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The related effector proteins SopD and SopD2 from *Salmonella enterica* serovar Typhimurium contribute to virulence during systemic infection of mice

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Summary

***Salmonella* resides within host cells in a vacuole that it modifies through the action of virulence proteins called effectors. Here we examined the role of two related effectors, SopD and SopD2, in *Salmonella* pathogenesis. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) mutants lacking either *sopD* or *sopD2* were attenuated for replication in the spleens of infected mice when competed against wild-type bacteria in mixed infection experiments. A double mutant lacking both effector genes did not display an additive attenuation of virulence in these experiments. The double mutant also competed equally with both of the single mutants. Deletion of either effector impaired bacterial replication in mouse macrophages but not human epithelial cells. Deletion of *sopD2* impaired *Salmonella*'s ability to form tubular membrane filaments [*Salmonella*-induced filaments (Sifs)] in infected cells; the number of Sifs decreased, whereas the number of pseudo-Sifs (thought to be a precursor of Sifs) was increased. Transfection of HeLa cells with the effector SifA induced the formation of Sif-like tubules and these were observed in greater size and number after co-transfection of SifA with SopD2. In infected cells, SifA and SopD2 were localized both to Sifs and to pseudo-Sifs. In contrast, deletion of *sopD* had no effect on Sif formation. Our results indicate that both SopD and SopD2 contribute to virulence in mice and suggest a functional relationship between these two proteins during systemic infection of the host.**

Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a facultative intracellular pathogen capable of causing a variety of diseases in different hosts. In humans these bacteria remain a leading cause of food poisoning whereas in mice they cause a systemic disease that resembles typhoid fever (Tsolis *et al.*, 1999a). After ingestion of contaminated food or water, *S.* Typhimurium penetrate the intestinal barrier through invasion of several cell types, including columnar epithelial cells (Takeuchi, 1967), M-cells at Peyer's patches (Jones *et al.*, 1994) and dendritic cells (Rescigno *et al.*, 2001). In the mouse model of infection, *S.* Typhimurium colonizes macrophages in the liver and spleen during systemic phases of disease (Richter-Dahlfors *et al.*, 1997; Salcedo *et al.*, 2001). Throughout the course of infection, these bacteria occupy a vacuolar niche inside host cells known as the *Salmonella*-containing vacuole (SCV). Maturation of the SCV is distinct from that of model phagosomes and phagosomes containing dead or virulence-attenuated bacteria, indicating that *S.* Typhimurium actively modulates endosome transport to subvert innate immune mechanisms in the host cell (Knodler and Steele-Mortimer, 2003; Linehan and Holden, 2003; Brumell and Grinstein, 2004).

S. Typhimurium uses two type III secretion systems (TTSS) to alter host cell machinery and cause disease. These specialized protein delivery systems mediate the transfer of virulence proteins, termed effectors, into the host cell where they manipulate host cell function to promote infection (Hueck, 1998). The *Salmonella* Pathogenicity Island (SPI)-1-encoded TTSS plays an essential role in gastroenteritis (Galyov *et al.*, 1997; Jones *et al.*, 1998; Watson *et al.*, 1998; Wood *et al.*, 1998; Wallis *et al.*, 1999) and mediates the invasion of non-phagocytic epithelial cells *in vitro* (Galan and Curtiss, 1989). Invasion is medi-

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ated by the actions of at least four SPI-1 effectors that initiate actin rearrangements (Galan and Zhou, 2000) and phospholipid signalling events (Zhou *et al*., 2001; Terebiznik *et al*., 2002; Brumell and Grinstein, 2003) that culminate in bacterial uptake. After invasion, a second TTSS encoded within SPI-2 delivers a distinct set of effector proteins across the SCV (Hensel, 2000). The SPI-2 TTSS is required for the establishment of systemic disease in mice and is necessary for bacterial survival and replication in murine macrophages (Hensel *et al*., 1995; 1997; 1998; Ochman *et al*., 1996; Shea *et al*., 1996; 1999; Cirillo *et al*., 1998; Pfeifer *et al*., 1999). It also plays a significant role in gastroenteritis and intestinal pathology (Tsolis *et al*., 1999b; Bispham *et al*., 2001).

