Activation of Akt by the Bacterial Inositol Phosphatase, SopB, is Wortmannin Insensitive

Kendal G. Cooper”, Seth Winfree, Preeti Malik-Kale, Carrie Jolly, Robin Ireland, Leigh A. Knodler, Olivia Steele-Mortimer

Laboratory of Intracellular Parasites, National Institutes of Allergy and Infectious Disease, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, Montana, United States of America

Abstract

Salmonella enterica uses effector proteins translocated by a Type III Secretion System to invade epithelial cells. One of the invasion-associated effectors, SopB, is an inositol phosphatase that mediates sustained activation of the pro-survival kinase Akt in infected cells. Canonical activation of Akt involves membrane translocation and phosphorylation and is dependent on phosphatidylinositol 3 kinase (PI3K). Here we have investigated these two distinct processes in Salmonella infected HeLa cells. Firstly, we found that SopB-dependent membrane translocation and phosphorylation of Akt are insensitive to the PI3K inhibitor wortmannin. Similarly, depletion of the PI3K regulatory subunits p85α and p85β by RNAi had no inhibitory effect on SopB-dependent Akt phosphorylation. Nevertheless, SopB-dependent phosphorylation does depend on the Akt kinases, PDK1 and rictor-mTOR. Membrane translocation assays revealed a dependence on SopB for Akt recruitment to Salmonella ruffles and suggest that this is mediated by phosphoinositide (3,4) P2 rather than phosphoinositide (3,4,5) P3. Altogether these data demonstrate that Salmonella activates Akt via a wortmannin insensitive mechanism that is likely a class I PI3K-independent process that incorporates some essential elements of the canonical pathway.

Introduction

Salmonella enterica is a facultative intracellular pathogen that causes a number of diseases ranging from self-limiting gastroenteritis to systemic typhoid fever. Like many other Gram-negative pathogens, Salmonella use Type III Secretion Systems (T3SS) to deliver bacterial effector proteins into host cells. T3SS1, also known as the invasion-associated T3SS, mediates efficient invasion of non-phagocytic eukaryotic cells, such as enterocytes in the intestinal epithelium. The invasion process has been extensively studied using cultured epithelial cells and S. enterica serovar Typhimurium (S. Typhimurium). It is characterized by the formation of localized membrane ruffles, which involves the co-operative activity of the T3SS1 effectors: SopE, SopE2 and SopB [1]. These effectors act in concert to activate the Rho family GTPases, Cdc42 and Rac, either directly, by acting as GTPase exchange factors (SopE and SopE2), or indirectly, by the generation of phosphoinositides on the cytosolic face of the plasma membrane (SopB).

In addition to its role in invasion, SopB has a number of other roles in establishing the intracellular niche [2,3,4,5,6,7]. One of the major targets of SopB in mammalian cells is the prosurvival kinase Akt (also known as PKB) [5,6], a serine/threonine kinase that plays central roles in a variety of cellular functions. Other bacterial pathogens also target Akt in epithelial cells, suggesting that manipulation of this kinase may be an important step in establishing infection [8,9,10,11,12,13]. Canonical Akt activation, as illustrated by growth factor stimulation of epithelial cells, involves two sequential steps: (1) Class I PI3K-dependent membrane-translocation, followed by; (2) phosphorylation at Thr308 and Ser473, that occurs in the cell membrane [14]. The PH domain of Akt binds with high affinity to the 3'-phosphorylated lipid products of PI3K, PtdIns(3,4,5)P3 and PtdIns(3,4)P2 [15,16,17]. Once at the membrane, Akt is phosphorylated on Thr308 by the serine-threonine kinases PDK1 (phosphoinositide-dependent kinase 1) [18] and subsequently on Ser473 by mTORC2 (mammalian target of rapamycin complex 2) [19]. Akt phosphorylation is typically short-lived due largely to the rapid hydrolysis of PtdIns(3,4,5)P3 and PtdIns(3,4)P2 by a number of phosphoinositide phosphatases including PTEN, inositol polyphosphate 4-phosphatases and inositol polyphosphate 5-phosphatase 4-phosphatase [20,21,22,23].

The mechanism of activation of Akt by SopB is not well understood. Both SopB and IpgD, a homolog from Shigella flexneri [24], are phosphoinositide phosphatases with homology to mammalian inositol 4-phosphatases as well as the inositol 5-phosphatase synaptojanin [25]. Phosphoinositide phosphatase activity is essential for Akt activation by either effector [5,10], however, the mechanism by which this intersects with the canonical PI3K/Akt pathway to induce Akt activation remains unclear. Inhibition of SopB/IpgD-dependent Akt phosphorylation by the PI3K inhibitor LY294002 supports a role for PI3K [5,10], however, a different study found that this inhibitor did not
inhibit Salmonella-dependent accumulation of either PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ in membrane ruffles [26]. IpgD has been shown to activate the PI 3-kinase/Akt pathway activation via a process that involves conversion of PI(4,5)P₂ into PtdIns(5)P [10,24]. And more recently it was shown that PtdIns(3,5)P₂ may act indirectly to increase Akt phosphorylation by inhibiting the PP2A phosphatase [27]. In vitro phosphatase assays have shown that SopB and IpgD have relatively low specific activity compared to mammalian homologues and that phosphatase assays have shown that SopB and IpgD have relatively low specific activity compared to mammalian homologues and that they have slightly different substrate specificities. Sop B has a preference for PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ whereas IpgD has a preference for PtdIns(4,5)P₂ followed by PtdIns(3,4,5)P₃ [24,28]. Thus the involvement of PI3K in Salmonella-dependent Akt activation has not been definitively established.

SopB-dependent Akt activation in epithelial cells has important implications for Salmonella pathogenesis, particularly during the gastrointestinal phase of infection where the intestinal epithelium is targeted. To gain a better understanding of how Salmonella activates this critical cellular kinase in epithelial cells, we have investigated the role of PI3K, and other known components of the PI3K/Akt pathway, in SopB-dependent Akt phosphorylation and membrane localization in Salmonella-induced membrane ruffles.

