

Salmonella Modulates Vesicular Traffic by Altering Phosphoinositide Metabolism

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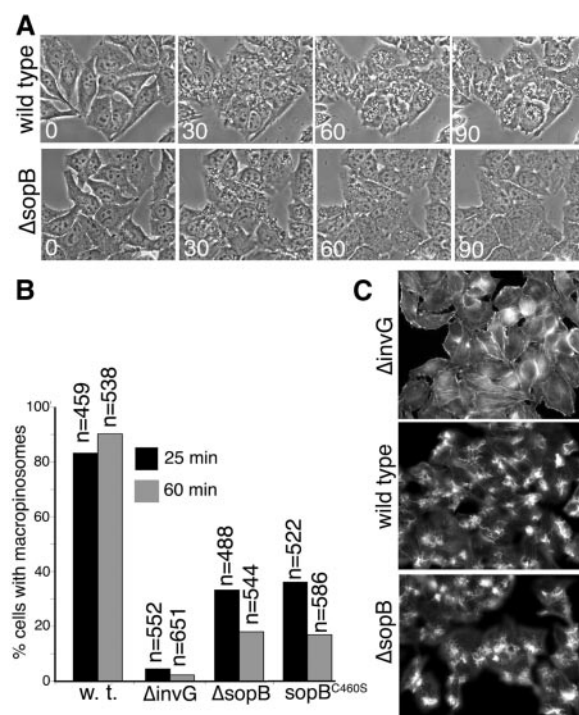
Salmonella enterica, the cause of food poisoning and typhoid fever, induces actin cytoskeleton rearrangements and membrane ruffling to gain access into nonphagocytic cells, where it can replicate and avoid innate immune defenses. Here, we found that SopB, a phosphoinositide phosphatase that is delivered into host cells by a type III secretion system, was essential for the establishment of *Salmonella*'s intracellular replicative niche. SopB mediated the formation of spacious phagosomes following bacterial entry and was responsible for maintaining high levels of phosphatidylinositol-three-phosphate [PtdIns(3)P] in the membrane of the bacteria-containing vacuoles. Absence of SopB caused a significant defect in the maturation of the *Salmonella*-containing vacuole and impaired bacterial intracellular growth.

Salmonella enterica can invade nonphagocytic cells through the activity of bacterial proteins delivered into host cells by a type III secretion system (TTSS) encoded within pathogenicity island 1 (SPI-1) (1, 2). Immediately after internalization, *Salmonella* resides within a spacious, membrane-bound compartment that transiently exhibits features of early endosomes (3, 4). However, this compartment rapidly segregates from the normal endocytic pathway, thereby avoiding fusion with degradative compartments (5–9). The formation of a mature *Salmonella* compartment that supports bacterial replication requires initially the activity of the SPI-1 TTSS (10, 11) and later the activity of a second TTSS encoded within pathogenicity island 2 (SPI-2) (10, 12, 13), which is expressed only after ~3 hours of infection. At this time, *Salmonella* begins to replicate within a specialized compartment that can be stained with antibodies directed to lysosomal glycoproteins such as Lamp-1 but that does not exhibit features of lysosomes (6, 14–16).

The requirement of the SPI-1 TTSS for the intracellular proliferation of *Salmonella* (11) suggests that one or more proteins delivered by this system promote the formation of the bacterial intracellular repli-

cative niche. SopB, a phosphoinositide phosphatase, is delivered into host cells by the SPI-1 TTSS (17–19). Because phosphoinositides are important in vesicular trafficking (20, 21) and *Salmonella* modulates phosphoinositide metabolism to facilitate entry into host cells (19, 22), we examined the role of SopB in the generation of the characteristic *Salmonella* phagosomes. We infected intestinal Henle-407 cells with either wild-type *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) or an isogenic deletion mutant

