Activation of Akt/Protein Kinase B in epithelial cells by the Salmonella typhimurium effector SigD

Running title: Activation of Akt by Salmonella

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Summary

The serine-threonine kinase Akt is a proto-oncogene involved in the regulation of cell proliferation and survival. Activation of Akt is initiated by binding to the phospholipid products of phosphoinositide 3-kinase at the inner leaflet of the plasma membranes followed by phosphorylation at Ser^{473} and Thr^{308}. We have found that Akt is activated by *Salmonella enterica* serovar Typhimurium in epithelial cells. A bacterial effector protein, SigD, which is translocated into host cells via the specialized type III secretion system is essential for Akt activation. In HeLa cells, wild type *S. typhimurium* induced translocation of Akt to membrane ruffles, phosphorylation at residues Thr^{308} and Ser^{473} and increased kinase activity. In contrast, infection with a SigD deletion mutant did not induce phosphorylation or activity although Akt was translocated to membrane ruffles. Complementation of the SigD deletion strain with a mutant containing a single Cys to Ser mutation (Cys462Ser), did not restore the Akt activation phenotype. This residue has previously been shown to be essential for inositol phosphatase activity of the SigD homologue, SopB. Our data indicate a novel mechanism of Akt activation in which the endogenous cellular pathway does not convert membrane associated Akt into its active form. SigD is also the first bacterial effector to be identified as an activator of Akt.
Introduction

The serine/threonine kinase Akt (also known as PKBα) is a key regulator of cellular survival which is activated by a variety of extracellular signals including mitogens [for review (1)]. In the current model for Akt activation Akt is first recruited from the cytoplasm to cellular membranes by interaction of its PH (Pleckstrin Homology) domain with the phosphatidylinositol 3-kinase (PI3-K) lipid products phosphatidylinositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol-3,4-biphosphate {PI(3,4)P2} (2). Akt activity is not stimulated by translocation itself, but is dependent upon phosphorylation at two residues (Ser473 and Thr308). Thr308 is phosphorylated by the phosphoinositide-dependent kinase-1, (PDK1), which also contains a PH domain (3,4). The mechanism of phosphorylation of Ser473 remains elusive, while PDK1 is a possible candidate, the integrin-linked kinase and most recently autophosphorylation have also been implicated (5-7).

The facultative intracellular pathogen Salmonella enterica serovar Typhimurium (S. typhimurium) is an important causative agent of food-borne gastroenteritis in humans. Penetration of the intestinal epithelium and survival within a variety of mammalian cell types is essential for pathogenicity and is dependent on a number of virulence factors. The genes encoding these factors are located within five discrete Salmonella pathogenicity islands (SPIs) on the bacterial chromosome [reviewed in (8)]. Encoded in SPI1 is a type III secretion system

3 The abbreviations used are: Cyto D, Cytochalasin D; EGF, epidermal growth factor; GSK3, glycogen synthase kinase 3; LY29, LY294002; NF-kB, nuclear factor kappa beta; PCR, polymerase chain reaction; PDK1, phosphoinositide-dependent kinase-1; PI3-K, phosphoinositide 3-kinase; PI(3,4)P2, phosphatidylinositol-3,4-biphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; SPI, Salmonella-pathogenicity Island; TTSS, Type three secretion system;
(TTSS), which translocates effectors directly into the cytoplasm of host cells and is required for bacterial invasion of nonphagocytic cells (9). This remarkable process is instigated by interactions between bacterial effectors, components of eukaryotic signaling pathways and the actin cytoskeleton, resulting in membrane ruffling on the cell surface, and subsequent bacterial internalization (10-13). Two GTPases, Cdc42 and Rac-1, which are key regulators of eukaryotic actin dynamics, are directly targeted by Salmonella. For example, the SPI1 effector protein SopE, when microinjected into eukaryotic cells, catalyzes the exchange of GDP for GTP on Cdc42 and Rac-1 thus activating them, to induce membrane ruffling (11).