Results

SopB is sufficient for Akt phosphorylation

Several features of Salmonella pathogenesis require the concerted actions of multiple T3SS1 effectors. In particular, SopB cooperates with SopE and SopE2 to induce the actin rearrangements leading to invasion [29]. To investigate whether these, or other effectors, contribute to SopB-dependent Salmonella-mediated Akt phosphorylation, HeLa cells were infected with mutant S. Typhimurium strains that lacked either specific effectors or the ability to translocate them. Akt phosphorylation was then assessed by immunoblotting using phospho-specific antibodies that recognize Akt when it is phosphorylated at Ser473 or Thr308 (Figure 1A). As shown previously, wild type (WT) Salmonella induces Akt phosphorylation whereas a sopB deletion mutant, ΔsopB, does not [5]. A strain lacking SopE and SopE2 (ΔsopE/ΔsopE2) induced Akt phosphorylation levels comparable to WT, whereas the triple mutant ΔsopE/ΔsopE2/ΔsopB was indistinguishable from the ΔsopB strain. A ASPI1 mutant, which lacks the T3SS1 structural and regulatory components and is unable to translocate any T3SS1 effectors into host cells, also did not induce Akt activation. Since several of these mutants are invasion defective, we confirmed that invasion per se is not required for Akt activation by pretreating cells with cytochalasin D to disrupt the actin cytoskeleton. Cytochalasin D inhibits bacterial infection (not shown and [30]) but not in cells infected with Salmonella (Figure S1). These experiments were repeated in human (FHs 74 Int) and rat (IEC-18) intestinal epithelial cells that are physiologically relevant for Salmonella pathogenesis (data not shown). In these cell lines Salmonella-induced Akt phosphorylation was also insensitive to wortmannin, thus wortmannin-insensitivity seems to be a characteristic of this pathway in epithelial cells.

The Akt phosphorylation defect of ΔsopB Salmonella can be rescued by plasmid expressed SopB or the Shigella homologue IpgD [25]. Using the plasmids pACDE, which encodes both SopB and its chaperone SigE, and pACIpgDE, which encodes IpgD and its chaperone IpgDE, we directly compared SopB- and IpgD-dependent Akt phosphorylation in infected HeLa cells. In both plasmids, expression is under the transcriptional control of the sopB promoter [25]. Like SopB, IpgD efficiently induced Akt phosphorylation, which was inhibited by LY294002 but not wortmannin (Figure 1C). Thus SopB and IpgD induce Akt phosphorylation via a similar wortmannin-insensitive mechanism.

Since the differential sensitivity to the pharmacological inhibitors wortmannin and LY294002 was both unexpected and difficult to interpret, we next sought to verify whether or not class I PI3K is required for Salmonella-induced Akt activation. To do this we used RNAi-mediated knockdown to deplete the p85α and p85β regulatory subunits of class I PI3K. Cells were transfected with siRNA 48 hr prior to infection with Salmonella for 15 min. As shown in Figure 2, depletion of p85 resulted in significant inhibition of EGF-induced Akt-phosphorylation but had no effect on Salmonella-induced Akt-phosphorylation. Furthermore, a time course experiment showed no requirement for PI3K in Salmonella-induced Akt-phosphorylation up to 3 hr post-infection (Figure S2). Together the above experiments indicate that the Salmonella-induced phosphorylation of Akt is not dependent on class I PI3K.

Differential effects of Akt inhibitors on SopB- and EGF-induced phosphorylation of Akt

Having shown a difference between Salmonella-mediated and EGF-mediated Akt activation using the PI3K inhibitor wortmannin, we next targeted post-PI3K steps in the Akt-activation pathway using a panel of pharmacological inhibitors. These included: SH-6, a phosphatidylinositol analog that prevents phosphorylation of Akt [31,32]; Triciribine (TCN), a cell-permeable tricyclic nucleoside that selectively inhibits the cellular phosphorylation/activation of Akt
Akti-1/2, a PH domain dependent allosteric inhibitor that preferentially inhibits Akt1 and Akt2; and Akt inhibitor 6 [10-(4-nitro-(N-diethylamino)butyl)-2-chlorophenoxazine, HCl] (AIX), a PH domain independent inhibitor of Akt kinase activity. HeLa cells were treated with Akt inhibitors for 30 min then either infected with *Salmonella* for 30 min or treated with EGF for 2 min. AIX was the only one of these inhibitors that inhibited *Salmonella*- and EGF-stimulated Akt phosphorylation with similar efficiency (compare Figure 3A and B). Two of the inhibitors, SH-6 and TCN, had no significant effect on *Salmonella*-induced Akt phosphorylation when used at concentrations that caused inhibition of EGF-stimulated Akt phosphorylation. In contrast, Akti-1/2 had no effect on EGF-stimulated Akt phosphorylation at the concentrations used here (0.05 mM–0.1 mM) but did significantly reduce *Salmonella*-induced Akt phosphorylation at 0.1 mM. Altogether, these results confirm our initial findings with the PI3K inhibitor wortmannin; that SopB-dependent Akt phosphorylation is occurring via a mechanism distinct from the canonical PI3K/Akt pathway.