SPI-2 effectors are encoded both within SPI-2 and elsewhere in the bacterial chromosome (Beuzon *et al*., 1999; Brumell *et al*., 2000; 2001a; Miao and Miller, 2000; Nikolaus *et al*., 2001; Hansen-Wester *et al*., 2002; Knodler *et al*., 2002). Members of the *Salmonella* Translocated Effector (STE) family are encoded in low G+C islets on the bacterial chromosome and are unique in that they share a conserved N-terminal domain of ≈140 amino acids that directs their translocation into host cells (Miao and Miller, 2000). Members of this family include SspH2 and SseI, which are targeted to vacuole-associated actin polymerizations induced by intracellular *S.* Typhimurium (Miao *et al*., 2003). Another family member, SifA, is targeted to the SCV (Brumell *et al*., 2002) and plays an active role in maintaining the integrity of this compartment (Beuzon *et al*., 2000). In the absence of *sifA*, the SCV is degraded in a manner that requires SseJ, another member of the STE family thought to possess lipolytic activity (Ruiz-Albert *et al*., 2002). SifA also contributes to the formation of *Salmonella*-induced filaments (Sifs), a unique intracellular phenotype observed in infected epithelial cells (Garcia-del Portillo *et al*., 1993; Stein *et al*., 1996) and macrophages (Knodler *et al*., 2003). Sifs are tubular extensions of the SCV that associate with microtubules and are enriched in lysosomal glycoproteins (Brumell *et al*., 2002). Fusion of late endosomal compartments with the SCV is required for Sif formation, as many subcellular markers of these compartments, including the Rab7 GTPase, are localized to Sifs (Brumell *et al*., 2001b). Deletion of *sifA* blocks Sif formation (Stein *et al*., 1996) whereas expression of SifA in epithelial cells is sufficient to induce aggregation and swelling of late endocytic compartments (Brumell *et al*., 2001b) and the formation of Sif-like tubules (Brumell *et al*., 2001a). While these findings demonstrate that SifA is essential for Sif formation and sufficient to induce endosomal alterations in the host cell, other SPI-2 effectors were recently shown to be required for this phenotype: SseF and SseG, both encoded within SPI-2, are localized to Sifs and are required for their formation (Guy *et al*., 2000; Kuhle and Hensel, 2002). Other SPI-2 effectors localized to Sifs include SifB (Freeman *et al*., 2003), PipB (Knodler *et al*., 2002) and PipB2 (Knodler *et al*., 2003), although it is unclear whether these or other bacterial factors directly contribute to Sif formation.

We recently identified SopD2, a novel member of the STE family of effectors (Brumell *et al*., 2003). SopD2 plays a significant role in the virulence of *S.* Typhimurium and contains an N-terminal membrane-binding domain that causes its localization to late endocytic compartments in host cells (Brumell *et al*., 2003). SopD2 has a very high identity to a second STE family protein, SopD (43% identity, 63% similarity), strongly suggesting an evolutionary relationship between these two effectors. SopD was originally identified as an effector of the SPI-1 TTSS and plays a significant role in gastroenteritis in bovine models of infection (Jones *et al*., 1998). Deletion of *sopD* in this model leads to a reduction of both fluid secretion and inflammatory responses during infection (Jones *et al*., 1998). However, expression of the *sopD* gene is maintained at later stages of infection when other SPI-1 effectors are not expressed, indicating this effector may also play a role in systemic infection of the host (Brumell *et al*., 2003). Despite the high degree of identity between SopD and SopD2, their behaviour is markedly different. SopD2 is translocated by the SPI-2 TTSS, and the expression of *sopD2* is dependent upon the SPI-2 regulators *ssrA/B* and *phoP/Q*. In contrast, SopD is probably translocated by both the SPI-1 and SPI-2 TTSSs, but expression of *sopD* is not dependent on either of the SPI-2 regulators, nor on the SPI-1 regulator *hilA*. Furthermore, SopD2 localizes to late endocytic compartments in transfected cells and to the SCV in infected cells, whereas SopD is found in the host cell cytosol under both conditions (Brumell *et al*., 2003). These data suggest that although the two proteins are highly homologous, they are not performing the same function. In this report, we have further characterized the roles of SopD and SopD2. We demonstrate that both effectors promote pathogenesis by *S.* Typhimurium in the mouse model of systemic infection. We also demonstrate that SopD2 contributes to the formation of Sifs in infected epithelial cells.

