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ORIGINAL ARTICLE

Salmonella type III effector SopB modulates host cell exocytosis

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Salmonella enterica pathogenesis is dependent on its ability to enter and replicate inside host cells. Replication occurs inside the *Salmonella*-containing vacuole (SCV), a vacuolar compartment that is modified by bacterial effectors secreted through the two type III secretion systems (T3SS-1 and T3SS-2). Type III effectors interact with the host cell endocytic pathway to aid replication. We investigated whether *Salmonella* effector proteins may also interact with the host's exocytic pathway. A secreted alkaline phosphatase (SEAP) assay indicated three *Salmonella* effectors inhibited the secretory pathway, although only *Salmonella* outer protein B (SopB) was confirmed to block exocytosis using a vesicular stomatitis virus glycoprotein-green fluorescent protein (VSVG-GFP) transport assay. The 4-phosphatase activity of SopB was crucial to its effect on exocytosis. The interaction with the secretory pathway could potentially be important for providing replicating *Salmonella* with nutrients, contributing membrane material necessary for SCV biogenesis, altering antibacterial peptide/protein secretion or manipulating cell surface proteins important in the host response to infection.
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INTRODUCTION

Salmonella enterica species are among one of the main bacterial causative agents of gastroenteritis in humans and animals, including livestock, and the cause of the systemic disease Typhoid fever. Recent *Salmonella* outbreaks in the United States were attributed to contaminated peanut butters, cantaloupes and raw vegetables. *Salmonella* is therefore a re-emerging zoonotic pathogen, with a considerable public health burden and economic cost to society. Consequently, much research has been conducted to understand *Salmonella* and its pathogenesis, and develop mechanisms by which to eliminate it and prevent transmission.

A characteristic of *Salmonella* infection is the uptake of the bacterium into non-phagocytic intestinal epithelial cells.[1,2] Genes within a region of the *Salmonella* chromosome known as *Salmonella* pathogenicity island-1 (SPI-1) encode a type three protein secretion system (T3SS-1),[3,4] which drives bacterial invasion. T3SS-1 translocates SPI-1-encoded effector proteins, and effectors encoded elsewhere in the *Salmonella* chromosome, into the host cell cytoplasm.[5] The coordinated actions of these effector proteins, including SipA, SipC, SopB/SigD, SopE, SopE2 and SptP, modulate the host cell actin cytoskeleton to promote internalisation of *Salmonella* into the non-phagocytic cell in a characteristic membrane 'ruffle'.[6–10]

Upon entrance to the host cell, *Salmonella* exists within a *Salmonella*-containing vacuole (SCV).[2] *Salmonella* successively modifies the SCV using effectors secreted by T3SS-1[11] and the *Salmonella* pathogenicity island-2 (SPI-2)-encoded type three secretion system (T3SS-2).[12] These effector proteins facilitate interactions of the SCV with the host cell endocytic pathway leading to maturation of the SCV,[13,14] and also serve to position the SCV towards the Golgi where replication is initiated.[15]

For successful invasion and replication inside host cells, *Salmonella* must spatially and temporally regulate its effector proteins. This is particularly important in the case of effectors, such as SopB, which perform more than one role. *Salmonella* outer protein B (SopB) is a phosphatidylinositol phosphatase,[6,16] being composed of a C-terminus that possesses 4-phosphatase motifs[16] and a synaptojanin-like 5-phosphatase domain.[17] SopB uses its phosphatidylinositol phosphatase activity to (i) activate the Rho GTPases RhoG and Rho to mediate actin-dependent and myosin II-dependent bacterial invasion respectively;[18,19] (ii) modulate the phosphatidylinositol composition of the plasma membrane to allow SCV formation;[20–22] (iii) modulate the phosphatidylinositol composition of the SCV to allow maturation, in part through the recruitment of host proteins such as Rab5 and Vps34[23] and sorting nexins-1 and sorting nexins-3,[24,25] and avoidance of SCV-lysosome fusion;[26] (iv) activate myosin II to place the SCV in a juxtanuclear position;[27] (v) activate serine protein kinase AKT to prevent host cell death via apoptosis;[28] and (vi) regulate host cell chloride channel function.[29–31]

The multiple roles of SopB are permitted by regulating its activity through its N-terminal domain and its half-life. Ubiquitination of the N-terminal leads to translocation of SopB from the plasma membrane to the SCV, potentially switching the role of SopB from invasion to intracellular survival.[32,33] SopB can also bind Cdc42 through its N-terminal,[34,35] and this too appears to be important for SopB localisation to the SCV and its spatial regulation.[32,35] Although, only translocated by T3SS-1,[36,37] SopB is detected in cells for up to 12 h post-invasion.[38] This relatively long half-life for an effector protein allows SopB to extend its role from the early stages of invasion through to the intracellular phase of *Salmonella* survival.