### Rictor and PDK1 are involved in SopB-dependent Akt phosphorylation

To verify the above data and also determine the requirement for other known components of the PI3K/Akt pathway in SopB-mediated Akt phosphorylation, we used RNAi-mediated knockdown to deplete proteins directly involved in Akt regulation (Figure 4). First, we performed targeted knockdown using isoform-specific siRNAs to compare the roles of Akt1 and Akt2, the two Akt isoforms present in HeLa cells. Cells were transfected with siRNA 48 hr prior to infection with *Salmonella* for 30 min. The levels of total Akt (Akt1 and Akt2), phospho Akt (Akt1 and Akt2) and actin were then assessed by immunoblotting. In HeLa cells the pan Akt antibody that we used to detect total Akt, recognizes both Akt1 (upper band) and Akt2 (lower band). Knockdown efficacy was better for Akt2 than Akt1. Negative control siRNA targeting Akt3, an isoform not expressed in HeLa cells, did not affect Akt1 and Akt2 levels and had no effect on *Salmonella*-dependent Akt phosphorylation. Depletion of either Akt1 or Akt2 resulted in reduced levels of Akt phosphorylation although Akt2 depletion had a more pronounced effect (Figure 4A). Depletion of both Akt1 and Akt2 caused almost complete abrogation of Akt phosphorylation as previously shown [6], but also caused loss of cell growth and/or viability as indicated by the decrease in actin. These data show that *Salmonella* can induce phosphorylation of both Akt1 and Akt2 in infected HeLa cells.

### Down-regulation of growth factor mediated Akt phosphorylation is dependent on phosphatase and tensin homologue deleted on chromosome 10 (PTEN) which dephosphorylates PtdIns(3,4,5)P3

However, targeted knockdown of PTEN with siRNA had no effect on *Salmonella*-dependent Akt phosphorylation in infected HeLa cells.
apparent effect on the amount of Akt phosphorylation in HeLa cells infected with Salmonella for 30 min (Figure 4A) or in extended (3 hr) time-course experiments (data not shown).

Phosphorylation of Akt at Thr308 and Ser473 is mediated by the Akt kinases, PDK1 and mTORC2 respectively [19,36]. We assessed the role of these kinases using siRNA targeting PDK1 or Rictor, the defining component of the multisubunit complex mTORC2. In cells depleted of PDK1 and then infected with WT Salmonella for 30 min, we observed a strong reduction in Thr308 phosphorylation as well as a detectable reduction in Ser473 phosphorylation (Figure 4B). In contrast, in mTORC2 depleted cells Ser473 phosphorylation was preferentially reduced. As an additional control, we also depleted raptor, which is complexed with mTOR in mTORC1, but this had no effect on Akt phosphorylation. Collectively, these data demonstrate a requirement for both PDK1 and mTORC2 in the Salmonella-induced activation of Akt.

PDK1 and rictor, are recruited to Salmonella-induced ruffles independent of SopB

Having shown that Salmonella-induced phosphorylation of Akt is dependent on PDK1 and rictor we next sought to confirm that these kinases are translocated to the plasma membrane during infection. The dominant characteristic of Salmonella invasion of epithelial cells is the formation of membrane ruffles and Akt is specifically translocated to the ruffle where it is phosphorylated [6]. To determine whether the Akt kinases are also translocated to the ruffles we used transiently expressed myc-tagged PDK1 and rictor fusion proteins since the endogenous proteins were below the levels of detection in our system (not shown). As shown in Figure 5 both PDK1-Myc and Myc-rictor were recruited to ruffles induced by WT Salmonella.

Intriguingly, although SopB is required for Salmonella induced phosphorylation of Akt, no requirement has been demonstrated for SopB in membrane translocation. On the contrary, Akt is apparently enriched in ruffles induced by ΔsopB Salmonella (Figure 5 and [6]). Here we found that PDK1 and rictor are also translocated to ruffles induced by the ΔsopB strain (Figure 5). These experiments indicate that Akt, PDK1 and rictor are translocated to Salmonella-induced ruffles independent of SopB activity. This does not explain why Akt phosphorylation is strictly SopB dependent. One possibility is that a negative regulator of Akt phosphorylation could be involved in the absence of SopB. We analyzed the localization of CTMP, a 27-kDa protein that has been shown to regulate the activity of Akt by associating with it at the plasma membrane [37,38]. However, in

Figure 2. The class I PI3K regulatory subunits p85α and p85β are not required for SopB-mediated Akt phosphorylation. HeLa cells were transfected with siRNAs, specific for p85α and p85β, for 72 hr then either treated with EGF or infected with Salmonella WT. For siRNA control siRNA specific for AKT3 was used (cont.). Monolayers were then solubilized in sample buffer and processed for immunoblotting using antibodies to detect phospho Akt (Ser473), total Akt or p85α. (A) Representative immunoblot showing p85α knockdown efficacy and effect on Akt phosphorylation in infected or EGF treated cells. (B) Quantification of Akt phosphorylation estimated by densitometry. Shown are the means ± SD from three independent experiments.
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HeLa cells co-expressing FLAG-CTMP and GFP-Akt, CTMP co-localized with Akt in ruffles induced by either WT *Salmonella* or the ΔsopB mutant. Altogether these experiments did not reveal any requirement for SopB in localization of Akt kinases or CTMP to plasma membrane ruffles.

**Semi-quantitative analysis of SopB-dependent Akt recruitment and phospholipid changes in *Salmonella*-induced membrane ruffles**

Although the visual comparison of ruffles did not reveal a requirement for SopB in localization of Akt kinases or CTMP to plasma membrane ruffles.

**Figure 3. PI3K/Akt inhibitors differentially affect *Salmonella*-induced and EGF-induced Akt phosphorylation in epithelial cells.** HeLa cells were pretreated with PI3K/Akt inhibitors for 30 min and then infected with *Salmonella* for 30 min (A) or treated with EGF (50 ng/ml) for 2 min (B). Monolayers were then solubilized in sample buffer and processed for immunoblotting using antibodies to detect phospho Akt (Ser473) and total Akt. Inhibitors used were; Wortmanin (WTM:100 nM), LY294002 (LY29: 50 μM), SH-6 (20 μM, 10 μM), TCN (20 μM, 10 μM), Akti-1/2 (0.1 μM, 0.05 μM) and AIX (10 μM, 5 μM). The graphs below each panel show the quantification of Akt phosphorylation estimated by densitometry. Shown are the means ± SD from three independent experiments (*P*<0.05, significantly different from untreated).