Results

Presence of sopD2 *in different* Salmonella *serovars*

Genomic analysis has previously demonstrated that *sopD* is located at the same chromosomal location in all *Salmonella* serovars, indicating its acquisition preceded divergence of contemporary *Salmonella* subspecies from a common ancestor (Prager *et al*., 2000; Mirold *et al*., 2001). A search of the available genomic resources (http://www.sanger.ac.uk; McClelland *et al*., 2001; Parkhill

et al., 2001) revealed *sopD2* to be present in *S. gallinarum*, *S. enteriditis* and three *S.* Typhimurium serovars (LT2, DT104 and SL 1344). To further examine the distribution of *sopD* and *sopD2*, we first generated DNA probes specific for each effector. Because of the high degree of nucleotide homology between the two genes, we performed controls using plasmid-derived *sopD* and *sopD2*, as well as *Escherichia coli* genomic DNA, to ensure there was no cross-hybridization of the probes with the two genes (Fig. 1A). Next, we performed Southern dot blotting of genomic DNAs isolated from a variety of *Salmonella* serovars. Genomic DNA from *E. coli* was included in the dot blot as a negative control. Both the *sopD* and *sopD2* genes were present in all 32 serovars that were tested (Fig. 1B; only a small number of strains are shown as all strains contain both genes), including clinical and environmental isolates representative of *S. enterica* subspecies I and IIIa (*S. arizonae*). A complete list of the serovars tested can be found in *Experimental procedures*.

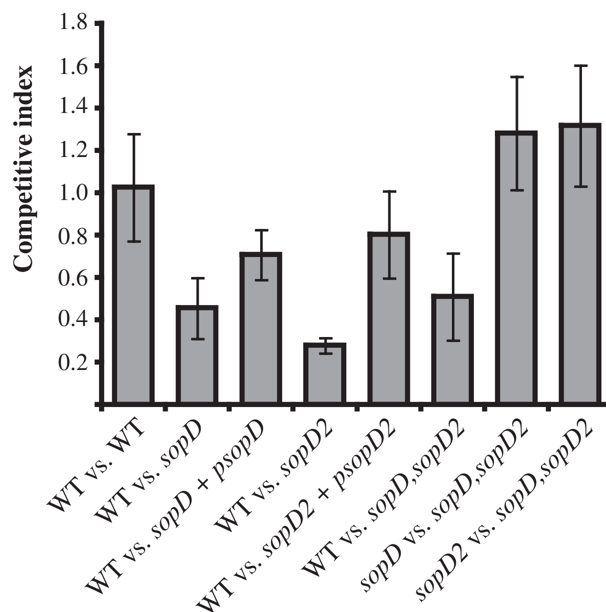

Fig. 2. Competitive index (CI) analysis of *sopD* and *sopD2* mutants in mice. Strains used in mixed infections are referred to by their genotype. Mice were infected by intraperitoneal injection with 10^5 total bacteria. The CI was calculated as the output ratio of the mutant or double mutant to wild-type (WT) or single mutant bacteria, divided by the input ratio, as previously described (Beuzon and Holden, 2001). The CIs shown are the means of at least three independent infections of mice ± the standard deviation.

SopD and SopD2 contribute to the virulence of S. *Typhimurium* in vivo

To examine the virulence functions of *sopD* and *sopD2 in vivo*, we performed mixed infections of BALB/c mice (Beuzon and Holden, 2001). In these experiments, mice were infected by intraperitoneal injection with equal numbers of wild-type and mutant bacteria and the number of each strain present in the spleen was enumerated 2 days after infection. By comparing the replication of wild-type bacteria to isogenic mutants, a competitive index (CI) was determined. The CI is a ratio reflecting how well a mutant strain is able to replicate within the spleen of the infected mouse, with respect to wild-type bacteria; therefore, the CI gives a measure of how much a strain is attenuated as a result of a mutation. Control infections using two wild-type strains provided a CI value of ≈1, indicating that the two strains are able to replicate equally well in the mouse spleen and are therefore recovered in equal numbers (Fig. 2). In contrast, mixed infections using wild-type and a *sopD* mutant strain yielded a decreased CI value, indicating that the *sopD* mutant was significantly reduced in its ability to replicate in the mouse spleen. Partial complementation of the replication defect was achieved by expression of *sopD* on a plasmid (Fig. 2). Consistent with previous observations (Brumell *et al.*, 2003), the *sopD2* mutant also displayed decreased replication in the spleen – a defect that could be partially complemented by expression of the *sopD2* gene on a plasmid. These findings provide the first evidence that both *sopD* and *sopD2* contribute to virulence in the mouse model of systemic infection.