Although Salmonella-induced membrane ruffling is growth-factor receptor independent (14), and invasion is not blocked by inhibitors of PI3-K (15), we show that S. typhimurium rapidly activates Akt in epithelial cells. One translocated bacterial effector protein, SigD, was found to be absolutely required for both phosphorylation and activation of Akt. A single point mutation in a conserved inositol phosphatase catalytic domain of SigD (16) abolished both Akt phosphorylation and activation. SigD was not required for Salmonella-induced membrane translocation of Akt, indicating that in this novel pathway the membrane translocation of Akt does not lead to its activation unless an exogenous bacterial factor is present.
Experimental Procedures

Materials

Except where indicated chemicals were obtained from Sigma. Cytochalasin D stock, 1 mg/ml in DMSO, was stored at -20°C. LY294002 (BioMol) stock, 100mM in DMSO, was stored at -80°C. EGF (Upstate Biotechnology) stock, 100µg/ml in water, was stored at -20°C.

Cell culture and bacterial strains

HeLa cells, salmonella typhimurium 1344 wild type and the SB111/pRI203, A1A1 and E12A2 mutants were as described previously (17,18)

Plasmid construction and generation of S. typhimurium sigD mutant

To construct the sigD mutant (missing amino acids 34-465), we used the primers D1 (5'-GCG AAT TCT ATC TGT TCA AGC ATG-3) and D2 (5' -GTC GAC TGA GAG AAT CTG CAT TCC-3) to amplify by PCR a 514 base pair fragment including the region upstream of sigD and the first 33 codons of the gene. The primers D3 (5'-GTC GAC AAA GAT CGT ACA GGG ATG-3') and D4 (5'-CTG CAA AGT CAG GAT GTC GTC AGG-3') were used to amplify a 484 bp fragment including the last 99 codons of sigD and the downstream region. PCR products were confirmed by sequencing. These two PCR products were fused at a SalI site (underlined in D2 and D3) and ligated into pCRTOPO2.1 (Invitrogen). The resulting deletion cassette was released by digestion with SacI and XbaI, ligated into the corresponding sites of the positive selection suicide vector pRE112 (CmR) and transformed into SY327 λpir (19). The SL1344 sigD deletion mutant (sigD) was then constructed by allelic exchange as described (20).

The pMWDE plasmid was constructed by amplification of the sigDE operon starting 46 bp upstream of the ATG start codon (includes the ribosome binding site but not the sigD promoter)
using the primers D5 (5’-TGT GGA TCC TGT TGA ATG TTC CCA C-3’) and D6 (see above), and inserted as a BamHI-EcoRI fragment into pMW119 (AmpR) (Nippon Gene Co, Ltd., Japan). The resulting plasmid, pMWDE, expresses the sigDE open reading frame under the control of the lacZ promoter. The C462S SigD mutant was constructed by site-directed mutagenesis converting TGT (C462) to TCG (S462) and creating an EcoRI site. Primer SIGD-EV (5’-AAA AGA TAT CTT TCC CAG TGC TTA TG-3’) and primer C462S-Rev (5’-ATT CGA ATT CCA GGC GGG TAC CG-3’) were used to amplify a 780 bp fragment including the C462 codon. Primer C462S-For (5’-CTG GAA TTC GAA AAG CGG CAA AGA TCG-3’) and primer SIGD-StuI (5’-TCG AGG CCT AAC GCG TCA TAT AAA C-3’) were used to amplify a 300 bp fragment including the C462 codon and the downstream region. These two fragments were inserted into pBluescript SKII (+) (Stratagene) and ligated at the new EcoRI site. The resulting plasmid contains part of the sigD ORF from the EcoRV site to the StuI site in sigE. The corresponding fragment of pMWDE was removed and replaced with the mutated fragment, producing pMWDE*.

