The Annexin A2/p11 complex is required for efficient invasion of Salmonella Typhimurium in epithelial cells

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Summary

The facultative intracellular pathogen, Salmonella enterica, triggers its own uptake into non-phagocytic epithelial cells. Invasion is dependent on a type 3 secretion system (T3SS), which delivers a cohort of effector proteins across the plasma membrane where they induce dynamic actin-driven ruffling of the membrane and ultimately, internalization of the bacteria into a modified phagosome. In eukaryotic cells, the calcium- and phospholipid-binding protein Annexin A2 (AnxA2) functions as a platform for actin remodelling in the vicinity of dynamic cellular membranes. AnxA2 is mostly found in a stable heterotetramer, with p11, which can interact with other proteins such as the giant phosphoprotein AHNAK. We show here that AnxA2, p11 and AHNAK are required for T3SS-mediated Salmonella invasion of cultured epithelial cells and that the T3SS effector SopB is required for recruitment of AnxA2 and AHNAK to Salmonella invasion sites. Altogether this work shows that, in addition to targeting Rho-family GTPases, Salmonella can intersect the host cell actin pathway via AnxA2.

Introduction

A variety of bacterial pathogens invade, or form intimate attachment to, eukaryotic host cells by utilizing processes involving the actin cytoskeleton (Haglund and Welch, 2011). Some of these organisms, including Salmonella, enteropathogenic Escherichia coli (EPEC), entero-haemorrhagic E. coli (EHEC), Shigella and Chlamydia, use contact-dependent type 3 secretion systems (T3SS) to inject bacterial effector proteins into the host cell where they cause extensive actin remodelling. EPEC and EHEC adhere to the surface of the cell, where they induce the formation of long-lived actin rich pedestals, in contrast Salmonella, Shigella and Chlamydia trigger the formation of short-lived actin-rich membrane ruffles and extensions that engulf the bacteria resulting in its internalization into a membrane-bound vacuole. T3SS effectors can modulate actin dynamics either directly, by binding actin, or indirectly by targeting host cell proteins, including the Rho-family GTPases, that regulate the actin cytoskeleton (Haglund and Welch, 2011).

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a common cause of gastroenteritis. This facultative intracellular pathogen readily invades cultured epithelial cells, by pathogen-induced plasma membrane ruffling, which results in the bacteria being internalized into the Salmonella-containing vacuole (SCV) (Galán, 2001). Membrane ruffling is induced by the cooperative activity of four effector proteins translocated by T3SS1, SopE/SopE2, SopB, SipA and SipC. There is some functional redundancy so that mutants lacking any one of these effectors only have minor defects in invasion. However, mutants lacking SopE/E2 and SopB are virtually unable to invade cultured epithelial cells (Zhou et al., 2001). SopE and SopE2 are guanine nucleotide exchange factors (GEFs), which induce membrane ruffling by activating the GTPases Cdc42 and Rac1 (Hardt et al., 1998; Stender et al., 2002; Patel and Galán, 2006; Burkinshaw et al., 2012). SopB interacts directly with Cdc42 (Alemán et al., 2005) but also activates an SH3-containing guanine nucleotide exchange factor (SGEF), an exchange factor for RhoG (Patel and Galán, 2006).

In addition to targeting the proteins involved in actin membrane dynamics Salmonella also targets membrane phospholipids. SopB (also known as SigD) is an inositol phosphatase, and can hydrolyse a variety of substrates including the membrane phospholipids phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], PI(4,5)P2 and PI(3,4)P2 (Norris et al., 1998; Marcus et al., 2001; Mason et al., 2007). During invasion of epithelial cells...
SopB phosphatase activity causes localized conversion of PI(4,5)P2 to PI(5)P (Terebiznik et al., 2002) as well as an increase in the amounts of PI(3,4)P2 in Salmonella-induced ruffles (Cooper et al., 2011). The effect of SopB phosphatase activity on protein recruitment to the ruffles has not been well studied. Two proteins have been shown to be recruited via a SopB-dependent process are the serine threonine kinase Akt and the Arf GEF Arno (Steele-Mortimer et al., 2000; Humphreys et al., 2012). Rapid and sustained phosphorylation of Akt is a hallmark of Salmonella invasion in epithelial cells and is dependent on SopB phosphatase activity (Steele-Mortimer et al., 2000).

One of the key players involved at the interface between actin and membrane dynamics in mammalian cells is Annexin 2 (AnxA2), a PI(4,5)P2-binding protein that is recruited to actin assembly sites at membranes (Rescher et al., 2004) and is involved in a variety of actindriven membrane processes such as phagocytosis, macropinocytosis, endocytosis, cell–cell adhesion, epithelial cell polarity and membrane ruffling (Hayes et al., 2009; Morel et al., 2009; Grieve et al., 2012). AnxA2 exists both as a monomer and in a stable heterotetramer consisting of two molecules each of AnxA2 and p11 (S100A10) (Hayes et al., 2006). The AnxA2/p11 complex has higher affinity for membranes than AnxA2 alone and is the predominant form of AnxA2 found at the plasma membrane of epithelial cells (Hayes et al., 2006). Several proteins have been shown to interact with the AnxA2/p11 complex including AHNAK, an exceptionally large protein (700 kDa) implicated in actin reorganization at membranes through the activation of small GTPases including Rac (Benaud et al., 2004; Lim et al., 2013). AHNAK is a substrate of Akt and its phosphorylation state appears to regulate its subcellular localization and perhaps function in epithelial cells (Sussman et al., 2001). In Salmonella-infected cells, AHNAK was identified as a potential Akt substrate that is phosphorylated via a SopB-dependent mechanism (Rogers et al., 2011).