Fig. 1. The formation of large macropinosomes in *Salmonella*-infected cells requires the bacterially encoded phosphoinositide-phosphatase SopB. **(A)** Intestinal Henle-407 cells were infected with wild-type *S. typhimurium* or an isogenic Δ sopB strain and observed by time-lapse video microscopy. Frames obtained at the indicated times (in minutes) after infection are shown. (The entire sequence is shown in Movie S1.) **(B)** Intestinal Henle-407 cells were infected with wild-type *S. typhimurium*, the mutant strains Δ sopB or Δ invG, or a mutant strain expressing a phosphatase-inactive SopB (SopB^{C460S}). The percentages of cells containing large macropinosomes were enumerated at the indicated times. Equivalent results were obtained in several repetitions of this experiment. The *n* values represent the number of cells examined. **(C)** Intestinal Henle-407 cells were infected for 20 min with wild-type *S. typhimurium* or the Δ sopB or Δ invG isogenic mutants, then fixed and stained with rhodamine-labeled phalloidin to visualize the actin cytoskeleton.



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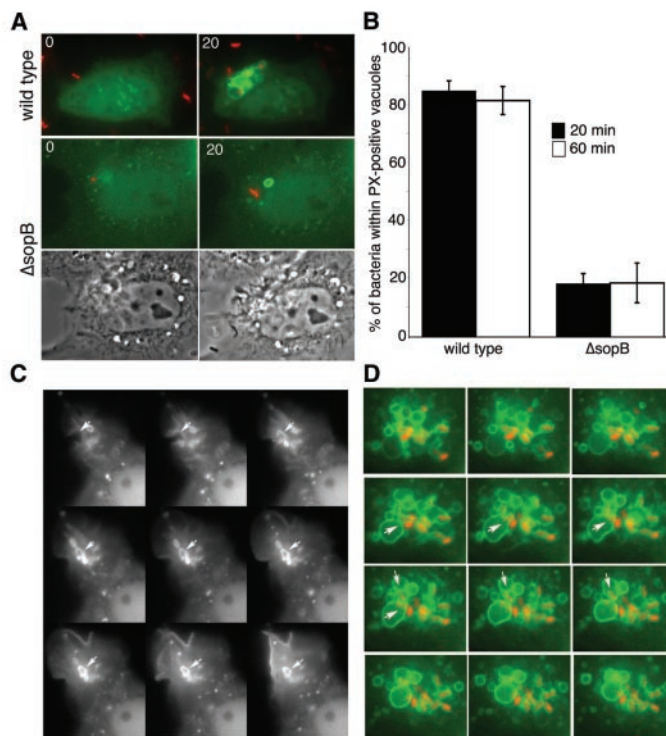
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lacking *sopB* and examined them by time-lapse video microscopy. Cells infected with the wild-type strain contained large macropinosomes, which persisted for up to 90 min (Fig. 1, A and B, and Movie S1). In contrast, cells infected with the Δ sopB strain or a strain expressing a phosphatase-inactive mutant of SopB (SopB^{C460S}) had significantly smaller macropinosomes, which disappeared shortly after infection (Fig. 1, A and B, and Movie S1). The difference in the size and persistence of the macropinosomes was not due to differences in the degree of membrane ruffling and actin cytoskeleton rearrangements because these cellular responses were equivalent in cells infected with either the wild-type or Δ sopB mutant strains (Fig. 1, A and C, and Movie S1). In fact, absence of *sopB* results in only a minor delay in bacterial entry (19), which cannot account for the severe reduction of the size of the bacterially induced macropinosomes. Thus, the formation and persistence of the large macropinosomes following *Salmonella* infection required the phosphoinositide phosphatase activity of SopB.

We investigated the role of SopB in phosphoinositide metabolism during infection. We used chimeric proteins consisting of the yellow fluorescent protein (YFP) fused to the PH domains of phospholipase C δ (23), the general receptor for phosphoinositides protein-1 (GRP1) (24), phosphatidylinositol-four-phosphate adaptor protein-1 (FAPP1) (25), or the PX domain of the 40-kD subunit of the nicotinamide adenine dinucleotide phosphate oxidase (26) to monitor the presence

