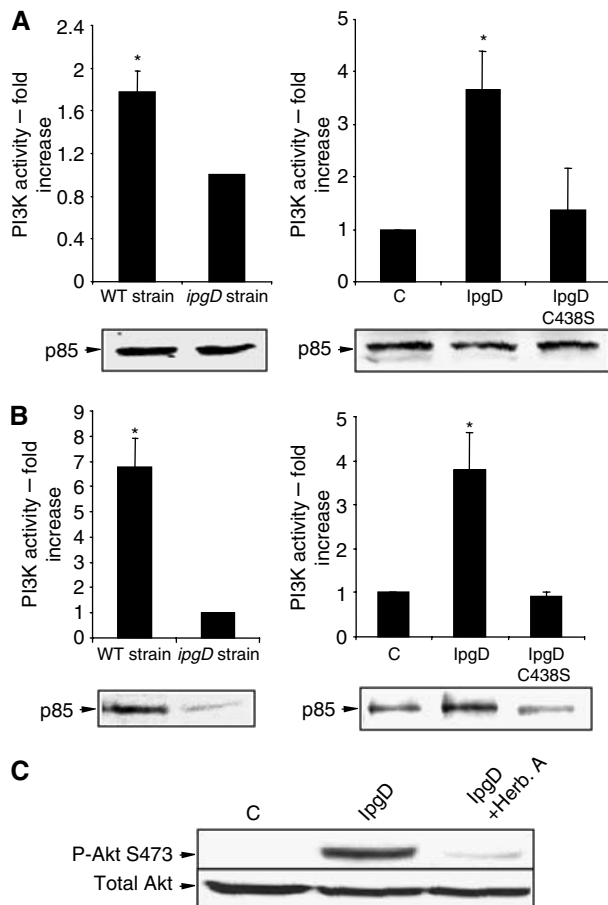


Figure 8 IpgD induces PI 3-kinase activation. HeLa cells were infected for 10 min with the WT M90T or the *IpgD*-deficient (*ipgD*) strains (left panel) or transfected with IpgD or its inactive mutant (right panel). The p85 subunit of PI 3-kinase (A) or tyrosine-phosphorylated proteins (B) were immunoprecipitated and PI 3-kinase was assayed. The amount of immunoprecipitated p85 was probed by Western blotting. For the experiment with transfected IpgD, the effect has been normalised to the percentage of transfected cells, which was routinely around 40%. * $P < 0.05$, $n = 4$. (C) HeLa cells transfected with IpgD were treated by 10 μ M herbimycin A (Herb.A) for 5 h and Akt phosphorylation was assessed using the anti-phospho-S473 antibody. Representative Western blots are shown.

evaluated a potential allosteric activation mechanism. However, we did not find any significant effect of PtdIns(5)P, compared to PtdIns(3)P or PtdIns(4)P, on the capacity of PI 3-kinase to produce PtdIns(3,4,5)P₃ *in vitro* and no interaction of PtdIns(5)P with p85 α could be observed by 'fat-blot' assays (not shown). Interestingly, the p85 subunit of PI 3-kinase was recovered in the antiphosphotyrosine immunoprecipitates performed from cells infected with *S. flexneri*, but not from cells infected with the *IpgD*-deficient mutant (Figure 8B). This was accompanied by a seven-fold increase in PI 3-kinase activity, indicating a recruitment of this enzyme to tyrosine-phosphorylated proteins. An increase in PI 3-kinase activity was also observed in antiphosphotyrosine immunoprecipitates from cells expressing IpgD, but not from cells expressing the inactive mutant (Figure 8B, right panel). Consistent with these results, we found that a 5 h treatment with the tyrosine kinase inhibitor herbimycin A (10 μ M) strongly inhibited Akt phosphorylation (85 \pm 9% of inhibition, $n = 3$) in IpgD-expressing cells (Figure 8C).