Based on the above observations we hypothesized that the AnxA2/p11 complex and AHNAK could be important players in the actin-based membrane ruffles induced during Salmonella invasion. To investigate this we used cultured epithelial cells, HeLa and MDCK, that are readily invaded by Salmonella. Although HeLa cells are commonly used as a model for Salmonella–host cell interactions they are unable to polarize or form tight junctions, both functions that AnxA2 and AHNAK have been shown to participate in (Benaud et al., 2004; Grieve et al., 2012). We show that all three proteins are enriched at the Salmonella invasion site and are required for efficient Salmonella invasion in epithelial cells. Recruitment of AnxA2, and AHNAK where shown to be SopB-dependent. These findings indicate that the AnxA2/p11 complex is required for T3SS1-mediated invasion.

Results

Morphological analysis of Salmonella-induced ruffles in HeLa and MDCK cells

To investigate the possible role of the AnxA2/p11 complex in Salmonella invasion we started by selecting two cultured epithelial cell lines, HeLa and MDCK, which internalize S. Typhimurium via morphologically distinct ruffles. Cells were incubated with bacteria for 15 min then fixed and processed for scanning electron microscopy (Fig. 1). In MDCK cells the process is morphologically very similar to that described for macrophagosome formation, where cell-surface ruffles close first into open cups (ruffle closure) and then into discrete intracellular vesicles (cup closure) (Swanson, 2008). The Salmonella-induced ruffles in MDCK cells were distinguished by distinct radial symmetry and were usually formed around single bacteria. In contrast, HeLa cell ruffles were diffuse and asymmetrical and there are often multiple bacteria associated with each one.

To compare the recruitment of host cell proteins (actin, Rac1, tubulin) to Salmonella-induced ruffles in MDCK and HeLa cells the cells were infected for 15 min with S. Typhimurium and then fixed and processed for immunofluorescence. To visualize membrane ruffles the plasma membrane was stained with Alexa 647-conjugated wheat germ agglutinin (WGA). Polymerized actin and Rac1, but not tubulin, are recruited to ruffles in HeLa (Fig. 2A–C) and MDCK (Fig. 2D–F) cells. In agreement with the SEM data (Fig. 1), actin and Rac1 staining in Salmonella-infected MDCK cells revealed remarkable radial symmetry of ruffles, which had an average diameter of ∼8.6 μm (∼45 px) and a range of ∼3.8–13.3 μm (∼20–70 px). In contrast, ruffles in HeLa cells are diffuse and have no apparent symmetry.

To obtain a more complete assessment of protein enrichment at the Salmonella invasion site we developed a method to analyse the 3D confocal data set (z-series). Quantitative Spherical Enrichment Analysis (QSEA) assesses enrichment at the point of invasion by measuring mean pixel intensity in a 3D sphere with the bacteria at its centre and dividing this by the mean pixel intensity at 10 pseudo-random positions within the cell. For proteins enriched at the Salmonella invasion site the ratio should be >1.0. As proof of principle we used QSEA to assess enrichment for Rac1, actin and tubulin (Fig. 2G and H). As expected actin and Rac1 are enriched at Salmonella invasion sites in both MDCK (1.5 ± 0.1 and 2.2 ± 0.3 respectively) and HeLa cells (1.4 ± 0.2 and 1.7 ± 0.2 respectively). No enrichment was detected for tubulin in either MDCK (1.2 ± 0.1) or HeLa cells (1.0 ± 0.1). These results show that, although MDCK and HeLa cells have morphologically distinct ruffles, the recruitment of host cell proteins can be assessed in both by QSEA.
We next used immunofluorescence confocal microscopy followed by QSEA to investigate whether AnxA2, p11 and AHNAK are enriched at *Salmonella* invasion sites (Fig. 3). AnxA2 (2.8 ± 0.1), p11 (2.5 ± 0.2) and AHNAK (1.9 ± 0.3) are all enriched in MDCK cells with similar results being obtained in HeLa cells AnxA2 (3.0 ± 0.4), p11 (2.2 ± 0.4) and AHNAK (1.7 ± 0.1).

**AnxA2, p11 and AHNAK are required for efficient invasion by *Salmonella***

To determine whether AnxA2 and p11 are required for *Salmonella* invasion we infected cells that had been depleted of each of these proteins individually by siRNA (Fig. 4). While we were unable to get consistently efficient depletion in MDCK cells (not shown) in HeLa cells AnxA2 and p11 were reduced by over 80% (Fig. 4A). Depletion of either AnxA2 or p11 markedly reduced, but did not completely abrogate, invasion when assessed either by a gentamicin protection assay or by immunofluorescence microscopy (Fig. 4B and C).