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![Image](image-url)
a SopB-dependent process (WT 7.1 ± 3.6; ΔsopB 2.6 ± 1.4; ΔsopB/pACDE 7.4 ± 3.9). Translocation to the plasma membrane of the Akt PH domain can be mediated by PtdIns(3,4,5)P 3 and/or PtdIns(3,4)P2 [40,41]. To determine whether both of these phosphoinositides are enriched in in ruffles we used GFP fusions to the PH domains of Btk and TAPP1 which bind PtdIns(3,4,5)P3 and PtdIns(3,4)P2 respectively [40,42]. Only EGFP-TAPP1-PH showed statistically significant recruitment to ruffles in a SopB-dependent manner (WT = 2.0 ± 1.0; ΔsopB = 1.4 ± 0.7; ΔsopB/pACDE = 2.6 ± 1.6). This suggests that, in Salmonella-induced ruffles, SopB activity leads to an enrichment of PtdIns(3,4,5)P3, rather than PtdIns(3,4,5)P2. Finally, we analyzed recruitment of the PH domain of phospholipase C delta (PLC-δ), a probe for PtdIns(4,5)P2 [42,43]. This probe (PLC-δ-GFP) confirmed that PtdIns(4,5)P2 is enriched in Salmonella-induced ruffles (WT = 3.6 ± 1.7; ΔsopB = 4.2 ± 1.7; ΔsopB/pACDE = 3.9 ± 1.6). Although we could not detect any statistically significant dependence on SopB, it should be stated that this technique assesses the total amount of probe in ruffles and would not reveal differences within sub-regions of the ruffles. For example, depletion of PtdIns(4,5)P2 at the apex of the phagocytic cup as has previously been shown [2].

Discussion

While it has been well established that the PI3K/Akt pathway is modulated by many viruses and plays an important role in the establishment of viral infection [44], the appropriation of Akt by pathogenic bacteria is less well understood [5,6,10,11,43,46,47]. Salmonella, and other intracellular bacteria [9], use Akt activation to block or delay apoptosis in infected cells. Given the diverse cellular roles of Akt, it is likely to have additional functions during bacterial infection.

In this study, we first showed that the Salmonella effector protein SopB is necessary and sufficient for Akt phosphorylation in HeLa cells. To gain a better understanding of the role of Akt in Salmonella pathogenesis we then compared SopB-mediated Akt activation with the canonical EGF signaling pathway common to all epithelial cells. Using different approaches we assessed the two essential steps in Akt activation i.e. membrane translocation and phosphorylation. The most striking difference that our study revealed is that the irreversible PI3K inhibitor wortmannin is unable to inhibit either of these steps in Salmonella-infected HeLa cells. An obvious interpretation of this is that SopB-dependent Akt activation is independent of class I PI3K, supported by the finding that depletion of the p85 regulatory subunit of class I PI3K had no effect on this pathway. Surprisingly, the more specific PI3K inhibitor LY294002 did inhibit both membrane translocation and phosphorylation of Akt in Salmonella infected cells. However, LY294002 does have other intracellular targets [48], including: casein kinase-2, GSK3α and GSK3β, as well as p97/VCP, a member of the type II AAA ATPase family [49]. Several other

Figure 4. Both PDK1 and rictor are required for Salmonella-induced Akt phosphorylation. A and B. HeLa cells were transfected with the indicated SMART pool siRNA for 48 hr then infected with Salmonella WT or ΔsopB. For siRNA control SMART pool siRNA specific for AKT3 was used (cont). After 30 min, monolayers were solubilized and processed for immunoblotting. Antibodies were used to detect phospho-Akt (Ser473 or Thr308), total Akt, PDK1, raptor, rictor or actin. Gray bars underneath the individual panels highlight the efficiency of each siRNA knockdown.

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Figure 5. Akt regulators accumulate in Salmonella induced ruffles. HeLa cells were transfected with plasmids encoding epitope-tagged proteins as indicated. After 20 hr they were infected with Salmonella for 30 min, then fixed and processed for immunofluorescence microscopy. GFP-HA-Akt transfected cells were stained for Salmonella LPS (Cy5) and phospho Akt Ser473 (AF568: A–C), Myc (AF568: D–F) or 3×FLAG (AF568: J–L). Cells expressing PDK1-Myc (G–I) were stained for LPS (Cy5), Myc (AF488) and actin filaments (phalloidin-AF568) to reveal the ruffles.

Figure 6. Accumulation of phospho Akt in Salmonella-induced ruffles is wortmannin insensitive. HeLa cells expressing EGFP-mAkt were infected with Salmonella for 20 min then fixed and processed for immunofluorescence microscopy. Cells were doubly stained for plasma membrane (Cy5-WGA) and phospho Akt Ser473 (AF568). (A) Representative images with ruffles outlined to show phospho Akt in ruffles induced by WT Salmonella. In comparison, phospho Akt levels are much lower in ruffles induced by the ΔsopB mutant, unless the mutant is complemented in trans with sopB (pACDE). (B) Semi-quantitative analysis of phospho Akt (Ser473) levels (R_{pAkt/Akt}) in membrane ruffles. Where indicated, cells were pretreated with wortmannin (WTM: 100 nM) or LY294002 (LY29: 50 μM) for 30 min prior to infection and maintained throughout. Data are the means ± SD from three independent experiments (*P<0.05).

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potential targets, DNA-PK, PI4K and mTOR, can be excluded since they are equally sensitive to wortmannin [50,51,52]. We also found that SopB-dependent Akt phosphorylation was less sensitive than EGF-induced phosphorylation to two small molecule inhibitors of AKT. SH-6 is a phosphatidylinositol analog that competes with PI3K for PtdIns(4,5)P2 [32] whereas TCN is a cell-permeable tricyclic nucleoside that inhibits Akt phosphorylation [33]. One possibility is that the SopB pathway engages a mammalian PI3K other than the canonical class I PI3K, although this is unlikely since WTM does not show significant isoform specificity. A final alternative is PI3K-independent activation of Akt. This is not without precedent since both cAMP/protein kinase A and dopamine have been shown to elicit wortmannin-insensitive Akt activation [53,54,55,56]. Despite the above differences between the SopB-mediated and EGF-mediated pathways of Akt activation our data suggest that the Akt kinases, PDK1 and mTORC2, are essential components in both cases.