**Bacterial infection of eukaryotic cells**

Bacteria were grown in Luria-Bertani (LB) broth overnight at 37°C with shaking. Overnight cultures were subcultured at a dilution of 1:33 in fresh LB and incubated at 37°C with shaking for a further 3 h. The culture was centrifuged at 1,000 g for 2 min at room temperature and then directly resuspended in phosphate buffered saline (PBS). Invasion was initiated by the addition of these bacteria directly to cultured cells. Cells were then incubated at 37°C in 5% CO₂ for 5 to 20 min as indicated, free bacteria were then removed by washing with PBS. In experiments requiring longer infection times, the culture media was supplemented with 50 µg/ml gentamicin at 20 min post-infection to kill extracellular bacteria. Invasion assays were carried out as
previously described (21).

**Immunoblotting**

Cell extracts were prepared by adding 80 µl of hot SDS sample buffer containing 50 mM DTT directly to the monolayer in 35 mm dishes. Samples were boiled for 5 min and proteins separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. Blots were blocked in Tris-buffered saline (TBS; 20 mM Tris-HCl pH 7.6, 0.14 M NaCl) containing 0.1% (v/v) Tween-20 and 5% skim milk powder (Carnation). Primary antibodies were used at 1:1,000 dilution (rabbit anti-PKB, rabbit anti-PKB-Ser473 and rabbit anti-PKB-Thr308 from New England Biolabs). HRP-conjugated goat anti-rabbit secondary antibodies (Jackson Laboratories) were used at 1:5,000. The PhotoTope®-HRP detection system (NEB) was used according to the manufacturer’s directions.

**Akt kinase assay**

Subconfluent HeLa cells in 60 mm dishes (Nunc) were incubated in serum-free media for 3 h. Bacteria (H200 cfu/cell) were added for 10 or 20 min as indicated. Cells were placed on ice and 1 ml of ice cold solubilization buffer was added (50 mM Tris-HCl, pH 7.7, 0.5% (v/v) Nonidet P-40, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na$_3$VO$_4$, 1 mM Na$_3$MoO$_4$, 1 µg/ml microcystin-LR, 0.25 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin, 0.5 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor). Nuclei and bacteria were removed by centrifugation (20,000 X g, 1 min) and supernatants were incubated with 2 µg anti-Akt antibody (Upstate Biotechnology) at 4°C for 1 h, with continuous mixing. Protein-G sepharose beads (20 µl) (Pharmacia) were then added and the samples incubated for a further hour. Beads were washed once with solubilization buffer, twice with solubilization buffer containing 500 mM NaCl and once with kinase buffer (20
mM Hepes, pH 7.2, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.25 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 0.5 µg/ml leupeptin). The beads were then resuspended in 25 µl kinase buffer containing 60 µM Crosstide (Upstate Biotechnology). ATP solution (5 µl) [200 µM ATP, 10 µCi ³²P-γ-ATP (Amersham) in kinase buffer] was added, followed by incubation at 30°C for 15 min. Reactions were stopped by spotting 20 µl onto 2 cm² pieces of P81 filter paper (Whatman), followed by extensive washing in 1% (v/v) phosphoric acid. Measurement of associated radioactivity was by liquid scintillation counting.

Immunofluorescence and confocal microscopy

HeLa cells grown on glass coverslips were transfected with GFP-Akt (22) using the TransIT-LT1 transfection reagent (Mirus Corp., Madison, WI) according to the manufacturer’s instructions. After transfection (18-24 hr), infection with bacteria was carried out as described above. Cells were then either fixed immediately or washed and incubated for a further 20 min (30 min time point). Fixation was in 2.5% formaldehyde for 10 min followed by extensive washing with PBS and permeabilization in PBS containing 10% (v/v) normal goat serum (Gibco BRL) and 0.1% (v/v) TX100 (TSB) for 10 min. Rabbit anti-LPS (Difco) diluted 1:400 in TSB was applied followed by goat anti-rabbit Alexa 594 (Molecular Probes) at 1:500 dilution. Antibodies were applied for 30 min each and after each incubation the monolayers were washed extensively with PBS, 0.1% TX100 (v/v). Coverslips were mounted onto glass slides using Mowiol (Calbiochem). Confocal microscopy was performed using a BioRad MRC 1024 laser scanning confocal microscope equipped with a Zeiss Axioplan upright microscope and a 63X oil immersion objective. Single optical sections through each cell were obtained in a 512 x 512 pixel format. Images were processed using Adobe Photoshop 5.5.
Results