The enormous size of AHNAK makes it difficult to assess protein levels by immunoblotting. However, it is possible to monitor depletion in individual cells by immunofluorescence microscopy (Fig. 5A and B). When a polyclonal anti-AHNAK antibody was used to stain AHNAK the mean integrated fluorescence intensity (arbitrary units) in control cells was 2.3 ± 0.8 per cell (range 1–5) compared with 1.3 ± 0.2 (range 0.4–6) in siRNA-depleted cells. Although AHNAK knock-down was incomplete *Salmonella* invasion was found to be reduced by 50 ± 4% using a gentamicin protection assay (Fig. 5C). Analysis of infected monolayers by immunofluorescence microscopy confirmed a requirement for AHNAK since bacteria were detected in only 9 ± 1% of AHNAK-depleted cells compared with 35 ± 10% of control cells (Fig. 5D). To validate these results in a knock-down system independent of siRNA, we used mouse embryonic fibroblasts (MEFs) generated from an AHNAK knockout (AHNAK−/−) mouse (Lee et al., 2008). By immunofluorescence microscopy *Salmonella* infected only 7 ± 2% of AHNAK−/− MEFs compared with 38 ± 2% of AHNAK+/+ MEFs (Fig. 5D), correlating very well with the siRNA depletion results.

**SopB phosphatase activity is required for enrichment of AnxA2, p11 and AHNAK at the *Salmonella* invasion site**

Having found that the AnxA2, p11 and AHNAK are recruited to the site of infection and are required for optimal invasion we hypothesized that one or more of the T3SS1 effector proteins must be involved in recruitment of these host cell proteins. We focused on the three effectors, SopB, SopE and SopE2, which induce ruffling without directly binding actin. We included highly homologous SopE and SopE2 effectors since they act as GEFs for the small GTPases Cdc42 and Rac1 (Bakshi et al., 2008).
Annexin A2 is involved in Salmonella invasion

Fig. 2. Quantification of host protein enrichment at the Salmonella invasion site.
A–F. HeLa (A–C) or MDCK (D–F) cells were incubated with mCherry-Salmonella (cyan) for 15 min then fixed and immunostained (green) for actin (A, D), Rac1 (B, E) or tubulin (C, F). The plasma membrane was stained with fluorescent WGA (red). Shown are representative confocal projections (scale bar = 10 μm). Salmonella entry sites (arrowheads) are enlarged in the insets (scale bar = 5 μm).
G and H. QSEA analysis of confocal image stacks was performed on at least 10 independent bacteria–host interactions in at least 10 infected cells and in each of three independent experiments, means ± SD.

2000; Friebel et al., 2001), and Rac1 has been shown to interact with AnxA2 (Hansen et al., 2002). In agreement with earlier observations (Zhou et al., 2001; Hänisch et al., 2011), isogenic mutants lacking either SopB (ΔsopB) or both SopE and SopE2 (ΔsopE/E2) invaded MDCK cells with approximately half the efficiency of WT bacteria while a mutant lacking all three effectors (ΔsopB/E/E2) was essentially non-invasive (Fig. 6). Scanning electron and fluorescence confocal microscopy of invasion sites revealed dramatic differences in ruffle morphology (Fig. 7). For these experiments bacteria were incubated with cells for 15 min before fixation so that different stages of ruffle formation could be represented depending on their longevity. The non-invasive ΔsopB/E/E2.
Annexin A2 is involved in Salmonella invasion

Fig. 3. AnxA2, p11 and AHNAK localize to the Salmonella invasion site.

A–F. HeLa (A–C) or MDCK (D–F) cells were incubated with mCherry-Salmonella (cyan) for 15 min then fixed and immunostained (green) for AnxA2 (A, D), p11 (B, E) or AHNAK (C, F). The plasma membrane was stained with fluorescent WGA (red). Shown are representative confocal projections (scale bar = 10 μm). Salmonella entry sites (arrowheads) are enlarged in the insets (scale bar = 5 μm).

G–J. QSEA analysis of confocal image stacks was performed on at least 10 independent bacteria–host interactions in at least 10 infected cells and in each of three independent experiments. The means ± SD for three independent experiments are shown (G, H) as well as the values for each individual ruffle in the three combined experiments (i, J).

$E/E2$ mutant made no ruffles as detected by either SEM or fluorescence microscopy. The ΔsopB and ΔsopE/E2 mutants both induced ruffles although they were morphologically distinct from the WT ruffles. Immunofluorescence microscopy showed that AnxA2 was prominently recruited at the sites of invasion although the staining pattern was distinct for the two mutants. The SopE/E2-dependent ruffles induced by the ΔsopB mutant appeared smaller and less distinct than the WT ruffles. In contrast the SopB-dependent ruffles induced by the ΔsopE/E2 mutant typically had a larger diameter than the WT and AnxA2 was found in larger ring like structures with bacteria often located at the edges rather than at the centre.

Because of the heterogenous size and morphology of ruffles induced by the ΔsopE/E2 mutant we were unable to use the QSEA method to measure enrichment of host cell proteins. However, we did compare enrichment of AnxA2, p11 or AHNAK in ruffles induced by the ΔsopB mutant (Fig. 8). AnxA2 enrichment at the Salmonella invasion site was reduced for the ΔsopB mutant (2.3 ± 0.3) compared with WT (3.4 ± 0.2) and complementation of the ΔsopB mutant with plasmid borne SopB (pWSKDE), but not the catalytically inactive SopB C460S (pWSKDE C460S), rescued enrichment (3.5 ± 0.5 versus 2.5 ± 0.4 respectively). Similarly, AHNAK enrichment was lower for the ΔsopB mutant (2.0 ± 0.2) compared with WT S. Typhimurium (2.8 ± 0.1) and could be rescued by complementation with plasmid borne SopB, but not the catalytically inactive C460S mutant (2.5 ± 0.1 versus 2.0 ± 0.22 respectively). The same trend was seen for p11, although without statistical significance. One concern was that these apparent differences in enrichment might be caused by differences in ruffle size since intensity is measured within a fixed 3D-volume (15-pixel radius from the bacteria). Indeed, analysis of the SEM images showed that WT ruffles were slightly larger (mean = 7.4 ± 2.1 μm, median = 7.1 μm, n = 28) than those induced by the ΔsopB mutant (mean = 5.7 ± 2.1 μm, median = 6.0 μm, n = 24) (Fig. S1). To determine whether this size difference could affect the QSEA analysis we repeated the analysis using smaller (5-pixel, 10-pixel) and larger (20-pixel) radii (Fig. S2). For AnxA2, no difference could be detected between the four strains tested when a 5-pixel radius was used; however, all the larger radii yielded very similar data. Analysis of p11 and AHNAK recruitment yielded similar results (not shown). Altogether these data suggest that, in MDCK cells, recruitment of AnxA2, p11 and AHNAK to the Salmonella invasion site is at least partially dependent on the phosphatase activity of SopB.