Figure 7. Enrichment of PH domain lipid-binding probes in Salmonella-induced ruffles. HeLa cells transfected with plasmids encoding EGFP-fusions to full length Akt (EGFP-mAkt) or isolated PH domains as indicated were infected with Salmonella for 30 min, then fixed and processed for immunofluorescence microscopy. Cells were stained for plasma membrane (Cy5-WGA) and Salmonella LPS (AF568). (A) Representative images to show EGFP-mAkt accumulation in ruffles induced by Salmonella WT, the ΔsopB mutant or ΔsopB complemented in trans with sopB (pACDE). Orthogonal sections show WGA (grayscale) and EGFP-Akt (grayscale converted to a heatmap using the “FIRE” look up table of ImageJ) corresponding to the red lines on the projections. (B) Analysis of GFP fusion enrichment in membrane ruffles. Shown is combined data from three independent experiments. Each dot represents one ruffle. P values were obtained by ANOVA and Tukey’s post hoc analysis (* P<0.05).

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To get a better understanding of the role of SopB in recruitment of signaling components we also investigated recruitment of proteins and phosphoinoside specific PH domains to membrane ruffles. This semi-quantitative method revealed that Akt enrichment is SopB dependent, whereas in a previous study where enrichment was simply assessed visually, we could not detect any requirement for SopB [5]. Furthermore, the PH domain translocation experiments indicated that SopB induces a localized increase in PtdIns(3,4)P2 rather than PtdIns(3,4,5)P3 in Salmonella-induced ruffles. This suggests that Akt phosphorylation in the Salmonella-induced ruffle is dependent on PtdIns(3,4)P2 rather than PtdIns(3,4,5)P3. Further studies are required to determine the roles of these phosphoinositides in SopB-dependent Akt activation. Interestingly, studies on the S. flexneri effector protein IpgD, a homolog of SopB, have shown that sustained Akt phosphorylation is mediated by IpgD-dependent generation of PtdIns(5)P [10] and indeed SopB causes localized conversion of PtdIns(4,5)P2 to PtdIns(5)P in
regions of Salmonella-induced plasma membrane ruffles [2,57]. One possible effect of increased PtdIns(3)P is to prevent the dephosphorylation of Akt by inhibiting the catalytic subunit of PP2A phosphatases [27]. However, these studies also found that PI3K played an essential role in IgD-dependent Akt-phosphorylation. Unfortunately, PtdIns(3)P is a rare phosphoinositide [40], making it very difficult to detect and it remains poorly understood.

In conclusion, we have shown that Salmonella induces Akt activation via a wortmannin insensitive mechanism that probably involves a novel class I PI3K-independent pathway. Why Salmonella have not simply tuned into the canonical pathway is unclear, but one possibility is that it could allow the targeting of different downstream proteins. The molecular mechanisms involved in this process remain unknown, however, the work presented here provides a foundation for future experiments that should lead to a better understanding of bacterial pathogenesis as well as the multi-faceted essential kinase Akt.

Materials and Methods

Materials

Primary antibodies, mouse monoclonal antibodies to Myc and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology unless otherwise noted. Fluorescently labeled Alexa Fluor (AF)-conjugated antibodies and phalloidin were from Invitrogen. Cy3-conjugated antibodies were from Jackson ImmunoResearch. Rabbit polyclonal antibodies to Salmonella LPS (Salmonella O Antiserum Group B Factors 1, 4, 5, 12) were from BD. Anti-FLAG antibodies were from Sigma. Other chemicals were from EMD Biosciences except where indicated. Cytochalasin D was from Sigma. LY294002 was from Enzo Life Sciences.

Cell Culture

HeLa (human cervical adenocarcinoma, ATCC CCL-2) cells were grown at 37°C in 5% CO₂ in Eagle's minimal essential medium (EMEM) (Mediatech) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen). Cells were passaged every three to four days and used for experiments within 15 passages of receipt from ATCC.

Bacterial strains and plasmids

Salmonella enterica serovar Typhimurium SL1344 wild type and the mutants ΔSPI-1::kan, ΔsopB and sopE::aphTΔ::M218 (Ter+) (M202Δ-E2+) were as previously described [5,58,59]. The ΔsopB/ΔsopE/Δ::M218 was constructed by sequential phage P22-mediated transduction of the ΔsopE::aphT and ΔsopE2::pM218 alleles of M202Δ-E2+ into SL1344 ΔsopB.

The complementing plasmids pACDE and pACDegDE have been described previously [25]. EGFP-mAkt [39], Myc-PDK1 [60], green fluorescent protein (GFP)-HA-Akt [41], enhanced GFP (EGFP::TAPP1-PH [61], Akt-PH-EGFP, PLCδ-PE-EGFP and Btk-PH-EGFP [43] have all been described previously. Myc-Rictor [62] was purchased from Adgene (plasmid #11367). To remove the PH domain of Akt, EGFP-mAkt was used as template to generate the ΔsopB version by PCR with the oligonucleotides hCTMP-3FLAG-Bgl5 (5’ GGA AGA TCA TCT GGT GAG GAG CTG CGC CGC G 3’) and hCTMP-3FLAG-Sal3 (5’ A CGG GTG GAC TTA TGT CAG ACT TTT AGG AGG ATT CAG 3’). The resulting amplicon was cloned into pCR2.1 TOPO (Invitrogen), released by EcoRI digestion and ligated into EcoRI-digested p3×FLAG-CMV 2.1 (Sigma) to create 3×FLAG-CTMP.