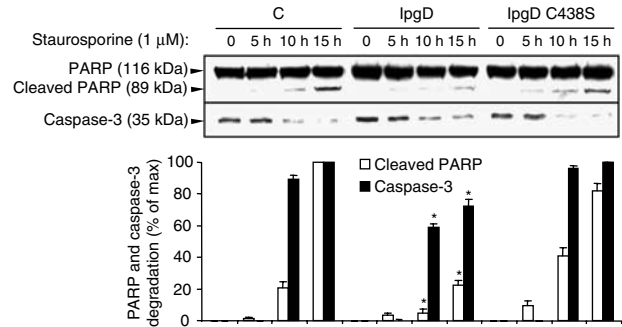


Figure 9 IpgD protects HEK293 cells from staurosporine-induced apoptosis. HEK293 cells were cotransfected with pCDNA3-HA-Akt and empty vector (C) or pRK5-Myc-IpgD and treated or not with 1 μ M staurosporine for 5, 10 or 15 h. Apoptosis was investigated by Western blotting experiments showing poly(ADP-ribose) polymerase (PARP) and caspase-3 degradation. The lower panel shows the quantification of Western blotting and results are expressed as percentage of cleaved PARP and caspase-3, 100% being the maximal degradation observed for each. * $P < 0.05$, $n = 3$.

IpgD plays a role in host cell resistance

IpgD-dependent phosphorylation of host cell FKHR and GSK3 suggests a function in cell survival. We therefore analysed the sensitivity of HEK293 cells overexpressing or not overexpressing IpgD to staurosporine, a classical inducer of apoptosis. Expression of IpgD protected HEK293 cells from staurosporine-induced apoptotic death, as demonstrated by the significant reduction of PARP and caspase-3 cleavages after 15 h of treatment (Figure 9). Consistent with these results, the IpgD-deficient mutant strain showed a decrease in intracellular growth (Supplementary Figure 3A) and induced an increased apoptotic phenotype of the host cells, as shown by phosphatidylserine exposure (Supplementary Figure 3B).

In the same way, *S. flexneri*-induced cytotoxicity of macrophages was decreased by IpgD, as shown by monitoring LDH release (Zychlinsky *et al*, 1992; Hilbi *et al*, 1998). Indeed, *S. flexneri* induced rapid phosphorylation of Akt in macrophages (not shown) and led to 2 \pm 3 and 12 \pm 4% cytotoxicities at 15 and 60 min, respectively, whereas the IpgD-deficient mutant, unable to induce Akt phosphorylation (not shown), led to 13 \pm 2 and 25 \pm 4% cytotoxicities at similar times.

Discussion

Besides effectors directly interacting with the cytoskeleton to modulate structure and size of the entry site (Tran Van Nhieu *et al*, 1999; Cossart and Sansonetti, 2004), *S. flexneri* also injects IpgD, which specifically transforms PtdIns(4,5)P₂ into PtdIns(5)P (Niebuhr *et al*, 2002). Consistent with a local effect of injected IpgD, we show here that PtdIns(5)P is produced at the entry site of the bacteria. This striking localisation suggests that the phosphoinositide is kept concentrated in these sites during the initial time of infection by interacting with proteins that prevent its lateral diffusion. Interestingly, we demonstrated that PtdIns(5)P is critical for the phosphorylation of host cell Akt at serine 473 and threonine 308 and localises with the active form of this kinase during the initial steps of infection. Once activated at the membrane, Akt is known to propagate its signal through the cytosol and ends up inside the nucleus, where it phos-

phorylates substrates including the forkhead transcription factors (Kunkel *et al*, 2005). Accordingly, *S. flexneri*-induced phosphorylation of Akt substrates such as GSK3 and FKHR require IpgD, while activation of other signalling pathways such as the MAP kinases is independent of PtdIns(5)P production. The demonstration that PtdIns(5)P is a key molecule in *S. flexneri*-induced Akt phosphorylation is based on three major observations: (i) ectopic expression of IpgD reproduces the activation of Akt, while Inp54, which transforms similar amounts of PtdIns(4,5)P₂ into PtdIns(4)P, has no effect; (ii) short-chain penetrating PdIns(5)P induces Akt phosphorylation, while the other phosphatidylinositol monophosphates are very poor activators; (iii) expression of PI5P4KIIβ, which transforms most of PtdIns(5)P into PtdIns(4,5)P₂ (Rameh *et al*, 1997), or sequestration of PtdIns(5)P by overexpression of GFP-PHDx3, strongly decreases IpgD-induced Akt phosphorylation.