Akt activation is not required for recruitment of AnxA2/p11 and AHNAK to the Salmonella invasion site

The phosphatase activity of SopB is required for Akt phosphorylation in Salmonella-infected HeLa cells (Steele-Mortimer et al., 2000). To confirm that this is also true in MDCK cells we infected cells with S. Typhimurium for 15 min then assessed the phosphorylation state of Akt by immunoblot analysis. While serum starvation of MDCK cells did not completely deplete phospho-Akt, infection with WT S. Typhimurium, but not the ΔsopB mutant, increased the amount of phospho-Akt (Fig. S3). Complementation of the ΔsopB strain with plasmid borne SopB, but not the inactive C460S mutant, restored the ability of Salmonella to induce Akt phosphorylation. To investigate whether this SopB-dependent Akt phosphorylation in MDCK cells is required for enrichment of AnxA2 or AHNAK to the Salmonella invasion site we used the phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 (LY29). However, although LY29 efficiently inhibits SopB-dependent Akt phosphorylation in both HeLa (Cooper et al., 2011) and MDCK cells, it had no effect on the enrichment of AnxA2, p11 or AHNAK at Salmonella invasion sites (Fig. S4). Thus SopB-dependent enrichment of AnxA2 and AHNAK at the Salmonella invasion site is not dependent on Akt phosphorylation.

Discussion

AnxA2 is a key player in actin-based events occurring at or near the plasma membrane including clathrin-mediated budding, cell–cell adhesion and the formation of macropinosomes and phagosomes (Grieve et al., 2012). Although AnxA2 can bind actin and membranes alone it is primarily found in a stable heterotetramer, consisting of two molecules each of AnxA2 and the small-EF hand protein p11. The heterotetramer forms prior to recruitment to the cytoplasmic face of the plasma membrane and endocytic vacuoles, where it associates preferentially with areas enriched in cholesterol and PI(4,5)P2 (Osborn et al., 1988). At the plasma membrane AnxA2/p11 can associate with the giant phosphoprotein AHNAK and it has been proposed that

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AHNAK contribute to Salmonella invasion of epithelial cells. We used two epithelial cell models, HeLa and MDCK, in which the Salmonella-induced ruffles have different morphologies. The consistent morphology of ruffles in MDCK cells, where they exhibit radial symmetry, may make them a better system than HeLa cells, where there is no apparent symmetry, for looking at subtle defects in ruffle formation. This appears to be true where we could detect ruffle defects in the MDCK cells for the ΔsopB mutant that were not apparent in HeLa cells.

AnxA2 is localized at apical plasma membranes and the microvillar brush border of intestinal epithelial cells (Hansen et al., 2003; Danielsen et al., 2003), which are targeted by several enteric bacterial pathogens that use contact-dependent T3SS to subvert the actin cytoskeleton at their site of contact. EPEC and EHEC form actin pedestals, organized cytoskeletal structures containing filamentous actin, beneath sites of bacterial attachment. The AnxA2/p11 complex is recruited to the attachment sites of both EPEC and EHEC (Zobiack et al., 2002; Rescher et al., 2004; Miyahara et al., 2009; Munera et al., 2012) via a process dependent on the translocated T3SS effector Tir (Translocated intimin receptor). Tir, which is required for pedestal formation, binds directly to the C-terminal region of AnxA2 and recruits it to the site of adherence via an actin-independent process (Munera et al., 2012). Also associated with AnxA2 at the EPEC attachment site is NHERF2 (Na+/H+ exchange regulatory factor 2) and it has been proposed that AnxA2 and NHERF2 may form a scaffold complex involved in the retention and dissemination of other effectors at the attachment site (Munera et al., 2012).

A defining characteristic of annexins is their Ca	extsuperscript{2+}-regulated affinity for negatively charged membrane phospholipids, and AnxA2 binding to Pi(4,5)P2- and cholesterol-rich regions of the plasma membrane is at least partially regulated by Ca	extsuperscript{2+} ions (Gerke et al., 2005). What role this plays in Salmonella invasion is unclear. Both Salmonella and EPEC cause rapid increases in intracellular Ca	extsuperscript{2+} when they interact with epithelial cells but it has not been conclusively demonstrated that this is required for invasion or pedestal formation (Ruschkowski et al., 1992; Pace et al., 1993; Bain et al., 1998; Gewirtz et al., 2000; Brown et al., 2008; Figueiredo et al., 2009). Recent experiments with Shigella, which invades via a similar process to Salmonella, have shown that a localized, rather than global, Ca	extsuperscript{2+} response may be important in invasion (Tran Van Nhieu et al., 2013). Tran Van Nhieu and colleagues showed that the bacterially induced actin reorganization at the site of invasion can limit diffusion of small solutes leading to sustained local Ca	extsuperscript{2+} increases at the invasion site. These effects could not be attributed to any single