For ectopic expression of SopB, sopB was amplified from pACDE [25] or pACDE C460S [6] with the oligonucleotides 6His-SigD-F (5’ GCC GGA TTC AAA TAC AGA GCT TCT CTC GAG TCA AGA 3’) and 6His-SigD-R (5’ CCC CAG TCA AGA TGT GAT TAA TGA AGA 3’) (engineered restriction sites are underlined). The resulting amplicons were digested with BamHI and XhoI and ligated into the corresponding sites of pcDNA3.1/His A (Invitrogen) to create 6His-SopB and 6His-SopB C460S, respectively.

Bacterial Infection of Mammalian Cells

Cells were seeded into 6-well plates (2.0×10⁵ cells/well), 10 cm tissue culture dishes (1.6×10⁵ cells/dish) or glass coverslips in 24-well plates (5.0×10⁴ cells) to yield monolayers of 75–85% confluency after 16–20 h. SPI1-induced bacteria were prepared by diluting 0.3 ml of overnight LB-Miller culture in 10 ml of fresh LBMiller and incubating at 37°C with shaking (225 rpm). At late log phase (3.5 h), the bacteria were pelleted at 8,000 × g for 2 min and resuspended in an equal volume of Hanks' balanced salt solution (Mediatech) or phosphate-buffered saline. This suspension of invasive bacteria was then used to inoculate HeLa cells (multiplicity of infection = 100) and invasion was allowed to proceed for 10 min at 37°C in 5% CO₂. Following invasion, extracellular bacteria were removed by washing with HBSS and the cells were then incubated with serum-free EMEM for 20 min. For experiments requiring longer incubations, gentamicin (50 µg/ml) was added 30 min post-infection and reduced to 10 µg/ml at 90 min post-infection, to kill any extracellular bacteria. Where indicated, cells were treated with inhibitors for 30 min immediately before infection and drugs were maintained in media thereafter. For serum starvation cells were incubated in serum-free EMEM for 3–3.5 h immediately before infection and also for subsequent steps.

Immunoblotting

Monolayers of infected HeLa cells in 6 well plates were solubilized in hot SDS-PAGE sample buffer (100 µl/well) at the indicated times and SDS-PAGE and immunoblotting were performed as described previously [4]. Rabbit polyclonal anti-Akt, rabbit monoclonal anti-total Akt (pan) (11E7) (cat # 4685), rabbit polyclonal anti-phyospho-Akt Ser473, rabbit monoclonal anti-phospho-Akt (Ser473) (193H12), rabbit monoclonal anti-phyospho-Akt (Thr308) (D9E), rabbit polyclonal anti-PDK1, rabbit polyclonal anti-raptor, rabbit polyclonal anti-rictor (BL2181, Bethyl Laboratories), mouse monoclonal anti-PI3K p85α (clone AB6, Millipore) or mouse monoclonal anti-actin (C-2, Santa Cruz Biotechnology) were used at a 1:1000 to 1:20,000 dilution in blocking buffer [Tris buffered Saline, 0.1% (v/v) Tween 20, 1% bovine serum albumin]. Secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit or horse anti-mouse IgG, were diluted 1:5000 in Tris buffered saline, 0.1% (v/v) Tween 20, 5% (w/v) skim milk powder. For chemiluminescent detection the SuperSignal West Femto Substrate Kit or SuperSignal West Pico Substrate Kit were used according to the manufacturer’s instructions (Thermo). Immunoblotting with rabbit polyclonal antibodies to SopB was as previously described [63,64].

ELISA

HeLa cells in 10 cm dishes were infected with Salmonella as described above. Samples were prepared and the level of Akt phosphorylation was assessed using the PathScan® Phospho-Akt1
Experimental procedures

Cell culture

HeLa cells were seeded in 6-well or 24-well plates and 6–8 h later were transfected with plasmids using Fugene® 6 according to the manufacturer’s instructions. After 20 h cells were infected with Salmonella or processed directly for immunoblotting following solubilization in 150 μl hot 1.5 × SDS-PAGE sample buffer.

RNA-mediated interference

Small interfering RNA (siRNA) SMARTpool (Dharmacon) sequences targeting human Akt1, Akt2, Akt3, PTEN, PDK1, raptor and rictor were diluted and stored according to the manufacturer’s instructions. Cells were transfected with siRNA using RNAfect (Qiagen) transfection reagent according to the manufacturer’s instructions and infected with Salmonella 48 h later. For experiments in which knockdown of p85α was carried out ON-TARGETplus SMARTpool siRNA targeting human AKT3, p85α and p85β was used. HeLa cells were seeded in 6-well plates at a density of 9 × 10^4 cells/well and incubated for 16–20 h to yield a monolayer of 50–60% confluency. Cells were transfected using DharmaFect1 reagent (Dharmacon) according to the recommended protocol with 50 nM siRNA for a single target and 25 nM siRNA each for dual targets. Cells were treated with EGFR or infected with Salmonella 72 h later.

Imaging of phospho Akt in membrane ruffles

HeLa cells grown on glass coverslips were transfected with plasmid EGFP-mAkt 16–18 hrs prior to infection with Salmonella. After 15 min coverslips were fixed in 2.5% paraformaldehyde for 10 min at 37 °C, washed in PBS and stained with 1.0 μg/ml WGA-Alexa Fluor 647 (AF647-WGA) for 5 min, washed and fixed for 5 min at 37 °C in 2.5% paraformaldehyde. Cells were permeabilized for 5 min with 0.1% saponin, 10% normal goat serum in PBS, processed for immunofluorescence using a mouse anti-Akt phospho-serine 473 antibody, followed by AF568-conjugated anti mouse secondary. Coverslips were mounted onto glass slides with Prolong Gold and imaged within 1 week. Z-stacks were acquired for each secondary. Coverslips were mounted onto glass slides with Prolong Gold and imaged within 1 week. Image acquisition and analysis was performed with using a spinning disk confocal microscope as previously described [65]. Image analysis and maximum intensity projections were performed with ImageJ v.1.4.1 (written by Wayne Rasband at the U.S. National Institutes of Health and available by anonymous FTP from zippy.nih.gov) and figures assembled using Adobe Photoshop Cs2.