*Salmonella typhimurium* induces phosphorylation of Akt in cultured epithelial cells

The activation state of Akt in infected epithelial cells was initially investigated by determining its phosphorylation state. HeLa cells were serum starved for 3 h and then infected with *S. typhimurium* for 2-10 min. After infection, the cells were washed to remove extracellular bacteria and incubated for varying times. Western blots were probed with phospho-specific antibodies recognizing either Thr^{308} or Ser^{473} phosphorylation of Akt. Phosphorylation on both residues was induced within 10 min of the addition of bacteria and peaked at approximately 60 min (Figure 1A and B). In comparison, activation of Akt by epidermal growth factor (EGF) produces a much more rapid and short lived phosphorylation response (Figure 1C).

Akt phosphorylation is PI3-K dependent and requires the SPI1 type III secretion system

To determine whether the observed Akt phosphorylation was PI3-K-dependent we used the PI3-K inhibitor LY294002 (LY29). Cells were pretreated for 20 min before infection and the drug was maintained throughout the course of the experiment. Ser^{473} phosphorylation was not detected in cells which had been pretreated with 40 µM LY29 and then infected with *S. typhimurium* for 20 min (Figure 2A). Similarly EGF-stimulated Akt Ser^{473} phosphorylation was completely abrogated by LY29 pretreatment (Figure 2B). These experiments indicate that Akt phosphorylation by *Salmonella* is PI3-K dependent.

The actin depolymerizing agent Cytochalasin D (CytoD) was used to inhibit *S. typhimurium* invasion of epithelial cells (23). Pretreatment of cells for 15 min with 1 µg/ml CytoD did not prevent *S. typhimurium* induced Akt phosphorylation (Figure 2C). Although the level of phosphorylation appears slightly reduced compared to that in untreated cells, this is not
proportional to the reduction in bacterial invasion. Under these conditions, invasion was less than 1% of that in cells without CytoD treatment (not shown). Thus *S. typhimurium*-induced Akt phosphorylation in epithelial cells does not require bacterial internalization.

We subsequently examined whether effector proteins secreted by the SPI1 TTSS of *S. typhimurium* were involved in Akt activation. A *S. typhimurium* strain with a nonpolar mutation in invA, a gene encoding an essential structural component of the SPI1 TTSS, was used (24). This invasion deficient strain has been complemented with the inv gene of *Yersinia pseudotuberculosis* to ensure the intimate interaction of bacteria with HeLa cells (17). Invasin is expressed on the bacterial surface of *Y. pseudotuberculosis*, or in this case the *S. typhimurium* SB111/pRI203 strain, and facilitates bacterial internalization by binding with high affinity to \(\beta_1\) integrin receptors (25). Under the conditions employed here, the mutant was internalized at approximately half the efficiency of the wild type bacteria. However, the mutant did not induce Akt phosphorylation even when extremely high inocula (320 cfu/cell) were used (Figure 2D). Thus Akt phosphorylation is dependent on one or more effectors secreted by the SPI1 TTSS.

**The SPI5 encoded type III effector SigD is required for Akt phosphorylation**

To identify effectors involved in Akt phosphorylation we screened several *S. typhimurium* mutants. These mutants were constructed by the random insertion of a promoterless luciferase gene cassette into the *S. typhimurium* chromosome, and had been selected in a previous screen for increased intracellular expression (18). One of these mutants, A1A1, was unable to induce Akt phosphorylation in HeLa cells (Figure 3A). In this mutant, the transposon is inserted into a previously described gene, *sigD* located in SPI5 the gene product of which, SigD, is translocated via the SPI1 TTSST (26). The effect of the *sigD* mutation is not polar, since the E12A2 mutant, in which the luciferase gene cassette is inserted in a downstream SPI5 gene, *pipB*, did induce Akt
phosphorylation (Figure 3A and B).