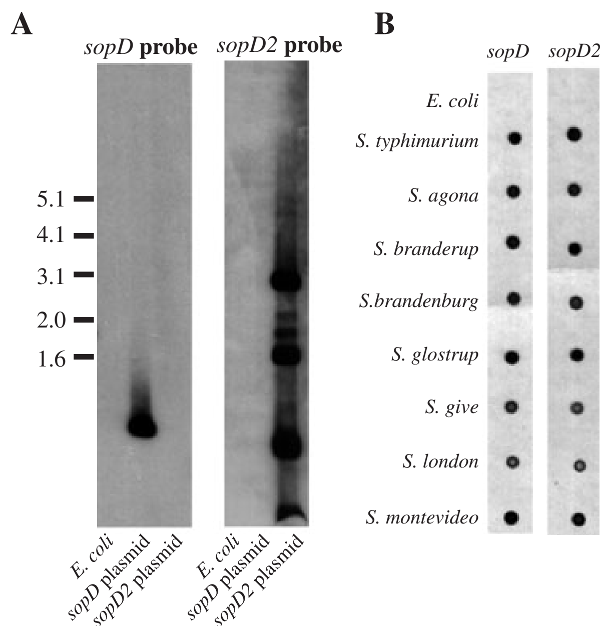

Fig. 1. Presence of *sopD* and *sopD2* in different *Salmonella* serovars.
A. Probes specific for *sopD* or *sopD2* were used to probe cellular DNA from *E. coli* or plasmids bearing each effector gene, as indicated. Molecular weight standards in kilobases are indicated to the left.
B. Gene-specific probes shown in (A) were used to assess the distribution of *sopD* and *sopD2* in different *Salmonella* serovars using a Southern dot blot assay.

To further examine the relationship between *sopD* and *sopD2*, a double mutant lacking both effectors was constructed and used in mixed infections of mice as above. Not surprisingly, this double mutant was attenuated for replication in the spleens of infected animals, as compared with wild-type bacteria (Fig. 2). However, the defect observed for the double mutant was not greater than that observed for either single mutant. To further understand the contribution of each effector to systemic virulence, we performed mixed infections with the *sopD,sopD2* double mutant against each of the single effector mutants. As shown, the double mutant did not display a virulence defect compared with either of the single mutants.

SopD and SopD2 promote replication in mouse macrophages

We next analysed the contribution of *sopD* and *sopD2* to intracellular replication of *S.* Typhimurium in different cell types *in vitro*. RAW macrophages were infected with wild-type bacteria or isogenic mutants and the number of intracellular bacteria was enumerated after 21 h (Fig. 3A). Deletion of *ssaR* (which is essential for secretion by the SPI-2 TTSS) blocked replication, consistent with previous studies (Pfeifer *et al*., 1999; Brumell *et al*., 2001a). Deletion of either *sopD* or *sopD2* impaired bacterial replication by approximately 50% and 60% respectively. As a control, the replication defect of both the *sopD* and *sopD2* mutants was complemented by expression of these genes on a plasmid. A *sopD,sopD2* double mutant was also attenuated for replication, but similar to the *in vivo* competition assays above, the defect observed for the double mutant was no greater than the defect of the single *sopD* and *sopD2* mutants. These data demonstrate that *sopD* and *sopD2* both contribute to replication in macrophages.

We also examined intracellular replication in HeLa cells. As shown in Fig. 3B, *S.* Typhimurium undergoes rapid intracellular replication after a 3–4 h lag period after invasion into this cell type. The *ssaR* mutant underwent a rapid, early replication after this lag period but stopped replicating after 6 h and bacterial numbers decreased thereafter. In contrast, intracellular replication of a *sopD,sopD2* double mutant was not impaired compared with wild-type bacteria. These findings demonstrate that both *sopD* and *sopD2* play an important role in intracellular replication of *S.* Typhimurium in macrophages but their function is dispensable in some cell types such as epithelial cells.