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Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
S. Typhimurium		
SL1344	Wild type	43, 44
ZP18	ΔssaV	22
SB923	ΔsopB	10
Plasmids		
pIRES2-EGFP		Clontech
pEGFP-N2		Clontech
pEX0322	pSEAP	Dr Zhao-Qing Luo
pEX0232	pEGFP-C1-Lpg2556	Dr Zhao-Qing Luo
pZP0029	pIRES2-*sopA*	This paper
pZP2137	pIRES2-*sopB*	This paper
pZP2138	pIRES2-*sopD*	This paper
pZP2157	pIRES2-*sopD2*	This paper
pZP2139	pIRES2-*sopE*	This paper
pZP2140	pIRES2-*sopE2*	This paper
pZP2136	pIRES2-*sipA*	This paper
pZP2141	pIRES2-*sptP*	This paper
pZP2142	pIRES2-*slrP*	This paper
pZP2150	pIRES2-*avrA*	This paper
pZP2159	pIRES2-*ssaB*	This paper
pZP2160	pIRES2-*sseA*	This paper
pZP2161	pIRES2-*sseB*	This paper
pZP2162	pIRES2-*sseC*	This paper
pZP2163	pIRES2-*sseD*	This paper
pZP2164	pIRES2-*sseE*	This paper
pZP2144	pIRES2-*sseF*	This paper
pZP2146	pIRES2-*sseG*	This paper
pZP2165	pIRES2-*sseI*	This paper
pZP2147	pIRES2-*sseJ*	This paper
pZP2166	pIRES2-*sseK*	This paper
pZP2167	pIRES2-*sseK2*	This paper
pZP2168	pIRES2-*sseK3*	This paper
pZP2169	pIRES2-*sseL*	This paper
pZP2148	pIRES2-*sifA*	This paper
pZP2149	pIRES2-*sifB*	This paper
pZP2171	pIRES2-*steA*	This paper
pZP2172	pIRES2-*steB*	This paper
pZP2173	pIRES2-*steC*	This paper
pZP2153	pIRES2-*pipA*	This paper
pZP2154	pIRES2-*pipB*	This paper
pZP2155	pIRES2-*pipB2*	This paper
pZP2156	pIRES2-*pipC*	This paper
pZP2151	pIRES2-*gogB*	This paper
pZP2152	pIRES2-*msgA*	This paper
pZP2243	pIRES2-*sspH1*	This paper
pZP2170	pIRES2-*sspH2*	This paper
pZP0074	pIRES2-*spvB*	This paper
pZP2158	pIRES2-*spvC*	This paper
pZP0211	pEGFP-N2-sopB	6
pZP0216	pEGFP-N2-sopB[C460S]	6
pZP2175	pEGFP-N2-sopB[R468A]	This paper
pZP2176	pEGFP-N2-sopB[C460S, R468A]	This paper
pZP2174	pEGFP-N2-sopB[K530A]	This paper
pZP2248	pEGFP-N2-sopB[C460S, K530A]	This paper
pZP02195	PI3K-RFP	Heather Piscatelli
pZP02194	PI4K IIβ-RFP	Heather Piscatelli
pZP02196	MTM1-RFP	Heather Piscatelli
pZP02197	PTEN-RFP	Heather Piscatelli
pZP02199	SKIP-GFP	Heather Piscatelli

As the SCV locates to a juxtanuclear position, close to the Golgi, it has been proposed that the SCV may also interact with the host's exocytic/secretory pathway,[15] as this occurs with several intracellular pathogens, e.g., *Legionella*, *Brucella* and *Chlamydia*.[39] Kuhle et al.[40] found that intracellular *Salmonella* could indeed recruit secretory vesicles from the trans-Golgi network (TGN) to the SCV in a SPI-2-dependent manner; the SPI-2-secreted effectors SseF, SseG and SifA play pivotal roles in post-Golgi vesicle recruitment.[40] The interaction of the SCV with the secretory pathway could potentially be important for providing replicating *Salmonella* with nutrients and/or to provide membrane material to the growing SCV. However, fusion between secretory vesicles and the SCV was not detected by Kuhle et al., although it has now been shown that secretory carrier membrane protein 3 (SCAMP3), which is typically located on the TGN, is also located on *Salmonella*-induced filaments and *Salmonella*-induced SCAMP3 tubules, dynamic structures which extend from the SCV membrane.[41] However, it is not yet clear how SCAMP3 is recruited to the SCV and whether the exocytic, endocytic or both pathways are used in this process. Additionally, it has been found that enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC), gastrointestinal pathogens that like *Salmonella* utilize a T3SS for pathogenesis, both reduce the efficiency of host cell secretion through effector protein Non-LEE-encoded effector A (NleA) which inhibits coat protein II complex (COPII)-dependent protein export from the endoplasmic reticulum.[42] Thus, interaction of bacterial pathogens, like *Salmonella*, with the host cell secretory system may not necessarily be important for maintenance of their replication niche inside the cell but by manipulating the cell's ability to secrete antibacterial peptides/proteins or by manipulating cell surface expression of proteins important in the host response to infection, be important for their survival once they leave the cell or to their extracellular counterparts.