A non-polar sigD deletion mutant, S. typhimurium sigD, was constructed by removing 430 codons from the sigD open reading frame. Like the A1A1 strain, S. typhimurium sigD did not induce Akt phosphorylation (Figure 3C). Under the conditions used in this study, the invasion efficiency of the SigD mutants did not differ significantly from the wild type S. typhimurium (not shown). Transcomplementation of the A1A1 and sigD mutants with a plasmid (pMWDE) encoding the sigD and sigE genes under the control of the lacZ promoter, rescued the ability to phosphorylate Akt ("DE" in Figure 3C). The sigE (pipC) gene was included in the pMWDE plasmid as it encodes a putative SigD chaperone which is essential for secretion of the effector (26).

**Akt kinase activity is increased in S. typhimurium infected cells in a SigD-dependent manner**

Having defined the phosphorylation status of Akt, we next investigated whether the enzymatic activity of Akt was increased by S. typhimurium. HeLa cells were infected with bacteria and then solubilized in lysis buffer after which total Akt was immunoprecipitated using a polyclonal antibody that recognizes both phosphorylated and nonphosphorylated forms. Immunoprecipitates were incubated with a synthetic peptide (Crosstide), containing the consensus sequence surrounding Ser9 of GSK-3β, a known target of Akt, and 32P-γ-ATP. Infection with wild type S. typhimurium for 10 min increased Akt kinase activity by approximately 12-fold compared to uninfected cells. Increasing the infection time to 20 min lead to a further increase (40-fold) in Akt activity (Compare Figures 4A and B). These results correlate well with the phosphorylation kinetics shown in Figure 1. Stimulation of kinase activity was almost completely abolished by the PI3-K inhibitor LY29 and was comparable to the level of stimulation obtained with EGF. Furthermore, no significant Akt activity was detected when HeLa cells were infected with either
the SPI1 TTSS mutant (SB111/pRI203) or with the sigD strain. Complementation of the sigD strain with pMWDE restored the bacterial stimulation of Akt activity (Figure 4A). These results demonstrate that S. typhimurium activates Akt and that this activation is SigD dependent.

SigD, and the S. dublin and Shigella flexneri homologues, SopB and IpgD respectively, all contain two inositol 4 phosphatase domains and a conserved cysteine residue in one of these domains is essential for the activity of mammalian inositol 4-phosphatases as well as in vitro phosphatase activity of SopB (16,27). To investigate whether this residue is also required for SigD-induced Akt phosphorylation/activation, we constructed the equivalent mutant (C462S), designated pMWDE*. When this plasmid was used to complement the sigD strain, no Akt activation was detected (Figure 4B) and neither Ser473 or Thr308 phosphorylation was induced (Figure 4C). Thus, a motif common to 4-phosphatases is required for Akt phosphorylation and activation by SigD.

**PI3-K activation and rapid translocation of Akt to the plasma membrane are independent of SigD**

Since LY29 inhibited Akt activation, the association of PI3-K with tyrosine phosphorylated proteins was examined. Such interactions lead to the activation of PI3-K by localization with its substrates at the plasma membrane and through allosteric modifications (28). Immunoprecipitation with anti-phosphotyrosine antibody showed that the p85 subunit of class 1α-PI3-K was recruited to tyrosine-phosphorylated proteins during infection with either wild type or sigD strains (Figure 4D). This demonstrates that while Salmonella invasion does activate PI3-K, this activation is independent of SigD.