AHNAK may cooperate with AnxA2 in mediating cytoskeletal and membrane rearrangements (Benaud et al., 2004; Rezvanpour et al., 2011; Dempsey et al., 2012). Here we have shown that AnxA2, p11 and
effector but seem instead to be due to production of InsP$_3$ as a response to the T3SS translocator components. Further work will be required to determine whether similar localized Ca$^{2+}$ responses are important for *Salmonella* invasion, but it is tempting to imagine that this could play a role in AnxA2/p11 recruitment.

In addition to the AnxA2/p11 complex we found that the giant phosphoprotein AHNAK was recruited to ruffles and required for efficient invasion. Like AnxA2, AHNAK is expressed prominently at the apical plasma membrane of polarized epithelial cells (Benaud et al., 2004). However, although there is substantial evidence linking it to AnxA2/p11, its role in actin driven processes is not well understood. It has been implicated in plasma membrane repair mediated by exocytic vesicles known as enlargeosomes, although functional evidence is lacking (Lorusso et al., 2006). AHNAK can bind directly to the AnxA2/p11 heterotrimer, via interactions with both AnxA2 and p11 (Rezvanpour et al., 2011), and may act as a scaffolding protein linking actin remodelling and signalling pathways (Benaud et al., 2004; Lim et al., 2013). AHNAK has also been shown to be involved in cell migration, specifically in the formation of actin-based protrusions such as pseudopodia and lamellipodia, where it may function by activating small GTPases including Rac (Shankar et al., 2010; Lim et al., 2013). Which, if any, of these roles is involved in *Salmonella* invasion remains to be determined. Interestingly, AHNAK is also involved in invasion by *Chlamydia trachomatis* (Hower et al., 2009) another pathogen that uses a T3SS to invade epithelial cells.

**Fig. 5.** AHNAK is required for efficient *Salmonella* invasion.

A. Representative images of HeLa cells transfected with either non-targeting (NTP) or AHNAK siRNA. Cells were fixed and immunostained for AHNAK. The plasma membrane was stained with fluorescent WGA. Representative images are maximum z-projections of 0.2 µm sections (scale bar = 10 µm).

B. The intensity of AHNAK fluorescence per cell was measured from z-projections. Shown are the combined data from ≥ 10 fields of cells, each dot represents one cell, from three independent experiments with the mean indicated.

C and D. *Salmonella* invasion is decreased following AHNAK depletion in HeLa cells and in AHNAK−/− MEFs.

C. siRNA-treated HeLa cells or MEFs were infected with *Salmonella*, treated with gentamicin to kill extracellular bacteria then lysed at 1.5 h p.i. to recover the intracellular bacteria.

D. Cells were infected with mCherry-*Salmonella* for 15 min and then prepared for immunofluorescence microscopy. Extracellular bacteria were stained with anti-lipopolysaccharide antibodies in the absence of permeabilization.

C and D. Data are presented as the means ± SD from three independent experiments.
is appears that, like AnxA2, AHNAK may be targeted by multiple pathogens that form intimate interactions with polarized epithelial cells.

The *Salmonella* effector SopB is a bi-functional protein with an N-terminal Cdc42-binding domain and a C-terminal phosphoinositide phosphatase domain (Rodríguez-Escudero *et al.*, 2011). Here we have shown that SopB, together with the effector GEFs SopE and SopE2, is required for recruitment of AnxA2 and AHNAK to the site of invasion in MDCK cells. Although AHNAK has been identified as a SopB-dependent target of Akt kinase activity in infected cells (Rogers *et al.*, 2011) we did not find any requirement for Akt in recruitment of AnxA2/p11 and AHNAK and have not been able to detect SopB-dependent changes in the phosphorylation state of AHNAK (data not shown). AnxA2 could potentially be acting upstream of Akt, as has been shown in other systems (Su *et al.*, 2010; Kagawa *et al.*, 2012), and this together with the role of SopB phosphatase in recruitment of AnxA2 and AHNAK are subjects of our current research.

In summary, our findings demonstrate that AnxA2 and AHNAK are recruited to the *Salmonella* entry site in a SopB/SopE/SopE2-dependent manner and comprise an essential component of *Salmonella* invasion. We postulate that AHNAK and the AnxA2/p11 complex participate

![Fig. 6. Requirement for SopB and SopE/E2 in *Salmonella* invasion of MDCK cells. MDCK cells were infected with *Salmonella* strains as indicated, treated with gentamicin to kill extracellular bacteria, then lysed at 1.5 h p.i. to recover the intracellular bacteria. Serial dilutions were plated on LB-M agar to enumerate cfu. Data represent the means ± SD from three independent experiments.](image1)

![Fig. 7. The *Salmonella* effector proteins SopB and SopE/E2 are required for AnxA2 enrichment at the *Salmonella* invasion site. A. MDCK cells were incubated for 15 min with *Salmonella* strains as indicated then fixed and immunostained for LPS (red) and AnxA2 (grey scale). Nuclei were stained with DAPI (blue). Shown are representative fields of cells (scale bar = 20 μm) as well as enlarged individual ruffles (scale bar = 5 μm). B. Scanning electron microscopy images of MDCK cells infected for 15 min with WT *S.* Typhimurium. Representative ruffles are shown (scale bar = 2 μm).](image2)
in the reorganization of the actin cytoskeleton during *Salmonella* invasion but the exact mechanism remains to be elucidated. That *Salmonella*, EPEC and EHEC have all developed distinct mechanisms to harness the actin modulating activities of AnxA2 at the plasma membrane indicates that this is a critical step for each of these enteric pathogens. This highlights the remarkable adaptability of T3 effector proteins, which can manipulate host cell pathways via a variety of different mechanisms.