This study examined how *Salmonella* may interact with the exocytic pathway during the intracellular phase of survival, specifically looking at whether any *Salmonella* type III effectors were capable of inhibiting secretion. Three *Salmonella* effectors were identified with the potential to inhibit the host cell secretory pathway. SopB, as one of these effectors, was studied in further detail to determine its mode of action.

MATERIALS AND METHODS

Bacterial strains
Bacterial strains are listed in Table 1.[10,22,43,44] *Salmonella* serovar Typhimurium strains were routinely cultured in Luria–Bertani broth. For invasion experiments overnight cultures of *Salmonella* were diluted 1:30 in Luria–Bertani broth with 0.3 M NaCl and grown for 3 h with aeration on a rotating wheel. Antibiotics were used at the following concentrations: ampicillin at 120 μg/mL, streptomycin at 25 μg/mL, kanamycin at 40 μg/mL.

Construction of plasmids
Plasmids used in this study are listed in Table 1. Each *Salmonella* effector gene was cloned into the eukaryotic expression vector pIRES2-EGFP either by subcloning the gene from another plasmid or by amplifying the gene from the *Salmonella* genome by polymerase chain reaction using Deep Vent DNA polymerase (NEB). All plasmids were verified by restriction digests and sequencing.

The plasmids carrying *sopB* mutations were created by performing site-directed mutagenesis using the Pfu Turbo DNA polymerase (Stratagene) as per manufacturer's instructions. The primer for creating the SopB R468A mutation was 5′ GTA AAA GCG GCA AAG ATC TGA CAG GGA TGA TGG ATT CAG AA and for the K530A

mutation 5′ GGG CGG GAA ACA AAG TAA TGG CCA ATT TAT CGC CA. All point mutations were verified by sequencing.

Mammalian cell culture and transfections
HeLa cells (CCL-2; ATCC, Manassas, VA, USA) and 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All cells were maintained at 37 ℃ and 5% CO_2. Cells were transfected with plasmids by using TransIT-express transfection reagent (Mirus) according to the manufacturer's instructions. 24 h after transfection, cells were infected with *Salmonella* as indicated.

Secreted alkaline phosphatase (SEAP) assay
293T cells were plated in 24-well dishes at 1×10^5 cells/well, allowing three wells per effector. After an overnight incubation, the cells were cotransfected with plasmids encoding corresponding proteins and the plasmid encoding the SEAP. 24 h later, cells were washed, and fresh serum-free tissue culture medium was added. SEAP activity was measured in triplicate wells 7 h later using the Phospha-Light System (Applied Biosciences, Grand Island, NY, USA) following the manufacturer's instructions. Data are presented as a secretion index, which is the ratio of SEAP activity detected in the culture medium to the cell-associated SEAP activity. Total SEAP inside cells was not affected by coexpression of any of the effector plasmids.

Vesicular stomatitis virus glycoprotein (VSVG) transport assay
293T cells were plated in 100 mm dishes at 6.5×10^5 cells/dish. After an overnight incubation, cells were cotransfected, using calcium phosphate, with plasmids encoding the indicated proteins and a plasmid encoding a ts045-VSVG-GFP plasmid. 4 h after transfection the cells were washed and placed overnight at 40 ℃. Next, 100 μg/mL cycloheximide was added to each dish which was then transferred to 32 ℃ for the appropriate time period. Cells were then transferred to ice, and washed twice with ice-cold PBS before being collected. Cells were lysed with lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 1:100 protease inhibitor cocktail (Calbiochem, Billerica, MA, USA)) on ice for 10 min, before the lysate was collected in an Eppendorf tube. After protein concentration determination using the Bradford assay (Bio-Rad, Hercules, CA, USA), equal amounts of protein from all samples were treated or mock-treated with Endoglycosidase H (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the samples, boiled for 10 min and subjected to electrophoresis on an 8% SDS–PAGE gel. Samples were transferred to nitrocellulose membrane for immunoblot analysis using anti-GFP and anti-actin antibodies.