Having shown that SigD is essential for the phosphorylation and activation of Akt in epithelial cells, we also wished to define the role of SigD in the membrane recruitment of Akt. The
localization of Akt in infected epithelial cells was studied using confocal microscopy and GFP-Akt (22). HeLa cells were transfected with the GFP-Akt plasmid and then infected with S. typhimurium. Localized translocation of Akt in the vicinity of both S. typhimurium wild type and ΔsigD mutant was clearly evident within 2 min of adding bacteria (Figure 5A and B). At this time point extensive membrane ruffling, characteristic of Salmonella invasion, is evident where the bacteria are in contact with the host cell (29). Bacterial-induced translocation of Akt is still evident 30 min post-infection, which is similar to the localization pattern induced by cell-cell or cell-matrix contact in epithelial cells (22). These results show that Akt is translocated to membrane ruffles induced by Salmonella and, furthermore, that this translocation is independent of SigD.
Discussion

The remarkable ability of pathogens such as Salmonella to intricately interact with host cell signalling pathways is key to their survival. Defining the nature of these interactions is not only important for deciphering the role of bacterial effectors, but can also provide vital information on the intricate signalling cascades present in eukaryotic cells. One of the central regulators of eukaryotic cellular survival is the proto-oncogene Akt. We have shown conclusively that the S. typhimurium SPI1 TTSS translocated effector induces Akt activation. Intriguingly both wild type Salmonella typhimurium and a mutant which does not express SigD, stimulate both the translocation of Akt to sites of Salmonella-induced membrane ruffles and the association of PI3-K with phospho-tyrosine containing proteins. Thus another bacterial factor must be involved in the initial activation of the PI3-K/Akt pathway upstream of the actions of SigD. Our data indicate that SigD is required for the phosphorylation and activation of Akt after it has been recruited to the cellular membrane. Previously it has been demonstrated that SopB, the Salmonella enterica serovar Dublin SigD ortholog (93% amino acid identity), is an inositol phosphatase. Furthermore, both SigD/SopB and the highly homologous S. flexneri effector IpgD contain two motifs also found in mammalian inositol 4-phosphatases (16). Mutation of a single conserved cysteine residue in one of these motifs abrogates inositol phosphatase activity in all cases (16), and we found that the equivalent SigD mutant does not induce Akt activation. However, recombinant SopB has broad substrate specificity and dephosphorylates several inositol phosphatases and phosphatidylinositol phosphates including PIP3 and PI(3,4)P2 (16). Moreover, the recombinant protein hydrolysed phosphates at the C-3, -4, -5 and -6 positions. The localization of Akt to Salmonella-induced ruffles indicates that there are high concentrations of PIP3 and/or PI(3,4)P2 in the membrane at these sites (22). Thus, in vivo, the

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increased local concentration of 3’ phosphoinositides may be an important determinant for SigD activity and/or substrate specificity especially as translocated SopB/SigD appears to associate with cellular membranes (30). Identification of the substrates of SopB/SigD in vivo will help define the roles of PIP_3 and PI(3,4)P_2 in Akt activation, which to date are unclear (28,31-33).

It is well documented that Akt is a general mediator of survival signals and that Akt is both necessary and sufficient for survival of eukaryotic cells (for review see(34)). Thus it is possible that Salmonella-induced activation of Akt in epithelial cells would increase host cell survival, perhaps allowing the pathogen a greater intracellular time frame within which to replicate. Anti-apoptotic mechanisms are employed by at least two intracellular bacterial pathogens Chlamydia trachomatis (35) and Rickettsia rickettsii (36), although the bacterial factors mediating these processes have not been identified. In macrophages, Salmonella and other bacterial pathogens including Shigella, specifically induce apoptosis (12,37). The S. typhimurium TTSS effector, SipB, cleaves caspase-1 leading to rapid induction of the apoptotic cascade in macrophages but not in non phagocytic cells (12). This dichotomy may reflect the specific roles played by different cell types in host cell defense, giving those bacteria which can induce apoptosis in macrophages a distinct advantage in the establishment of infection (38).

While SigD does not appear to be required for virulence in a mouse model for typhoid fever (18,26), it is required for both increased fluid secretion and recruitment of subepithelial neutrophils, which correlates with an increase in intracellular Ins(1,3,4,5)P_4, in a ligated bovine intestinal loop model of diarrhea (16,30,39). The transcription factor NF-κB, which is activated by S. typhimurium, is an important mediator of inflammation in the intestinal epithelium, and also transmits both pro- and anti-apoptotic signals (40-42). Since Akt can regulate the transcriptional activity of NF-κB (43-45), SigD may also regulate NF-κB induction in
epithelial cells.