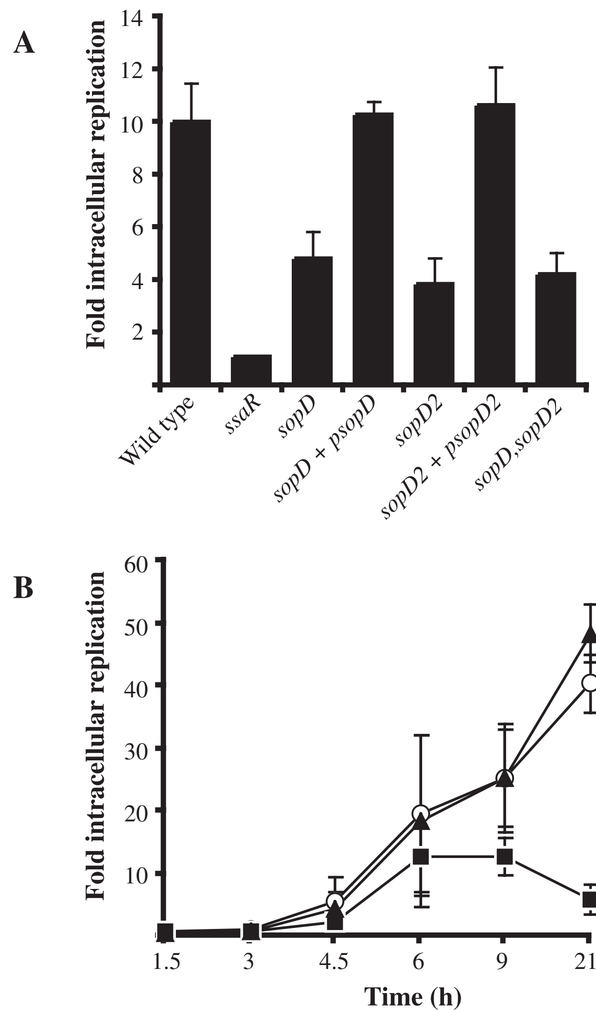

Fig. 3. *SopD* and *sopD2* promote replication of *S.* Typhimurium in mouse macrophages.
A. RAW macrophages were infected for 21 h with either wild-type *S.* Typhimurium or isogenic mutants deficient in *ssaR* (SPI-2 secretion system defective), *sopD*, *sopD2*, or a double mutant lacking both *sopD* and *sopD2*, as indicated. Infected cells were then lysed and intracellular bacteria were enumerated by plating on selective medium (see *Experimental procedures*). Expression of *sopD* or *sopD2* on a plasmid restored normal replicative potential to mutants lacking the corresponding effector gene. Values shown are the average ± the standard deviation for three experiments.
B. HeLa cells were infected with wild-type *S.* Typhimurium (open circles) or isogenic mutants deficient in *ssaR* (closed squares), or a double mutant lacking both *sopD* and *sopD2* (closed triangles) and intracellular replication was enumerated as in (A) over the time course depicted.

SopD2 contributes to Salmonella*-induced filament formation*

Salmonella-induced filament formation involves the extensive fusion of late endosomal compartments along microtubules (Brumell *et al*., 2001b; 2002). Sif tubules are rich in lysosomal glycoprotein and extend from SCVs. Originally identified in epithelial cells, Sifs have recently been observed in *S.* Typhimurium-infected macrophages (Knodler *et al*., 2003). While the function of Sifs is not known, all evidence to date suggests that this unique phenotype is essential to *Salmonella* pathogenesis.

As SopD2-GFP (green fluorescent protein) associates with late endosomal compartments in transfected cells (Brumell *et al*., 2003), we hypothesized that this effector mediates endosomal alterations contributing to Sif formation. To test this hypothesis, we infected HeLa cells with a *sopD2* mutant of *S.* Typhimurium and analysed Sif formation. As shown, wild-type *S.* Typhimurium induces the formation of these tubular endosome structures (Fig. 4A) in ≈55% of infected cells (Fig. 4E). This phenotype was not observed in cells infected with either *ssaR* (Fig. 4B and E) or *sifA* mutants of *S.* Typhimurium (Fig. 4E). Interestingly, deletion of *sopD2* led to a significant decrease (≈50% of wild-type levels) in the number of infected cells with Sifs (Fig. 4E), and the Sifs observed in these cells tended to be shorter in length. While Sif formation was impaired, the majority of *sopD2* mutant bacteria were observed in LAMP-1$^+$ vacuoles (Fig. 4C), indicating that overall integrity of the vacuole was not compromised. Sif formation was normal in cells infected with the single *sopD* mutant and was not further attenuated in the *sopD,sopD2* double mutants (Fig. 4E), demonstrating that SopD does not play a role in the Sif phenotype.