Data analysis
All statistical tests were performed using GraphPad Prism version 6. One-way analysis of variance was performed with three or more data sets, with Dunnett's multiple comparison test being applied *post hoc*.

RESULTS
Salmonella effectors inhibit the host cell secretory pathway
To determine whether *Salmonella*, like EHEC and EPEC which also use a T3SS in their virulence, may inhibit the host cell exocytic pathway during the infection process, known *Salmonella* effector proteins were screened for inhibition of the secretory pathway. A plasmid encoding a secreted form of human placental alkaline phosphatase (SEAP) was cotransfected into 293T cells with a plasmid encoding a *Salmonella* effector protein. The ratio of SEAP activity detected in the culture medium to the cell-associated SEAP activity (secretion index) was then measured for each *Salmonella* effector and for a *Legionella* effector (Lpg2556) known to inhibit exocytosis and which acted as a positive control (Dr Zhao-Qing Luo, personal communication). Of the 30 *Salmonella* effectors tested, SopB, PipB2 and SspH2 appeared to affect the secretion of SEAP (Figure 1).

SopB requires its 4-phosphatase activity to inhibit the secretory pathway
Among the three *Salmonella* type III effectors tested, SopB appeared to be the most potent in inhibiting SEAP secretion. We decided to test whether the phosphatidylinositol phosphatase activity of SopB was required for this effect. Point mutations were made in catalytic residues known to be essential for the phosphatase activity of SopB. Mutation of the 420 cysteine (C420S; C/S) or mutation of the 468 arginine (R468A; R/A), both of which are conserved between *E. coli* and *Shigella* IpgD proteins and human inositol phosphatase sequences, have been shown to be essential for 4-phosphatase activity.[6,16] The lysine residue at position 530 lies within the putative synaptojanin-homology domain that is essential for the 5-phosphatase activity of SopB.[17] When examined in the SEAP secretion assay, mutation of either C420 or R468 restores SEAP secretion to levels comparable to the empty vector indicating the 4-phosphatase region but not the 5-phosphatase domain is necessary for SopB inhibition of exocytosis (Figure 2).

Role of phosphatidylinositol 4-phosphate (PI(4)P) in SopB-mediated inhibition of exocytosis
Since the 4-phosphatase activity of SopB appears critical to its ability to inhibit exocytosis, we investigated whether overexpression of specific phosphoinositide kinases or phosphatases could restore SopB-mediated inhibition of exocytosis, and thereby aid identification of the pathways that may be used to inhibit secretion. Cells were cotransfected with pSEAP, vector or SopB and then one of the phosphoinositide kinases or phosphatases listed (Figure 3). Exocytosis in cells coexpressing SopB and phosphatidylinositol 4-kinase type IIβ (PI4K IIβ) was not statistically different to the control cells. PI4K IIβ is a 4-kinase which appears to regulate the PI(4)P synthesis required for several cellular processes, most notably secretion.[45] Since SopB relies on its 4-phosphatase activity to inhibit exocytosis it may suggest 4-kinase activity can rescue the SopB-mediated defect. It is also interesting to note that PI4K IIβ significantly increases the secretion of cells transfected with the vector. Thus, it appears PI(4)P levels may be important in the control of exocytosis.