Although several bacterial effectors have shown to interact with eukaryotic signalling molecules this is the first time that one has been shown to activate the Akt pro-survival pathway. While the effects of this activation in pathogenesis remain undefined it is certain that, by targeting this one central mediator of signal transduction, *Salmonella* have greatly expanded their access to potential cellular targets.
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Figure Legends

1. *S. typhimurium* induces Akt phosphorylation in HeLa cells. Cells were incubated with *S. typhimurium* for 10 min, after which extracellular bacteria were removed by washing and the cells further incubated as indicated. Western blot analysis was with phospho-specific antibodies (upper panels) after which the blots were stripped and probed with anti-Akt to detect total Akt (lower panels). (A) Induction of Akt Ser\(^{473}\) phosphorylation. (B) Induction of Akt Thr\(^{308}\) phosphorylation. (C) Induction of Akt Ser\(^{473}\) phosphorylation in cells stimulated with EGF (100 ng/ml) for 2 min. Molecular weight standards (kDa) are indicated.

2. Characterization of requirements for Akt phosphorylation in *S. typhimurium* infected HeLa cells. Cells were infected with *S. typhimurium* as described. Western blot analysis was performed as described in Figure 1. (A) Akt phosphorylation is PI3-K dependent. Cells were treated with 20 µM LY29 for 15 min, then infected with *S. typhimurium* for 20 min. (B) EGF stimulation of Akt. Cells were treated with 20 µM LY29 for 15 min, then treated for 2 min with EGF (100 ng/ml). (C) Bacterial internalization is not required for Akt phosphorylation. Cells were treated with CytoD for 15 min, then infected with *S. typhimurium* for 20 min. (D) The SPI1 TTSS is required for Akt phosphorylation. Cells were infected with wild type *S. typhimurium* or the SB111/pRI203 mutant strain for 20 min.

3. Akt phosphorylation is dependent on the *S. typhimurium* type III secreted effector protein SigD. (A) Comparison of the ability of two type III effector mutants to induce Akt activation. Cells were infected with *S. typhimurium* wild type or the mutant strains for 20 min. (B) Organization of genes, and position of the A1A1 and E12A2 insertions, in the *sigDE* operon of *S. typhimurium*. (C) Complementation of mutant strains with the plasmid pMWDE. The
A1A1 and *sigD* mutants do not induce Akt phosphorylation in HeLa cells unless transcomplemented with pMWDE (DE). pMW119 vector alone (V).

4. **Akt kinase activity is stimulated in *S. typhimurium* infected HeLa cells and is dependent on the presence of SigD.** (A) Cells were infected for 10 min with *S. typhimurium* wild type (wt), a SPI1 mutant (*SB111/pRI203*), *sigD*, or *sigD* complemented with either the pMW119 vector alone or pMWDE. The cells were then lysed and immunoprecipitated with anti-Akt antibodies. Kinase activity was measured by incubating the immunoprecipitated protein with Cross tide and $^{32}$P-$\gamma$-ATP. The results are shown as the fold stimulation of Akt kinase activity relative to uninfected cells and are the mean ± SEM from three experiments. (B) SigD, with a single point mutation (C462S)(pMWDE*), does not complement the *sigD* mutant. Cells were infected for 20 min, or treated with EGF (100 ng/ml) for 2 min. Thereafter the cells were lysed and kinase activity measured as above. Mean ± SEM from three experiments. (C) SigD, with a single point mutation (C462S), does not induce Akt phosphorylation. Cells were infected for 20 min and the Akt phosphorylation state was analyzed using phospho-specific antibodies. (D) PI3-K is translocated to membranes in infected cells independently of SigD. Cells were infected for 20 min with *S. typhimurium* wild type (wt) or the *sigD* strain. Alternatively they were treated with EGF (100 ng/ml) for 2 min. Immunoprecipitation was performed using anti-phosphotyrosine antibodies and the immunoprecipitated p85 subunit of PI3-K was detected on blots using anti-p85 antibodies.