Although the direct role of PtdIns(5)P in the Akt pathway has never been addressed before, a recent study provides evidence that activation of the pathway of conversion of PtdIns(5)P into PtdIns(4,5)P₂ by overexpression of PI5P4KIIβ decreases insulin-mediated PtdIns(3,4,5)P₃ production, possibly through downregulation of a PtdIns(3,4,5)P₃ 5-phosphatase, with a consequence on the activation of Akt (Carricaburu *et al*, 2003). Consistent with these data, mice lacking PI5P4KIIβ show increased level of Akt activation in response to insulin in skeletal muscles (Lamia *et al*, 2004). The extent of conversion of PtdIns(5)P into PtdIns(4,5)P₂ by PI5P4KIIβ was not evaluated in these studies, and a recent report suggests that this kinase is not very efficient in converting basal PtdIns(5)P into PtdIns(4,5)P₂ (Roberts *et al*, 2005). It is therefore difficult to discriminate between the effect of a decrease in PtdIns(5)P and the effect of neosynthesised PtdIns(4,5)P₂, probably quantitatively minor compared to the constitutive amount of this phosphoinositide. Nevertheless, these studies suggest a regulatory function for the PtdIns(5)P pathway on PtdIns(4,5)P₂ synthesis in the regulation of insulin-mediated Akt activation.

Our data identify PtdIns(5)P as the active molecule responsible for Akt activation induced by *S. flexneri*, and this mechanism depends on the activation of a class IA PI 3-kinase via tyrosine phosphorylations. How PtdIns(5)P may modulate tyrosine phosphorylations remains an open question, but one can suggest that there is a specific recruitment and activation of a tyrosine kinase by this lipid or the inhibition of a tyrosine phosphatase. Src kinase is known to phosphorylate cortactin and to regulate cytoskeletal dynamics during *S. flexneri* entry (Nhieu *et al*, 2005). However, cortactin phosphorylation still occurs upon infection by IpgD-deficient strain (Figure 1) and IpgD-induced Akt phosphorylation is not affected by a Src inhibitor (SU6656) or by expression of a kinase-dead form of Src in a stable cell line (not shown). Although Src is involved in invasion, our results strongly suggest that it is not implicated in the PI 3-kinase/Akt activation. Other kinases sensitive to herbimycin A, involved in this process, are currently being investigated.

Besides PI 3-kinase activation, a concomitant inhibition of phosphoinositide phosphatases allowing further accumulation of PI 3-kinase products cannot be excluded. Recent data suggest a role for PtdIns(5)P as an *in vitro* activator of myotubularin (Schaletzky *et al*, 2003), and to a lesser extent

PTEN (Campbell *et al*, 2003). We found that PtdIns(5)P partly but significantly decreased the PtdIns(3,4,5)P₃ 5-phosphatase activity of SHIP2 *in vitro* (35 ± 5% inhibition in the presence of 20 μM PtdIns(5)P compared to the effect of 20 μM PtdIns(3)P, *n* = 3, *P* < 0.05). This effect was reproducibly observed using different assay conditions and methods. In infected cells, a decrease in SHIP2 activity would lead to an accumulation of PtdIns(3,4,5)P₃ and a reduced production of PtdIns(3,4)P₂. However, we always found elevated intracellular levels of PtdIns(3,4)P₂ either upon ectopic expression of IpgD or during infection. This indicates that, if SHIP2 inhibition occurs *in vivo*, it has only partial effect in the accumulation of PtdIns(3,4,5)P₃.