Fig. 2. Rapid generation and persistence of PtdIns(3)P in SCVs. (A and B) SopB is required for the generation and persistence of PtdIns(3)P in SCVs. (A) Henle-407 cells expressing the PtdIns(3)P probe p40PX-YFP were infected with wild-type *S. typhimurium* or an isogenic Δ sopB mutant expressing the dsRed protein, and visualized by time-lapse video microscopy. (This entire sequence and other sequences of equivalent experiments with different cell lines are shown in Movies S2, S3, S5, and S6.) (B) The percentage of SCVs labeled by the p40PX-YFP probe at different times after infection was determined by fluorescence microscopy; values represent the mean \pm SD of three experiments in which at least 450 bacteria were examined. (C) Generation of PtdIns(3)P at bacterial entry sites. Cos-2 cells expressing p40PX-YFP were infected with wild-type *S. typhimurium* and imaged by time-lapse fluorescence video microscopy. Frames were captured every minute. (The entire sequence is shown in Movie S4.) (D) Time-lapse video microscopy of Henle-407 cells expressing p40PX-YFP and infected with wild-type *S. typhimurium* expressing dsRed, showing fusion of empty macropinosomes with SCVs. Frames were collected every minute. (The entire sequence is shown in Movies S6 and S7.)



of PtdIns(4,5)P₂, PtdIns(3,4,5)P₃, PtdIns(4)P, and PtdIns(3)P, respectively. Intestinal Henle-407 cells expressing the different phosphoinositide probes were infected with either wild-type *S. typhimurium* or a Δ sopB isogenic derivative, each expressing the dsRed fluorescent protein (27). Changes in phosphoinositide metabolism during infection were examined by time-lapse fluorescence video microscopy. We found that probes specific for PtdIns(4)P, PtdIns(4,5)P₂, or PtdIns(3,4,5)P₃ did not label phagosomes containing either strain (28). The p40PX-YFP probe, which is specific for PtdIns(3)P, efficiently labeled phagosomes containing wild-type *S. typhimurium* immediately after bacterial infection (29, 30) (Fig. 2, A to D, and Movies S2 to S7). In fact, the p40PX-YFP probe labeled the ruffling membrane and the nascent phagosomes (Fig. 2C and Movie S4), indicating the formation of PtdIns(3)P at the site of bacteria-host cell interaction immediately after infection. The *Salmonella*-containing vacuole (SCV) retained the PtdIns(3)P labeling for at least 90 min of infection (Fig. 2, A to C, and Movies S2, S4, S5, and S6). In contrast, nascent phagosomes containing the Δ sopB mutant strain were not labeled by the p40PX-YFP probe, and only a few sealed phagosomes recruited the probe (Fig. 2, A and B, and Movie S3). Also, in contrast to wild-type

S. typhimurium phagosomes, the labeling of the Δ sopB-mutant-strain-containing phagosomes was transient, losing the p40PX-YFP probe shortly after its acquisition (Movie S3). Thus, SopB is required for the efficient generation and persistence of PtdIns(3)P in the SCV.

Infection of intestinal Henle-407 cells with wild-type *S. typhimurium* resulted in the formation of a large number of vesicles that did not contain bacteria but that were also specifically labeled with the PtdIns(3)P probe (Fig. 2D and Movies S2, S6, and S7). The empty vesicles frequently fused with each other and with vesicles containing bacteria, resulting in a net increase in their size (Fig. 2D and Movies S2, S6, and S7). Cells infected with the Δ sopB mutant strain did not contain empty vesicles, and the SCVs did not show this dynamic behavior (Movies S1 and S3). Thus, the fusion of the SCVs with the empty vesicles generated during bacterial entry may be important in forming and determining the size of the spacious phagosomes, and SopB is essential for this event.

Intestinal Henle-407 cells were infected with wild-type *S. typhimurium* or isogenic mutants that carried a deletion in *sopB* or in *invG*, which encodes for a core component of the SPI-1 TTSS (31). At different times