Microscopy

Confocal images were either captured on a Zeiss LSM510 with 488 nm, 543 nm and 643 nm laser lines or on a spinning disc confocal microscope as previously described [65]. Image analysis and maximum intensity projections were performed with ImageJ v.1.4.1 (written by Wayne Rasband at the U.S. National Institutes of Health and available by anonymous FTP from zippy.nih.gov) and figures assembled using Adobe Photoshop Cs2.

Statistical Analysis

Unless otherwise noted results are presented as the mean ± S.D. of n = 3 independent experiments. One way Analysis of Variance (ANOVA) combined with the Tukey post hoc test was used to determine statistical significance with Prism™ software (GraphPad Software Inc).

Supporting Information

Figure S1 Wortmannin is effective at inhibiting EGFR-mediated but not Salmonella-mediated Akt phosphorylation. HeLa cells were pretreated with wortmannin (WTM:100 nM) then infected with Salmonella for 30 min or 3 hr. For the EGFR treated cells agonist was added for 2 min immediately before solubilization at 30 min or 3 hr. Samples were processed for immunoblotting using antibodies to detect phospho Akt (Ser473) and actin. (TIF)

Figure S2 Depletion of the class I PI3K regulatory subunits p85α and p85β does not affect the kinetics of SopB-mediated Akt phosphorylation. HeLa cells were transfected with siRNAs, specific for p85α and p85β, for 72 hr then either treated then infected with Salmonella WT for 15 min. For time points greater than 15 min monolayers were rinsed to remove non-internalized bacteria and were further incubated in the presence of gentamicin to kill extracellular bacteria. Monolayers were solubilized in sample buffer at the indicated times and processed for immunoblotting using antibodies to detect phospho Akt (Ser473), total Akt or actin. (TIF)

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Author Contributions

Conceived and designed the experiments: KGC SW PMK CJ RI LK. Performed the experiments: KGC SW PMK CJ RI LK. Analyzed the data: KGC SW PMK CJ RI LK. OSN. Wrote the paper: OSN.
References

1. Zhou D, Galan J (2001) Salmonella entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect 3: 1293–1298.

2. Terebusz-Mark, Vieira OV, Marcus SL, Slade A, Yip CM, et al. (2002) Elimination of host cell PtdIns(4,5)P2 by bacterial SipG promotes membrane fusion during invasion by Salmonella. Nat Cell Biol 4: 766–773.

3. Hernandez LD, Huesler K, Wenk MR, Galan JE (2004) Salmonella modulates vesicular traffic by altering phosphoinositide metabolism. Science 304: 1803–1807.

4. Dreekarch D, Knodler LA, Galbraith K, Steele-Mortimer O (2005) The Salmonella SPI1 effector SopB stimulates nitric oxide production long after invasion. Cell Microbiol 7: 105–113.

5. Steele-Mortimer O, Knodler LA, Marcus SL, Scheid MP, Geh B, et al. (2000) Activation of Akt/protein kinase B in epithelial cells by the Salmonella typhimurium effector sigD. J Biol Chem 275: 37718–37724.

6. Knodler LA, Finlay BB, Steele-Mortimer O (2005) The Salmonella effector protein SopB regulates epithelial cells from apoptosis by sustained activation of Akt. J Biol Chem 280: 9058–9066.

7. Bertelsen LS, Paasold G, Marcus SL, Finlay BB, Eckmann L, et al. (2004) Modulation of chloride secretory responses and barrier function of intestinal epithelial cells by the Salmonella effector protein SigD. Am J Physiol Cell Physiol 287: C939–C948.

8. Zhou D, Galan JE (2001) Salmonella entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect 3: 1293–1298.

9. Acuto O, Maira C, Ayvazian S, Loewer A, Fadok VA, et al. (2002) p53-dependent apoptosis following caspase activation is suppressed by the Akt kinase. J Biol Chem 277: 26159–26169.

10. Mao S, Park Y, Hasegawa Y, Tribble GD, James CE, et al. (2007) Intrinsic phosphatase activity of mTORC2 modulates Akt activation. J Biol Chem 282: 23699–23708.

11. Verbeke P, Welter-Stahl L, Ying S, Hansen J, Hacker G, et al. (2006) Recruitment of BAD by the Chlamydia trachomatis vacuole correlates with host cell survival. PLoS Pathog 2: e45.

12. Mao S, Park Y, Haegewa Y, Tribble GD, James CE, et al. (2007) Intrinsic apoptotic pathways of gingival epithelial cells modulated by Porphyromonas gingivalis. Cell Microbiol 9: 1997–2007.

13. Li YP, Venge CS, Browned L, Madsen M, Ingmer H, et al. (2010) Campylobacter jejuni induces an anti-inflammatory response in human intestinal epithelial cells through activation of phosphatidylinositol 3-kinase/Akt pathway. Vet Microbiol 148: 73–83.

14. Alessi DR, Cohen P (1998) Mechanism of activation and function of protein kinase B. British Journal of Cancer 78: 525–528.

15. Balla T, Varnai P (2009) Visualization of cellular phosphoinositide pools with phalloidin conjugates labelled with Alexa Fluor 488. Methods 48: 189–196.

16. Bellacosa A, Chan TO, Ahmed NN, Dutta K, Malstrom S, et al. (1998) Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene 17: 513–525.

17. Andjelkovic M, Alessi DR, Meyer R, Fernandez A, Lamb NJ, et al. (1997) Role of translocation in the activation and function of protein kinase B. J Biol Chem 272: 31253–31262.

18. Franke TF, Kaplan DR, Cantley LC, Toker A (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4,5-trisphosphate. Science 275: 665–668.

19. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, et al. (1997) Characterization of a phosphatidylinositol-3,4,5-trisphosphate-dependent protein kinase which phosphorylates and activates protein kinase B. EMBO J 16: 4912–4921.