Two homologues of IpgD, SopB in *Salmonella dublin* and SigD in *Salmonella typhimurium*, are also inositol polyphosphate-phosphatases (Marcus *et al*, 2001). Whether these phosphatases are capable of transforming PtdIns(4,5)P₂ into PtdIns(5)P is unknown, but our recent data suggest that *Salmonella* induces PtdIns(5)P production during invasion in a SigD-dependent manner (Masson *et al*, 2006). SigD is also required for Akt activation in Hela cells infected with *Salmonella* (Steele-Mortimer *et al*, 2000; Knodler *et al*, 2005), but the molecular mechanism of this process is not characterised. It is tempting to propose that, as in the case of *S. flexneri* infection, *Salmonella* induces synthesis of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ via PtdIns(5)P production.

Once activated, PI 3-kinase/Akt may influence many events, including cap-dependent protein translation, highlighting the ability of pathogens to manipulate many aspects of the cellular machinery. Here, we show that IpgD-mediated pathways protect cells from apoptosis through direct and transcriptional control of antiapoptotic processes. Moreover, PtdIns(5)P may participate in actin cytoskeleton remodelling (Sbrissa *et al*, 2004). Recent data also suggest that PtdIns(5)P can bind to different actors regulating transcription, such as ING2 and ACF (Gozani *et al*, 2003), or the p62 (Tfb1) subunit of the general transcription factor IIIH (TFIIH) (Di Lello *et al*, 2005).

Altogether, our data demonstrate for the first time that PtdIns(5)P is an important signalling molecule generated

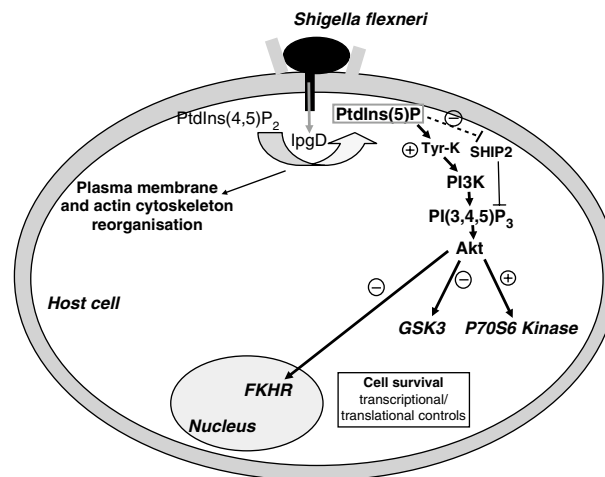


Figure 10 Roles of IpgD/PtdIns(5)P in *S. flexneri* infected cells. The local breakdown of PtdIns(4,5)P₂ into PtdIns(5)P at *S. flexneri* entry site results in dramatic plasma membrane and cytoskeleton rearrangements, while PtdIns(5)P production activates the PI 3-kinase/Akt pathway, leading to prolonged survival of the host cell and efficient replication of the bacteria.

during *S. flexneri* infection that plays a key role in class IA PI 3-kinase/Akt activation in the host cell (Figure 10). This mechanism is particularly important to regulate the survival of infected cells in order to maintain efficient bacterial colonisation.

Materials and methods

Materials

Several vectors were described previously, including the pRK5-Myc-IpgD WT and pRK5-Myc-IpgD-C438S mutant (Niebuhr *et al*, 2002); the pCDNA3-HA-Akt (Carricaburu *et al*, 2003); the pGEX-PHD2x, the pEGFP-PHD3x, the pEGFP-PHD3Kmt3x (Gozani *et al*, 2003) and the pCDNA3-GFP-Lyn-Inp54 vector (a kind gift from T Meyer) (Raucher *et al*, 2000). The antibodies used were P-Akt1/2/3 (S473 and T308), P-FKHR (S256), FKHR, P-GSK3 (GSK-3 α , S21 and GSK-3 β , S9), GSK-3 β , caspase-3 and P-p70S6K (T389) rabbit polyclonals (Cell Signaling Technology), P-MAP kinases1/2 monoclonal (Sigma-Aldrich), p85, cortactin (clone 4F11), phosphotyrosine (clone 4G10) (Upstate), P-cortactin (Y421) (Biosource International), PARP polyclonal (BD Biosciences Pharmingen), P-Akt1/2/3 (T308) used for immunofluorescence, Akt1/2 polyclonal, p110 β (S-19), Erk2, GFP, AnnexinV (FL319) (Santa Cruz Biotechnology), His6 (Clontech) and HA (clone 16B12, Covance). The anti-PtdIns(5)P 4-kinase II β polyclonal antibody was a gift from Lamia *et al* (2004). The *Shigella flexneri* serotype 5a LPS-monoclonal IgG C20 was generated by Phalipon *et al* (1997).