The phosphatidylinositol kinase PI3K had no affect on SopB-mediated inhibition, and therefore it is perhaps no surprise that PTEN, a 3-phosphatase, also had no affect since both enzymes play a role in the interconversion of phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$) and phosphatidylinositol (3,4,5)-triphosphate ($PI(3,4,5)P_3$). The PI(3)P phosphatase, MTMI (myotubularin I), and the PI(3)P inhibitors wortmannin and LY294002 were also found to have no effect (data not shown) and therefore PI(3)P does not appear to be involved in exocytosis inhibition which may be expected since PI(3)P is typically associated with early endosomes. The 5-phosphatase, skeletal muscle and kidney enriched inositol phosphatase (SKIP), appeared to in part restore exocytosis but the result was not statistically significant. Since one of the reactions catalyzed by SKIP is the conversion of $PI(4,5)P_2$ to PI(4)P
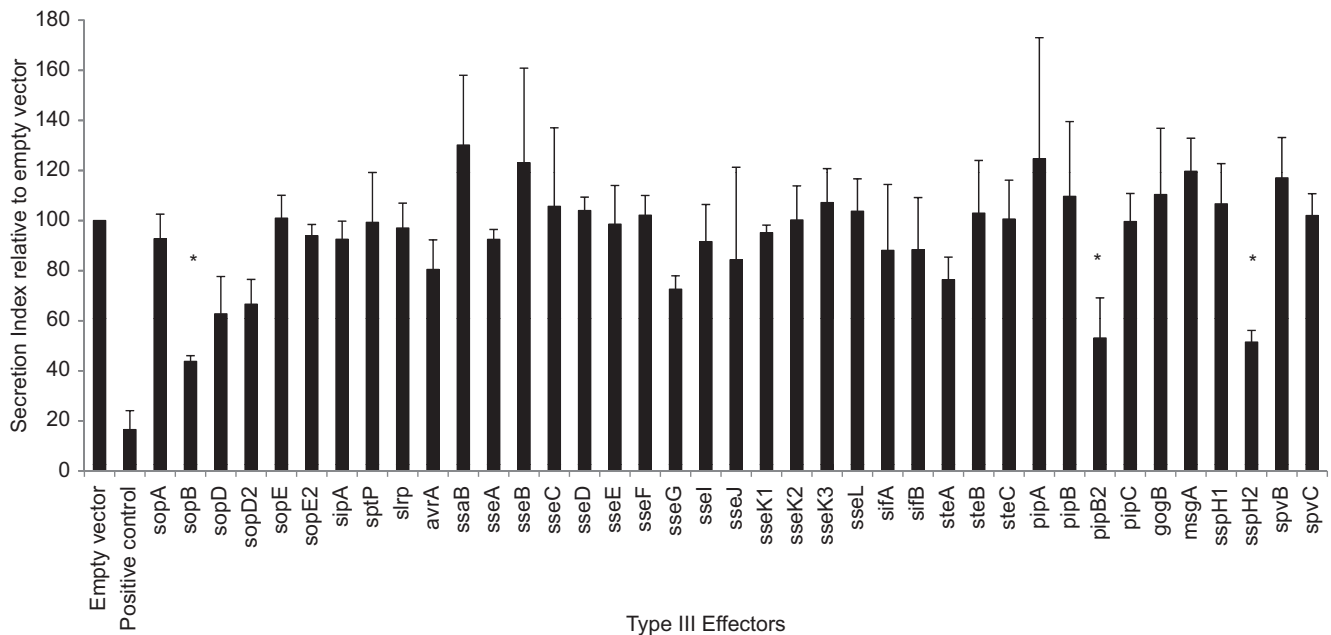
Figure 1 *Salmonella* effectors inhibit host cell exocytosis. A SEAP assay was performed on 293T cells transfected with plasmids encoding the indicated type III effectors. The protein secretion index is displayed. Values are the mean±SD for three independent experiments. Asterisks indicate a statistical significant difference compared to the empty vector.

and PI4K IIβ, which also had an effect in this assay, regulates PI(4)P, it is possible that PI(4)P is the key intermediate in SopB-mediated exocytosis inhibition. This is more probable given that PI(4)P is a prominent phosphatidylinositol in the Golgi membrane, particularly the TGN, and plays an important role in the secretory pathway.

As regulation of PI(4)P concentrations at the TGN are used to regulate secretion,[46] a FAPP1-PH-GFP probe, specific to PI(4)P,[47] was used in HeLa cells during *Salmonella* infection to determine whether PI(4)P levels were altered and may explain the effect of SopB. No change in PI(4)P levels were detected (data not shown).

However, since detecting phosphatidylinositols and their fluctuating concentrations is not always accurate by such probes, we cannot rule out that PI(4)P is one of the mediators of SopB secretion inhibition.

SopB delays VSVG-GFP between the endoplasmic reticulum (ER) and Golgi

To determine at which stage of the secretory pathway SopB may act, we employed a temperature-sensitive variant of vesicular stomatitis virus glycoprotein tagged with green fluorescent protein (ts045-VSVG-GFP). At 40 ℃, ts045-VSVG-GFP misfolds and is retained in the ER, but upon
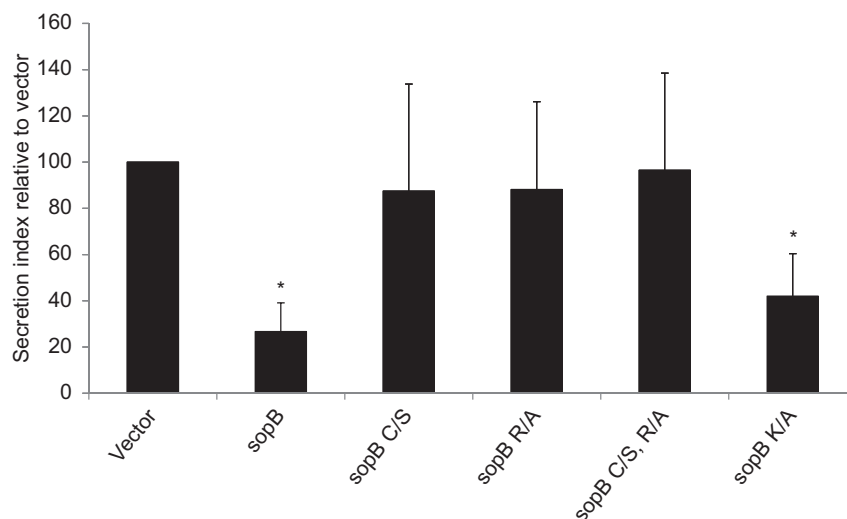