**Experimental procedures**

**Bacterial strains and growth conditions**

*Salmonella enterica* serovar Typhimurium strain SL1344 (Hoiseth and Stocker, 1981) was the wild-type strain used here and all other strains are isogenic to this strain. SL1344 constitutively expressing the mCherry fluorescent protein (Drecktrah et al., 2008), the ΔsopE/E2 mutant (Stender et al., 2002), the ΔsopE/E2/B mutant (Cooper et al., 2011), the ΔsopB-sigE mutant (Knodler et al., 2006) and ΔsopB-sigE mutant complemented with the pWSKDE plasmid (Knodler et al., 2009) were as previously described. Bacteria were routinely grown at 37°C in LB-Miller (LB-M) with streptomycin (100 μg ml⁻¹), kanamycin (50 μg ml⁻¹) or carbenicillin (50 μg ml⁻¹) when appropriate.

**Cell culture**

HeLa adenocarcinoma (ATCC #CCL-2) and Madin-Darby canine kidney (ATCC #CCL-34) epithelial cells were grown at 37°C in 5% CO₂ in Eagle’s minimum essential medium (Mediatech) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Life Technologies), 1 mM sodium pyruvate (Mediatech) and 2 mM L-glutamine (Mediatech). Primary mouse embryonic fibroblasts (MEFs) from AHNAK+/+ and AHNAK−/− C57BL/6 mice were provided by Dr Yun Soo Bae (Ewha Womans University, Seoul, Korea). Animal study protocols conformed to the Institutional Animal Care and Use Committee (IACUC) of Ewha Womans University (protocol number 2012-01-001) and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). MEFs were grown at 37°C in 5% CO₂ in Dulbecco’s modification of Eagle’s medium (Mediatech) supplemented with 10% (v/v) heat-inactivated fetal calf serum and 2 mM L-glutamine. Antibiotic-antimycotic [1% (v/v)] (Life Technologies) was added to the medium for the first two to four passages. MEFs were immortalized by serial passage as described previously (Xu, 2005). Briefly, 3–4 × 10⁵ cells were seeded in 25 cm² tissue culture flasks then incubated at 37°C in 5% CO₂. MEFs were passaged every fourth day until reaching passage 25–30, by which time they had reached a consistent growth rate.

**Scanning electron microscopy**

Samples were fixed in 2.5% glutaraldehyde (v/v) with 0.05 M sucrose in 0.1 M sodium cacodylate buffer for 2 h. Subsequent post-fixation with 1% OsO₄ was performed with microwave irradiation in a Pelco 3451 microwave processor (Ted Pella), in cycles of 2 min on–2 min off–2 min on at 250 W under 15 in.Hg vacuum. Specimens were dehydrated in a graded ethanol series for 1 min under vacuum. Samples were then dried in a Bal-Tec cpd 030 critical point drier (Leica). Cells were then coated with 75 Å of iridium in an IBS ion beam sputter (South Bay Technology). Samples were imaged on a Hitachi SU-8000 SEM (Hitachi).

**Quantification of bacterial invasion**

Cells were passaged 16–20 h prior to infection into 24-well tissue culture treated plates (HeLa and MDCK cells) or 24-well tissue culture treated plates coated with collagen type I (BD Biosciences) (MEFs). Bacteria were grown under conditions known to optimize T3SS1-dependent invasion (Steele-Mortimer, 2008). Briefly, an overnight culture of bacteria was subcultured in 10 ml of LB-Miller broth (1:33 dilution) without antibiotics, shaking, at 37°C for 3.5 h. Bacteria were pelleted at 8000 g for 2 min, resuspended in an equal volume of Hanks’ Balanced Salt Solution with Mg²⁺ and Ca²⁺ (HBSS⁺⁺, Mediatech), and used to inoculate monolayers in triplicate wells at an moi of ~50–100. Invasion was allowed to proceed for 10 min at 37°C in 5% CO₂.

Fig. 8. SopB phosphatase activity is required for AnxA2 and AHNAK enrichment at the *Salmonella* invasion site. Cells were incubated for 15 min with *Salmonella* strains as indicated then fixed and immunostained for AnxA2, p11 or AHNAK. The plasma membrane was stained with fluorescent WGA. QSEA of confocal image stacks was performed on at least 10 infected cells (at least 10 *Salmonella* invasion sites) in each of three independent experiments for AnxA2, p11 or AHNAK.
then extracellular bacteria were removed by washing three times with HBSS−. Infected cells were incubated in antibiotic-free growth medium until 30 min post invasion gentamicin (50 μg ml−1) was added for 1 h to kill any remaining extracellular bacteria. At 1.5 h post invasion, the monolayers were washed twice in HBSS− then lysed in phosphate-buffered saline (PBS) with 0.2% sodium deoxycholate and viable bacteria were enumerated by platting on LB-M agar.