5. **Both *S. typhimurium* wild type and *sigD* induce translocation of Akt to the plasma membrane.**

HeLa cells transfected with GFP-Akt were infected with bacteria as indicated. Cells were then fixed and processed for confocal microscopy using anti-LPS antibodies followed by Alexa 594 conjugated secondary antibodies to localize the bacteria. Shown in the main
panels (A-F) are two colour overlays obtained by merging the single colour images from the red (A'-F', bacteria) and green (A''-F'', GFP-Akt) channels. Arrows indicate areas of Akt accumulation. Representative cells from a single experiment are shown and the experiment was repeated four times with comparable results. Bar = 10µm.
Figure 1

A

Uninfected

S. typhimurium

| Time (min) | Uninfected | 2 | 5 | 10 | 20 | 30 | 60 | 90 | 120 | 150 |
|------------|------------|---|---|----|----|----|----|----|-----|-----|
| Ser473     |            |   |   |    |    |    |    |    |     |     |
| Akt        |            |   |   |    |    |    |    |    |     |     |

B

Uninfected

S. typhimurium

| Time (min) | Uninfected | 2 | 5 | 10 | 20 | 30 | 60 | 90 | 120 | 150 |
|------------|------------|---|---|----|----|----|----|----|-----|-----|
| Thr308     |            |   |   |    |    |    |    |    |     |     |
| Akt        |            |   |   |    |    |    |    |    |     |     |

C

Untreated

EGF

| Concentration (ng/mL) | Uninfected | 1 | 2 | 5 | 10 | 20 |
|-----------------------|------------|---|---|---|----|----|
| Ser473                |            |   |   |    |    |    |
| Akt                   |            |   |   |    |    |    |
Figure 3

A

|                | uninfected | wt | E12A2   | A1A1   |
|----------------|------------|----|---------|--------|
|                |            |    | 60      | 120    | 240    |
|                |            |    | cfu/cell|        |

Ser473

B

A1A1

|                | sigD (sopB) | sigE (pipC) | pipB |
|----------------|-------------|-------------|------|

E12A2

C

|                | c | wt | A1A1 | ΔsigD |
|----------------|---|----|------|-------|
|                |   |    | -    |       |
|                |   |    | V    | V     |
|                |   |    | DE   | DE    |

Ser473

Akt
Figure 4

A

Akt activity (fold stimulation)

|                | 10 min       | 20 min       |
|----------------|--------------|--------------|
| uninfected     | <1           | <1           |
| wt             | 10 ± 2       | 70 ± 10      |
| wt + LY        | 12 ± 2       | 80 ± 15      |
| SB111/pRI203   | 5 ± 1        | 40 ± 5       |
| ΔsigD/pMW119   | 3 ± 0.5      | 20 ± 4       |
| ΔsigD/pMWDE    | 15 ± 3       | 75 ± 20      |

B

Akt activity (fold stimulation)

|                | 20 min       |
|----------------|--------------|
| uninfected     | <1           |
| wt             | 10 ± 2       |
| ΔsigD/pMWDE    | 50 ± 10      |
| ΔsigD/pMWDE+   | 80 ± 15      |
| EGF            | 25 ± 5       |

C

Time (min): 15, 30, 60

Akt phosphorylation levels:

- Ser473
- Thr308
- Akt

D

IP: α-phos tyr
WB: α-p85

|          | EGF | wt (no Ab) | uninfected | ΔsigD |
|----------|-----|------------|------------|-------|
| 105      |     |            |            |       |
| 76       |     |            |            |       |
| 57       |     |            |            |       |
Activation of Akt/protein kinase B in epithelial cells by the salmonella typhimurium effector SigD
Olivia Steele-Mortimer, Leigh A. Knodler, Sandra L. Marcus, Michael P. Scheid, Benjamin Goh, Cheryl G. Pfeifer, Vincent Duronio and B. Brett Finlay

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