Figure 2 The 4-phosphatase activity is necessary for SopB inhibition of exocytosis. SEAP assays were performed on 293T cells transfected with pEGFP-N2 plasmids encoding the indicated forms of SopB. *sopB* mutation abbreviations: C460S (C/S), R468A (R/A), and K530A (K/A). The protein secretion index is displayed. Values are the mean±SD for three independent experiments. Asterisks indicate a statistical significant difference compared to vector.
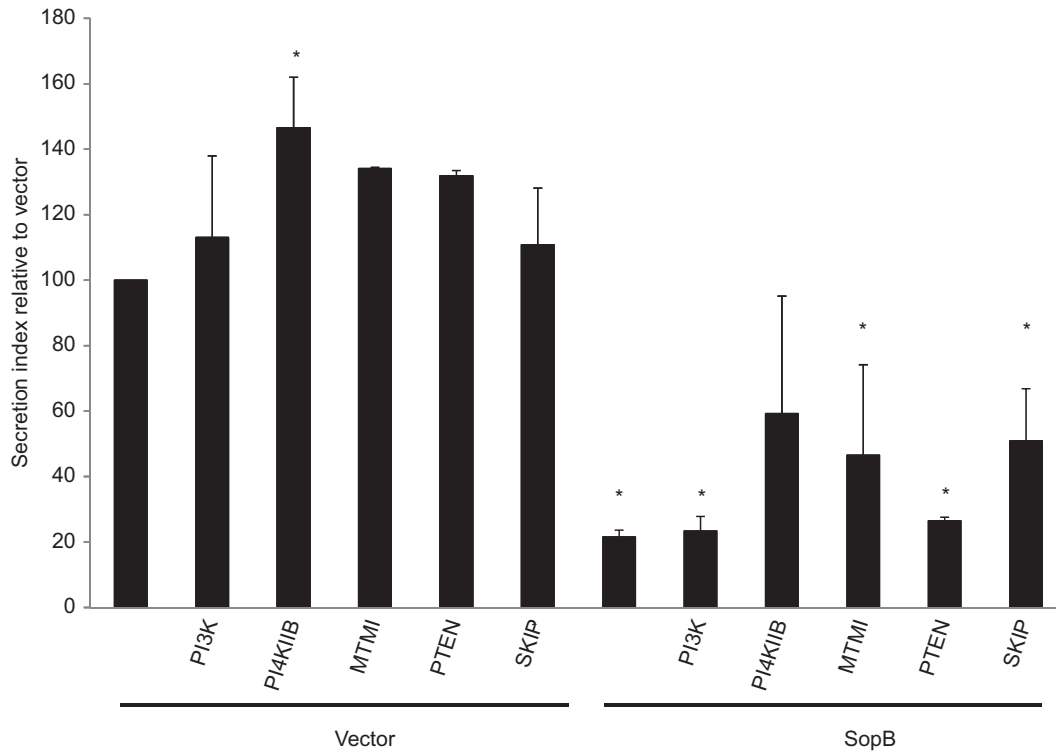
Figure 3 PI4K IIβ prevents SopB-mediated exocytosis inhibition. SEAP assays were performed on 293T cells transfected with pSEAP, either the pEGFP-N2 or pEGFP-N2-SopB plasmids, and one of the phosphoinositol kinase or phosphatases listed. The protein secretion index is displayed. Values are the mean±s.d. for three independent experiments. Asterisks indicate a statistical significant difference compared to cells transfected with vector only.

shifting cells to 32 ℃, the protein refolds and resumes transport to the Golgi and then to the plasma membrane.[48,49] Transport of VSVG from the ER to the Golgi is accompanied by a modification of glycosylation on the protein. The high-mannose glycosylation present on the ER form of ts045-VSVG-GFP is sensitive to the glycosidase Endoglycosidase H (Endo H), while glycan modification in the Golgi confers resistance to Endo H digestion.[48] By measuring the sensitivity of VSVG to Endo H at certain time periods after a shift from 40 ℃ to 32 ℃ the localization of VSVG within the secretory pathway can be determined and whether it is affected by a protein/drug of interest.