C16 and C4-PtdIns(5)P, C16 and C4-PtdIns(3)P and C4-PtdIns(4)P were from Echelon Biosciences Inc. They were resuspended after sonication in Dulbecco's modified Eagle's medium (DMEM) without foetal bovine serum (FCS) and used at 15 μ M for cell treatment. All chemicals used were of analytical grade and purchased from Sigma.

Cell culture, transient transfections of cDNA or siRNA treatments

HeLa, HEK293 and MEF cells (MEF WT; MEF6 p85 α +/–, p55 α +/–, p50 α +/–, p85 β –/–, p55 γ +/+ and MEF9 p85 α –/–, p55 α –/–, p50 α –/–, p85 β –/–, p55 γ +/+) (kind gifts of Dr S Brachman) were grown in DMEM supplemented with 10% FBS.

DNA transfections were performed using Effectene[®] (Qiagen) for HeLa cells or LipofectAmine Plus[™] (Invitrogen Life Technologies) for MEF cells. Cells were analyzed 12–24 h after transfection according to the experiment, as indicated.

SMARTpool[®] siRNA against Akt1 (# M-003000-01), PI 3-kinase (p110 β) (# M-003019-02) or siControl non-targeting siRNA #1 (# D-001210-01) was designed by Dharmacon siGENOME[™]. PI 3-kinase C2 α and C2 β siRNAs were previously described (Maffucci *et al*, 2005). siRNA transfections, performed according to the manufacturer protocol (Oligofectamine, Invitrogen Life Technologies), were repeated twice at 24 h of interval. In all experiments cells were lysed 48 h after the first siRNA treatment.

S. flexneri strains and cell infection

The WT invasive strain of *S. flexneri* serotype 5 (M90T) or the invasive IpgD-deficient strain (*ipgD*) (Niebuhr *et al*, 2002) was added to semiconfluent cells grown in 35-mm-diameter plastic tissue culture dishes (with coverslips for immunofluorescence experiments) with a multiplicity of infection of 100 bacteria per cell. After 15 min incubation at room temperature to allow bacterial adhesion, cells were washed and incubated at 37°C for the indicated times to allow bacterial entry. At given times, cells were washed twice in PBS and either fixed with paraformaldehyde for fluorescence labelling or scraped in Laemmli sample buffer for Western blot analysis.

The bacterial invasion assay of HeLa cells was performed as described previously (Sansonetti *et al*, 1986). Briefly, after 30 min of infection, HeLa cells were washed and 50 μ g/ml gentamicin was added to the medium in order to eliminate extracellular bacteria. After 30 or 300 min, cells were either submitted to immunofluorescence experiment or to a lysis with 0.5% sodium deoxycholate in PBS to liberate intracellular bacteria. The bacteria were then spread onto petri box overnight and the colony-forming units were counted.

Western blot analysis

Cells were scraped in Laemmli sample buffer and proteins were resolved by SDS-PAGE and then transferred onto nitrocellulose (membrane Hybond-C super, Amersham Pharmacia Biotech) using a liquid transfer apparatus. The membrane was blocked in Tris-buffer saline containing 0.1% Tween 20 and 5% bovine serum albumin and incubated using appropriate primary antibodies. The secondary antibodies were linked to HRP and revealed with the SuperSignal West Pico Chemiluminescence system (Perbio).