The percentage of infected cells within the monolayer was determined by immunofluorescence microscopy. HeLa and MDCK cells were seeded on 12 mm glass coverslips in 24-well plates. MEFs were seeded in collagen-coated eight-well μ-Chamber slides (ibidi). Duplicate wells were infected with mCherry-Salmonella as described above. At 1.5 h post invasion, the monolayers were washed twice in PBS then fixed with 2.5% (w/v) paraformaldehyde in PBS for 10 min at room temperature. Extracellular bacteria were labelled by incubating with the monoclonal anti-S. Typhimurium LPS (clone 1E6, Meridian Life Science) followed by Alexa Fluor-488-conjugated goat anti-mouse IgG (Life Technologies) diluted in 10% normal goat serum (Life Technologies) in PBS. To label the plasma membrane, the cells were incubated with 1.0 μg ml−1 Alexa Fluor-647-conjugated wheat germ agglutinin (WGA) (Life Technologies) for 10 min then washed with PBS and fixed again. Coverslips were mounted onto glass slides with ProLong Gold antifade reagent (Life Technologies). For μ-Chamber slides Mowiol mounting medium was added directly into the chamber to form a layer over the cells. Confocal imaging was performed using a spinning disk confocal system (Malik-Kale et al., 2012) comprised of a CSU10 spinning disk confocal (Yokogawa) with a custom laser launch (Prairie Technologies) attached to a TI-E microscope base (Nikon Instruments) with imaging on a Cascade II:512 EM-CCD camera with a pixel resolution of 0.19 μm × 0.19 μm (Photometrics). Unless otherwise stated a 60×1.4 NA oil immersion objective (Nikon, Japan) was used. Confocal images shown in Fig. 7 were captured on a Carl Zeiss LSM 710 confocal laser-scanning microscope equipped with Plan APOCHROMAT 63×1.4 NA objective and assembled into flat maximum intensity projections using Zen 2008 software (Carl Zeiss Microlmaging).

Immunofluorescence microscopy of host cell protein enrichment at the S. Typhimurium invasion site

HeLa and MDCK cells were seeded on 12 mm glass coverslips in 24-well plates 16–20 h prior to infection. Duplicate wells were infected with mCherry-Salmonella for 15 min. Cells were washed once with PBS then fixed with 2.5% (w/v) paraformaldehyde for 10 min at room temperature. Cell membranes were stained with Alexa Fluor-647-conjugated WGA as described above and fixed again. After three washes with PBS, the cells were permeabilized for 15 min with 0.2% saponin (EMD Chemicals) plus 10% normal goat serum (Life Technologies) in PBS. Monolayers were incubated with rabbit anti-AHNAK (Dr Ken Fields, University of Miami), mouse anti-AnxA2 (clone 5/Annexin II; BD Biosciences), mouse anti-p11 (clone 148/Annexin II light chain; BD Biosciences) or mouse anti-Rac1 (clone 102; BD Biosciences) antibodies followed by Alexa Fluor-488 goat anti-rabbit or anti-mouse IgG (Life Technologies) antibodies. Tubulin and actin were detected using Alexa Fluor-488-conjugated anti-tubulin antibody or Alexa Fluor-488-conjugated phalloidin (both from Life Technologies). Coverslips were mounted onto glass slides with ProLong Gold antifade reagent (Life Technologies). Images were collected using the spinning disk confocal microscope with a 60×1.4 NA oil immersion objective.

Quantitative spherical analysis

Cells were infected with mCherry-Salmonella for 15 min and fixed in 2.5% PFA for 10 min at room temp. Prior to permeabilization, cells were stained with Alexa Flour-647 WGA followed by a second fixation, permeabilization and staining as above. Confocal data sets of cell associated Salmonella and the invasion site were collected with a 60×1.4NA objective at 0.2 μm spacing on a spinning disk confocal as described above. To assess enrichment of target proteins and structures to the Salmonella induced ruffle, ruffles were first identified by the WGA staining and its position approximated by the location of the associated bacteria. In cases where multiple bacteria were associated with a ruffle, all bacteria were treated as independent bacteria–host interactions, and multiple enrichment analyses were performed. The average intensity associated within 15 pixels of the bacteria ($T_B$) was divided by the average intensity associated within 15 pixels of 10 pseudo-random positions within the cell ($T_R$).

\[
Enrichment = \frac{T_B}{T_R}
\] (1)

$T_B$ and $T_R$ were calculated by a custom program, ‘Enrichment v0.95’ written for ImageJ (Schneider et al., 2012) and Fiji (Schindelin et al., 2012). An approximate bacteria centroid position as determined from the mCherry channel, \( Position(x, y, z) \) \(_{bacteria} \), was entered manually for calculation of \( T_B \). Random positions, \( \{ Position(x_r, y_r, z_r) \}_{random} \), for calculation of \( T_R \) were generated by a pseudo-random number generator at run time and checked for localization within the cell per a 2D region of interest, ROIcell, and above a minimum intensity, \( I_{threshold} \). Enrichment was determined by thresholding on intensity and/or structures within the cell and used consistently across experiments. To ensure the comparison of comparable volumes for \( T_B \) and \( T_R \) a minimum number of pixels for determining \( T_B \) was set at 50% of the pixels measured to determine \( T_B \). Additionally, the pixels used to determine \( T_B \) were non-overlapping with the pixels measured for determining \( T_B \). Pixels included in average calculations were required to be within 15 pixels of \( Position(x, y, z) \) \(_{bacteria} \) or \( Position(x_r, y_r, z_r) \) \(_{random} \), where pixels are treated as equidistant Euclidean points. Furthermore, to exclude pixels outside of the cell from the average calculations, pixels were required to be within ROIcell and greater than or equal to \( I_{threshold} \). Enrichment was calculated in an Excel (Microsoft) spreadsheet per Equation 1.