To monitor the effect of SopB on VSVG trafficking, 293T cells were cotransfected with ts045-VSVG-GFP and either vector, SopB or SopB[c/s]. Cells were shifted to 40 ℃ overnight before cycloheximide was added to inhibit further protein synthesis and cells were shifted to 32 ℃ for various lengths of time to monitor VSVG transport. The vector- and SopB[c/s]-transfected cells exhibited similar behaviour, namely, that 1 h after the temperature shift the majority of the VSVG protein is Endo H-resistant and therefore has reached the Golgi (Figure 4). In contrast, cells transfected with SopB, show VSVG sensitivity to Endo H up to 2 h after the temperature shift, indicating that, in these cells, some of the VSVG protein remains localized to the ER (Figures 4B and 4C). Therefore, in the presence of SopB the transport of VSVG between the ER and Golgi is inhibited or delayed. The phosphatidylinositol phosphatase activity of SopB is important for this delay in transport between the ER and Golgi.

Determining whether SopB inhibition of ER to Golgi transport occurs during *Salmonella* infection

Although SopB has the potential to inhibit transport between the ER and Golgi, this may not occur during *Salmonella* infection, for

instance the spatial regulation of SopB or the presence of another effector protein may inhibit this activity. We therefore sought to determine if the SopB-dependent inhibition of secretion observed in transfected cells was observed during *Salmonella* infection. We tried to measure the secretion index of the SEAP reporter protein in 293T cells infected with wild type and *sopB* mutant *Salmonella*, however *Salmonella* proved cytotoxic to 293T cells which prevented accurate SEAP activity readings being taken. We therefore, performed the VSVG transport assay in cells that had been infected with *Salmonella*. For these experiments, cells were maintained at 40 ℃ overnight prior to infection and no cycloheximide treatment was performed. Once cells were infected with *Salmonella* the cells were either immediately shifted to 37 ℃, which was found to permit VSVG transport and would not affect the *Salmonella*, to monitor VSVG transport during infection or were maintained at 40 ℃ until 2 or 8 h post-invasion before the shift. In each instance wild type and *sopB* mutant *Salmonella* exhibited similar behaviour and neither affected the transport of VSVG (Supplementary information Figure S1).

DISCUSSION

For a bacterium to have an intracellular lifestyle, it must have the ability to do two things. First, the bacterium must be able to hide from or combat any anti-microbial defences the host cell possesses. Second, it must utilize the available cellular resources to ensure survival, and if possible, replication. *Salmonella* takes care of the first objective by using its T3SS-1 and T3SS-2 effectors to modulate the SCV membrane so that it avoids fusing with lysosomes or does so in a controlled fashion.[50–52] It is currently less clear how *Salmonella* supports its replication.
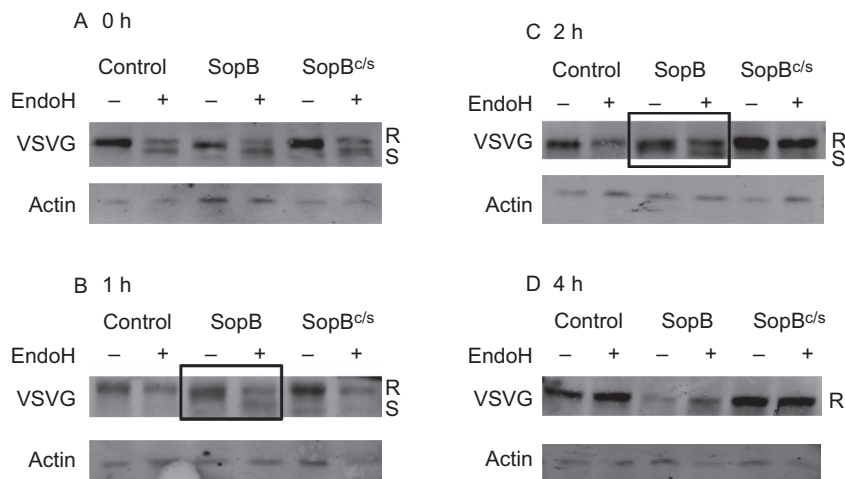
Figure 4 SopB delays transport of VSVG between the ER and Golgi. Immunoblot analysis of VSVG in cells cotransfected with ts045VSVG-GFP and either pEGFP-N2 (control), pEGFP-N2-SopB (SopB) or pEGFP-N2-SopB$^{c/s}$ (SopB$^{c/s}$) at various time points after the shift to 32 °C. Endoglycosidase H (Endo H)-treated (+) and mock-treated (−) samples are indicated. The mobility of the Endo H-resistant (R; upper band) and -sensitive (S; lower band) forms of the protein is indicated. The boxes in **B** and **C** indicate the different Endo H sensitivity pattern exhibited by SopB-transfected cells. Actin immunoblotting is included as a loading control.