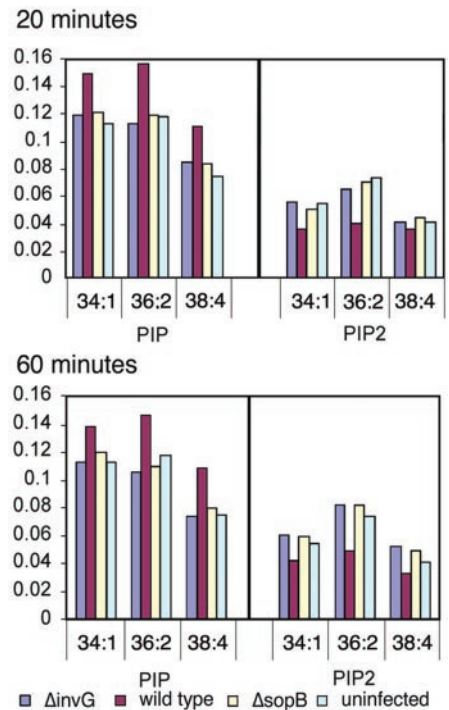
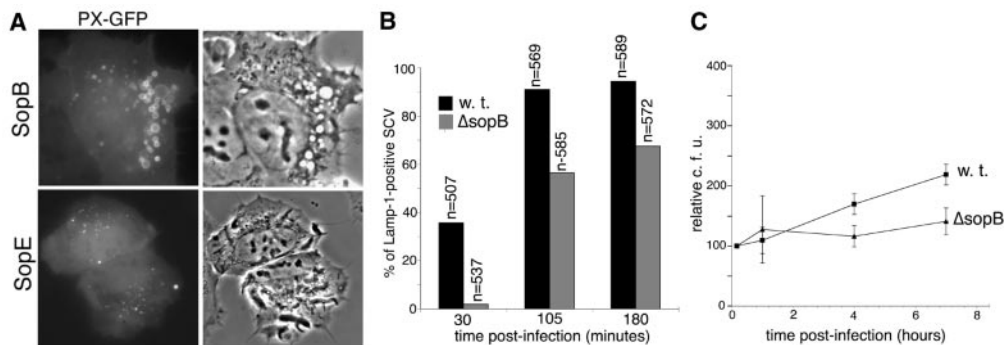


Fig. 3. Phosphoinositide levels in intestinal Henle-407 cells infected with wild-type or mutant *S. typhimurium* strains. Henle-407 cells were infected with wild-type *S. typhimurium* or the isogenic Δ sopB or Δ invG mutants, and 20 or 60 min after infection the levels of PtdInsP (PIP) and PtdInsP₂ (PIP₂) were determined by ESI-MS. The data are represented as relative levels normalized to phosphatidylinositol and represent one experiment of several repetitions with equivalent results. Variation of the relative values between experiments was less than 10%. Only the most abundant molecular species (xy), where x denotes the total number of fatty acid carbon atoms and y the number of double bonds, are shown.

after infection, the levels of PtdInsP and PtdInsP₂ in the samples were quantified by electrospray ionization mass spectrometry (ESI-MS) (32). Cells infected with wild-type *S. typhimurium* showed increased levels of PtdInsP both 20 and 60 min after infection (Fig. 3). In contrast, cells infected with either the Δ sopB or the TTSS-defective Δ invG mutant strains did not show a measurable increase in the levels of PtdInsP (Fig. 3), consistent with the observations using the phosphoinositide-specific YFP probes. This suggests that PtdIns(3)P contributes significantly to the total cellular PtdInsP. In addition, cells infected with wild-type *S. typhimurium*, but not those infected with either the Δ sopB or the Δ invG mutant strains, exhibited decreased levels of PtdInsP₂ (Fig. 3), consistent with the phosphoinositide phosphatase-specificity of SopB (18, 33) and the fact that wild-type *S. typhimurium* infection of cultured cells results in a localized consumption of PtdIns(4,5)P₂ (22).

To examine the ability of SopB to form large PtdIns(3)P-containing macropinosomes in