Fluorescence labelling

HeLa cells were grown on coverslips and transfected or not for 12–24 h depending on the vector. When indicated, cells were infected with *S. flexneri* or treated with lipids, washed in PBS, fixed with 3.7% (w/v) paraformaldehyde for 20 min and washed again in PBS. Cells were permeabilised by 0.05% (v/v) Triton X-100 in PBS for 10 min and then incubated in 3% (w/v) BSA/PBS for 30 min. Primary antibodies (1/100 in 3% (w/v) BSA/PBS) or the GST-PHDx2 biotinylated probe (40 μ g/ml in PBS/BSA 1%) were added for 1 h. After several washes in PBS, secondary antibodies (anti-rabbit fluorolink Cy5 PA45004 from Amersham, anti-rabbit-Alexa Fluor 594 and 350), streptavidin-Alexa Fluor 594 (Molecular Probes), phalloidin-Alexa Fluor 594 and 488 (Molecular Probes), all used at 1/250 in 3% (w/v) BSA/PBS, were added to the appropriate coverslips. After washes in PBS, the coverslips were mounted with Mowiol and the preparations observed by confocal (Zeiss LSM 510) or epifluorescence (Nikon Eclipse TE2000-U) microscopy.

Recombinant protein purification and biotinylation protocol for GST-PHD2x probe

After dialysis, the recombinant GST protein fused to a tandem of PHD (GST-PHD2x) (Gozani *et al*, 2003) produced in BL21 RIL⁺ bacteria (Stratagene) was concentrated with Vivaspin 500 Concentrator (Vivascience) and biotinylated using the BiotinTag Micro Biotinylation Kit (Sigma-Aldrich).

Phospholipid extraction and analysis

HeLa cells, grown in 100 mm dishes, were transfected with appropriate vectors and incubated in phosphate-free minimal essential medium containing [³²P]orthophosphate (Amersham) (200 μ Ci/ml) for 12 h. For infection experiments, HeLa cells were labelled with [³²P]orthophosphate as above and infected with *S. flexneri* for the indicated times. Lipids were then extracted and analysed by HPLC as described previously (Niebuhr *et al*, 2002). PtdIns(5)P was quantified by mass assay as described (Niebuhr *et al*, 2002).

In vitro PI 3-kinase assays

Cells were washed twice with PBS and lysed at 4°C in 20 mM Tris-HCl (pH 8), 150 mM NaCl, 4 mM EDTA, 2.7 mM KCl, 1 mM CaCl₂, 1 mM Na₃VO₄, 0.5 μ g/ml leupeptin and aprotinin, 1% NP40, 2% glycerol, 1 mM MgCl₂ and 20 mM NaF. Lysates were centrifuged at 13 000 g and the supernatants incubated for 1 h with anti-p85 or antiphosphotyrosine antibodies and for another hour with protein A-Sepharose beads or protein A-G mixed-Sepharose beads, respectively. The beads were washed once with the lysis buffer, twice with 0.1 M Tris-HCl (pH 7.4) containing 0.5 M LiCl and twice with 20 mM Hepes (pH 7.4) containing 5 mM MgCl₂. Half of the immunoprecipitate was analysed by Western blot with the anti-p85. The other half was resuspended in 50 μ l PI 3-kinase buffer (50 mM Tris-HCl pH 7.4, 1.5 mM DTT, 100 mM NaCl, 0.5 mM EDTA, 5 mM MgCl₂, 100 μ M ATP), 10 μ l of PtdIns/phosphatidylserine (1:2, w/w) vesicles and 15 μ Ci γ -[³²P]ATP were added, and after 10 min at 30°C, under gentle shaking, reactions were terminated and lipids quantified by HPLC.

PtdIns(3,4,5)P₃ 5-phosphatase SHIP2 assays

The [³²P]PtdIns(3,4,5)P₃ 5-phosphatase activity of SHIP2 was measured as described (Pesesse *et al*, 2001) after immunoprecipitation of His-tagged SHIP2, from HEK293-transfected cells, with the anti-His₆ antibody. Vesicles of [³²P]PtdIns(3,4,5)P₃ (20 000 counts per minute (c.p.m.)/samples) together with 50 μ g of phosphatidylserine and either 20 μ M of C16-PtdIns(5)P or of C16-PtdIns(3)P were used as a substrate. Similar results were observed using different assay conditions and methods (malachite green assay to measure the phosphate release, use of fluorescent PtdIns(3,4,5)P₃ substrate).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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