It is known that the SCV undergoes a series of interactions with the host endocytic pathway[53,54] and this may in part account for the increase in SCV membrane and the way in which the nutrients required for bacterial replication are obtained. However, other intracellular bacterial pathogens favour the exocytosis pathway to obtain the materials they need for replication. For example, *Legionella* tethers host-derived transitional ER-derived vesicles to its vacuolar compartment (Legionella-containing vacuole), which subsequently fuse with the Legionella-containing vacuole.[55,56] *Coxiella* employs a similar mechanism,[57] while the intermediate Brucella-containing vacuoles of *Brucella* interact with and subsequently fuse with the actual ER to generate ER-derived, replicative Brucella-containing vacuoles.[58,59] There is growing evidence that *Salmonella* may also utilize the secretory pathway to maintain and expand its replicative niche. The first evidence came in 2003, when it was found that not only do SCV accumulate near to the Golgi apparatus but that inhibition of ADP-ribosylation factor 1, a small GTPase required for the formation of COPI-coated vesicles that traffic between the ER and Golgi, inhibited *Salmonella* replication.[15] Kuhle *et al.*[40] have since found secretory vesicles from the TGN recruited to the SCV in a SPI-2-dependent manner, and a protein, SCAMP3, typically located at the TGN has been identified on *Salmonella*-induced filaments and *Salmonella*-induced SCAMP3 tubules.[41] However, direct interaction of secretory vesicles with the TGN still eludes visualisation, and thus while it appears likely that *Salmonella* interacts with the secretory pathway there is little direct evidence to indicate *Salmonella*, like its bacterial counterparts, uses this route to secure the materials it requires for replication.

The data presented here explores the alternative view that *Salmonella* may in fact use the secretory pathway in a different manner, that is, to disrupt secretion. In doing so, *Salmonella* may create a pool of material close to the SCV that could be harvested to support replication. Alternatively, inhibition of exocytosis may prevent particular proteins reaching the cell surface, including those that could alert the immune system to the infection. Several *Salmonella* effectors were identified that could inhibit exocytosis, as measured by the SEAP assay. Interestingly, one of these effectors was SopB, which through its phosphatidylinositol phosphate activity has been associated with manipulating membrane dynamics to allow fission of the SCV from the plasma membrane[20] and determine subsequent interactions of the SCV with host proteins.[23,24] The 4-phosphatase activity of SopB was found to play a critical role in the ability of SopB to inhibit exocytosis and one hypothesis that was explored was whether SopB used this activity to manipulate PI(4)P levels at the TGN. PI(4)P accumulates in regions of the TGN and is important to the creation and fission of secretory vesicles.[46] No direct evidence was obtained for SopB manipulating PI(4)P levels at the TGN and this is supported by the data obtained from the VSVG transport assay that indicates SopB acts earlier in the secretory pathway, i.e., on the transport pathway from the ER to Golgi. How exactly SopB acts at this stage of the pathway was not determined. It has been reported that SopB affects the production of the cytokines that cause inflammation of the gut, a characteristic of *Salmonella* infection.[7,60–62] Further studies are required to test whether SopB also plays a role in the secretion process.

Currently, the only other bacteria found to inhibit exocytosis are EHEC and EPEC, who use effector protein NleA to inhibit COPII-dependent protein export from the endoplasmic reticulum.[42] The reason for this inhibition is, as yet, unknown. It is interesting that EHEC, EPEC and *Salmonella*, which all appear to inhibit exocytosis, all possess a T3SS, and this may perhaps allude to a unique mechanism used by these bacteria to manipulate the host cell. The activity of the EHEC and EPEC effector, NleA, was partially determined after examining its location in the cell after translocation by the T3SS.[42,63] NleA was found to colocalize with mannosidase II and thus determined to localize to the Golgi.[63] SopB has predominantly been studied at the plasma membrane and early SCV membrane but this does not preclude the ability of SopB to associate with other membranes, such as the ER or Golgi, at other time points, and given that the SCV eventually orientates towards the Golgi, it may not need to, to have an effect on the secretory pathway, especially if its effects are mediated by changes in phosphatidylinositol concentrations. SopB has a relatively long half-life[38] and thus it could easily have multiple sites of activity during the infectious process, and which may play an important part in allowing *Salmonella* to successfully transition from its invasion to intracellular phase. This will require further study in the future to determine how exactly SopB affects exocytosis. To conclude, this work

may reveal yet another important role of SopB in *Salmonella* infection and perhaps another layer to the carefully orchestrated manipulation of the cell, and the host, by this bacterium.

ACKNOWLEDGMENTS

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