Fig. 4. (A) Expression of SopB results in the formation of large PtdIns(3)P-containing vesicles. Intestinal Henle-407 cells were co-transfected with plasmids encoding p40PX-YFP and either SopB-CFP or SopE-CFP and observed by time-lapse video microscopy. The initial frames of the time-lapse sequence are shown. (The entire sequence and sequences of other cell lines expressing the same constructs are shown in Movies S8 to S10.) **(B)** The *S. typhimurium* Δ sopB mutant shows delayed maturation of the bacteria-containing vacuole. Henle-407 cells were infected with wild-type *S. typhimurium* or the isogenic Δ sopB mutant; at the indicated times, the percentages of SCVs that were stained with an antibody to Lamp-1 were enumerated by fluorescence microscopy. *n* denotes the number of cells examined. Equivalent results were obtained in several repetitions of this experiment. **(C)** The *S. typhimurium* Δ sopB mutant shows decreased intra-



cellular growth within primary bone marrow-derived mouse macrophages. Macrophages were infected with either wild-type or Δ sopB *S. typhimurium*; at the indicated times, the numbers of intracellular bacteria were enumerated by determining colony-forming units (c.f.u.). Values were standardized considering the c.f.u. obtained 10 min after infection to be 100%.

the absence of other bacterial proteins, we coexpressed cyan fluorescent protein (CFP)-tagged SopB and p40PX-YFP in intestinal Henle-407 cells. Expression of SopB-CFP resulted in the formation of large macropinosomes that originated at the plasma membrane and moved to a perinuclear position (Fig. 4 and Movies S8 and S9). Furthermore, the ruffling plasma membrane and nascent vesicles were readily labeled by the PtdIns(3)P probe, which remained associated with the vesicles for at least 60 min. In contrast, expression of SopE, a *Salmonella* Rho-family guanine nucleotide exchange factor (34), in intestinal Henle-407 cells did not result in the formation of large PtdIns(3)P-containing macropinosomes, despite the induction of profuse membrane ruffling (Fig. 4 and Movie S10). Thus, membrane ruffling per se is not sufficient to induce the formation of large macropinosomes, and these results further support the role of SopB in the formation of *Salmonella*'s spacious phagosomes.

A key event in the maturation of the SCV is the rapid acquisition of the late endosomal or lysosomal marker Lamp-1 (6, 9). This event is not the result of fusion with lysosomes; instead, Lamp-1 is acquired from an unusual compartment in a Rab7-dependent manner (7). We found that there was a significant delay in the acquisition of Lamp-1 by the Δ sopB mutant when compared with wild type (Fig. 4B). In addition, the Δ sopB mutant showed a significant defect in its ability to grow within bone-marrow-derived mouse macrophages, consistent with a requirement of SopB for *Salmonella*'s efficient intracellular replication (Fig. 4C).

In vitro, SopB's phosphatase activity exhibits a predilection for phosphates at positions 4' and 5' of the inositol ring in PtdIns(3,5)P₂ and PtdIns(3,4,5)P₃ (33). We propose that SopB exerts its effect on the vesicular trafficking of *Salmonella* by at least two mechanisms. First, through its PtdIns(3,4,5)P₃ phosphatase activity, SopB may contribute to the generation of

PtdIns(3)P at the plasma membrane, thus resulting in the more rapid and efficient incorporation of this phosphoinositide into the SCV. Second, through its PtdIns(3,5)P₂ phosphatase activity, SopB may arrest the progression of the SCV down the vesicular trafficking pathway leading to lysosomes. In *Saccharomyces cerevisiae*, the balance between the levels of PtdIns(3)P and PtdIns(3,5)P₂, established by the activities of the PtdIns(3)P 5-kinase Fab1 and the PtdIns(3,5)P₂ phosphatase (Fig4), is key to the control of the size of the yeast vacuole, an equivalent compartment to mammalian multivesicular bodies or late endosomes (35–37). SopB may thus perform a function analogous to that of the yeast protein (Fig4) and through its PtdIns(3,5)P₂ phosphatase activity may prevent the transition of PtdIns(3)P to PtdIns(3,5)P₂, thus diverting the SCV from the endocytic pathway and resulting in enlarged vesicles. The persistence of PtdIns(3)P in the SCV may contribute to the enlargement of the SCV by stimulating homotypic fusion with other PtdIns(3)P-containing empty vesicles formed during bacterial infection. The resulting large vesicles may provide a favorable environment where *Salmonella* can reside and reprogram gene expression so that the SPI-2 TTSS machinery necessary for the building of its replicative niche can be assembled.

References and Notes

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Supporting Online Material

www.sciencemag.org/cgi/content/full/304/5678/1805/DC1

Materials and Methods

Movies S1 to S10

References

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