20. Sarbasoss DD, Guettin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098–1101.

21. Ivetac I, Gurum R, Hakam S, Horan KA, Sheffield DA, et al. (2009) Regulation of P53/Akt signalling and cellular transformation by insulin and phosphatidyl phosphate-4-phosphatase-1. EMBO Rep 10: 877–93.

22. Tamugney T, Stokoe D (2007) New insights into PTEN. J Cell Sci 120: 4071–4079.

23. Stephens LR, Jackson TR, Hawkins PT (1993) Agonist-stimulated synthesis of phosphatidylinositol[3,4,5]trisphosphate: a novel intracellular signalling system? Biochim Biophys Acta 1179: 27–75.

24. Dyson JM, Kong AM, Wiradjaja F, Aitie ML, Gurum R, et al. (2005) The SH2 domain containing inositol phospholipid 3-phosphatase-2: SHIP2. J Biol Chem 280: 22867–22873.

25. Niels Halvorsen B, Joseph RM, Pedersen T, Philpot DJ, Gain F, et al. (2002) Conversion of PtdIns(4,5)P2 into PtdIns(4)P by the Snail effector protein Idg organizes host cell morphology. EMBO J 21: 5069–5078.

26. Marcus SL, Wenk MR, Steele-Mortimer O, Finlay BB (2001) A sphingosynthetic homologous region of Salmonella typhimurium SigD is essential for inositol phospholipase activity and Akt activation. FEBS Lett 503: 55–62.

27. Damle N, Park Y, Hasegawa Y, Tribble GD, James CE, et al. (2007) Intrinsic phosphatase activity of mTORC2 modulates Akt activation. J Biol Chem 282: 23699–23708.

28. Zhou D, Chen LM, Hernandez L, Shears SB, Galan JE (2001) A Salmonella inositol polypolyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Mol Microbiol 39: 248–259.

29. Finlay BB, Ruschikowski S, Dedhar S (1991) Cytoskeletal rearrangements accompanying salmonella entry into epithelial cells. J Cell Sci 99: 203–206.

30. Krech T, Thiere M, Hälegren E, Schafer R, Jurchen K (2010) Characterization of Akt independent effects of the synthetic Akt inhibitors SH-5 and SH-6 using an integrated approach combining transcriptional profiling and signaling pathway perturbations. BMC Cancer 10: 267–298.

31. Gillis J, Castillo SS, Zhang C, Penikas P, Mennott RM, et al. (2007) Phosphatidylinositol effector lipids that inhibit Akt also independently activate the stress kinase, p3alpha, through MKK3/6-independent and -dependent mechanisms. J Biol Chem 282: 27020–27029.

32. Yang I, Dan HC, Sun M, Liu Q, Sun XM, et al. (2004) Atef (protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. Cancer Res 64: 4934–4939.

33. Barnett SF, Defeens-Jones D, Fu S, Hancock PFJ, Haskell KM, et al. (2005) Characterization and characterization of pleckstrin-homology-domain-independent and isoenzyme-specific Akt inhibitors. Biochem J 385: 399–408.

34. Thimmaiah KN, Easton JB, Germain GE, Morton CL, Kamath S, et al. (2005) Identification of N10-substituted phenazones as potent and specific inhibitors of Akt signalling. J Biol Chem 280: 31924–31935.

35. Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, et al. (1997) Role of translocation in the activation and function of protein kinase B. J Biol Chem 272: 31253–31262.
neurons: a new route to cAMP response element-binding protein phosphorylation. J Neurosci 22: 8911–8921.

54. Sable CL, Filippa N, Hemmings B, Van Obberghen E (1997) cAMP stimulates protein kinase B in a Wortmannin-insensitive manner. FEBS Lett 409: 253–257.

55. Konishi H, Matsuzaki H, Tanaka M, Takemura Y, Karoda S, et al. (1997) Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27. FEBS Lett 410: 493–498.

56. Filippa N, Sable CL, Filloux C, Hemmings B, Van Obberghen E (1999) Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase. Mol Cell Biol 19: 4989–5000.

57. Mason D, Mallo GV, Terebiznik MR, Payrastre B, Finlay BB, et al. (2007) Alteration of epithelial structure and function associated with PtdIns(4,5)P2 degradation by a bacterial phosphatase. J Gen Physiol 129: 267–283.

58. Drecktrah D, Knodler LA, Ireland R, Steele-Mortimer O (2006) The mechanism of Salmonella entry determines the vacuolar environment and intracellular gene expression. Traffic 7: 39–51.

59. Steender S, Friezel A, Linder S, Rohde M, Mirolid S, et al. (2000) Identification of SopE2 from Salmonella typhimurium, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Mol Microbiol 36: 1206–1221.

60. Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, et al. (1997) 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Curr Biol 7: 776–789.

61. Marshall AJ, Kazana AK, Ma K, Duronio V, Hou S (2002) TAPP1 and TAPP2 are targets of phosphatidylinositol 3-kinase signaling in B cells: sustained plasma membrane recruitment triggered by the B-cell antigen receptor. Mol Cell Biol 22: 5479–5491.

62. Sarbassov DD, Ali SM, Kion DH, Guertin DA, Latek RR, et al. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14: 1296–1302.

63. Marcus SL, Knodler LA, Finlay BB (2002) Salmonella enterica serovar Typhimurium effector SigD/SopB is membrane-associated and ubiquitinated inside host cells. Cell Microbiol 4: 435–446.

64. Knodler LA, Bertero M, Yip C, Strynadka JC, Steele-Mortimer O (2006) Structure-based mutagenesis of SigE verifies the importance of hydrophobic and electrostatic residues in type III chaperone function. Mol Microbiol 62: 920–940.

65. Drecktrah D, Levine-Wilkinson S, Dam T, Winfree S, Knodler LA, et al. (2008) Dynamic behavior of Salmonella-induced membrane tubules in epithelial cells. Traffic 9: 2117–2129.