RNA interference

Expression of human AHNAK (Gene ID: 79026), Annexin A2 (Gene ID: 302) and p11 (Gene ID: 6281) was silenced using synthetic ON-TARGETplus SMARTpool siRNA (Thermo Fisher Scientific). ON-TARGETplus non-targeting pool was used as a negative control. HeLa cells in six-well tissue culture treated plates at 50–60% confluence were transfected on two consecutive days with 50 nM of siRNA using DharmaFECT 1 Transfection
Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Knock-down efficiency was assessed by immunofluorescence microscopy or by immunoblotting of whole-cell lysates at 72 h post transfection. To evaluate *Salmonella* invasion, the transfected HeLa cells were re-plated 48 h after the initial transfection then infected 24 h later.

**Analysis of AHNAK expression by microscopy**

Confocal image stacks were taken on the spinning disk confocal as described above with 0.2 μm spacing with a 40×/1.3NA objective (Nikon Instruments) and analysed in ImageJ. The integrated intensities per cell were determined from a summed z-projection of the confocal image stack.

**Immunoblotting**

For confirmation of siRNA knock-down monolayers of HeLa cells in six-well tissue culture plates were washed twice with PBS then lysed in RIPA Buffer (Sigma) plus Protease Inhibitor Cocktail Set III (EMD Chemicals) according to the manufacturer’s instructions. Protein quantification was performed using the DC Protein Assay (Bio-Rad). Whole-cell lysates were prepared in SDS-PAGE sample buffer then – 10 μg of each sample was separated by SDS-PAGE. For analysis of Akt phosphorylation, MDCK cells in six-well tissue culture plates were infected for 15 min then immediately solubilized in hot SDS-PAGE sample buffer as previously described (Steele-Mortimer et al., 2000). Proteins were transferred to nitrocellulose (Bio-Rad) then incubated in blocking buffer [5% non-fat dry milk, Tris-buffered saline, 0.1% (v/v) Tween-20] for 1 h at room temperature. Blots were incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Dilutions and antibodies were: rabbit polyclonal anti-actin (1:10 000; Bethyl Laboratories), mouse monoclonal anti-annexin II (1:5000; 3D5, Santa Cruz Biotechnology), mouse anti-p11 (1:1000; clone 148, BD Biosciences), rabbit anti-Akt (1:1000, Cell Signaling Technology) and rabbit anti-phospho Akt Ser 473 (1:1000, Cell Signaling Technology). Blots were washed in blocking buffer then incubated with affinity-purified horse anti-mouse IgG (H&L) or goat anti-rabbit IgG (H&L) conjugated to horseradish peroxidase (1:20 000; Cell Signaling Technology) in blocking buffer for 1 h at room temp. Blots were washed then developed using the SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer’s instructions.

**Statistical analyses**

Results are mean ± SD from at least three independent experiments. Data were analysed for statistical significance by a one-way analysis of variance (ANOVA) with Tukey’s post hoc test. Student’s t-test was used if only two conditions were being compared. A P-value of ≤ 0.05 was considered significant.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** *Salmonella* lacking SopB generate smaller plasma membrane ruffles at the *Salmonella* invasion site. MDCK cells were incubated for 15 min with WT *Salmonella* or the ΔsopB mutant then fixed and processed for scanning electron microscopy (SEM). The diameter of at least 20 *Salmonella*-induced plasma membrane ruffles was measured from the SEM images.

**Fig. S2.** AnxA2 enrichment at a 5-, 10-, 15- and 20-pixel radius from the *Salmonella* at the invasion site. MDCK cells were incubated for 15 min with *Salmonella* strains as indicated then fixed and immunostained for AnxA2. The plasma membrane was stained with fluorescent WGA. QSEA of confocal image stacks was performed on at least 10 infected cells (at least 10 *Salmonella* invasion sites) at a 5-, 10-, 15- and 20-pixel radius from the *Salmonella* in each of three independent experiments.

**Fig. S3.** SopB-dependent Akt phosphorylation in MDCK cells. Cells were infected with *Salmonella* then, at 1 h post infection, solubilized and processed for immunoblotting to detect phosphorylated (A) and total Akt (B). Blots were subsequently stripped and incubated with anti-actin antibodies for estimation of protein loading. Molecular weight markers are indicated on the left.

**Fig. S4.** Akt phosphorylation is not required for AnxA2, p11 and AHNAK enrichment at the *Salmonella* invasion site. A. *Salmonella*-induced Akt phosphorylation is inhibited by LY29. MDCK cells were pre-treated with LY29 for 30 min prior to infection with WT *S. Typhimurium*. At 1 h post infection monolayers were solubilized and processed for immunoblotting to detect phosphorylated (A) and total Akt (B). B. Enrichment of AnxA2, p11 and AHNAK at the *Salmonella* invasion site is not dependent upon Akt activation. The plasma membrane was stained with fluorescent WGA. Cells were immunostained for AnxA1, p11 or AHNAK. QSEA of confocal image stacks was performed on at least 10 infected cells (at least 10 *Salmonella* invasion sites) in each of three independent experiments.