Normal Sif formation was restored to the *sopD2* mutant by expression of *sopD2* from a plasmid (Fig. 4D and E). Expression of SopD2 with two C-terminal HA epitope tags was also sufficient to complement loss of the chromosomal *sopD2* gene in Sif formation (Fig. 4E). Amino acids 1–200 of SopD2 have been previously shown to mediate type III secretion from *S.* Typhimurium and membrane association of this effector upon its translocation into host cells (Brumell *et al*., 2003). However, expression of this N-terminal domain alone was not sufficient to complement the Sif formation defect of the *sopD2* mutant (Fig. 4E). Furthermore, normal Sif formation was not restored to the *sopD2* mutant by overexpression of SifA on a plasmid (in tandem with the chromosomal *sifA* gene) (Fig. 4E). These findings demonstrate that SopD2 plays a contributory role in Sif formation and suggest that it functions in a manner distinct from that of SifA.

Deletion of sopD2 *arrests Sif formation at an intermediate stage*

Salmonella-induced filament formation requires the translocation of SifA into epithelial cells and its association with the SCV (Brumell *et al*., 2002; Boucrot *et al*., 2003). We have previously constructed a plasmid encoding SifA with two internal HA epitope tags, allowing for the visualization of SifA in infected cells (Brumell *et al*., 2002). To further examine the role of SopD2 in Sif formation, we infected cells with either wild-type or *sopD2* mutant bacteria, each expressing SifA-2HA. As shown in Fig. 5A, in cells

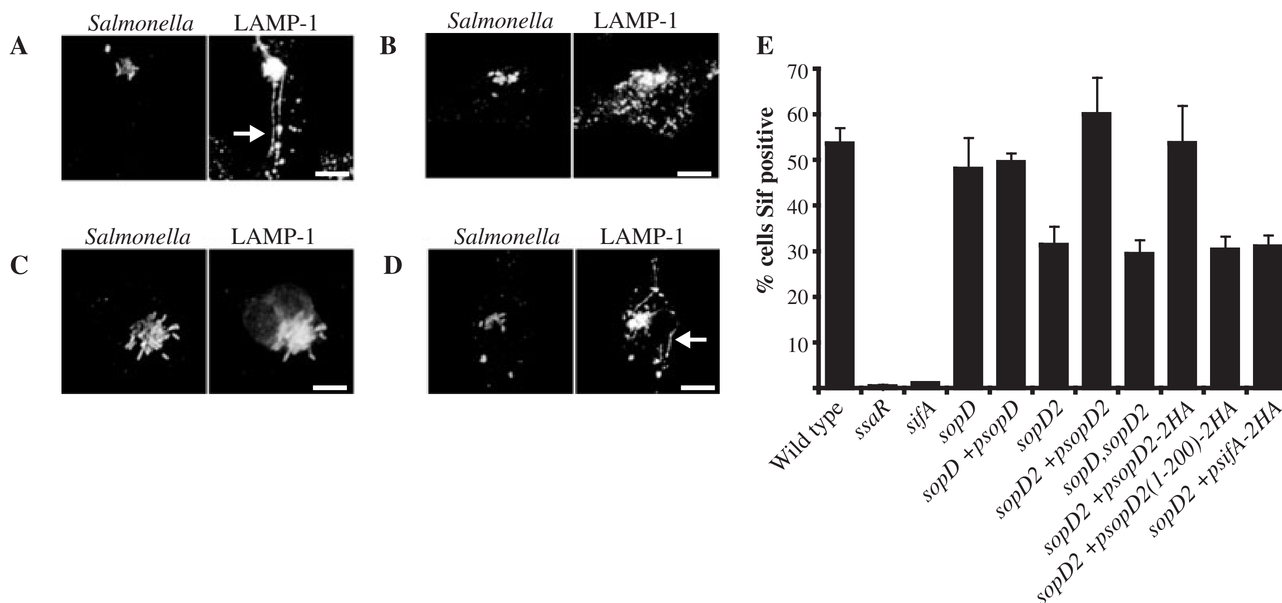

Fig. 4. *SopD2* contributes to *Salmonella*-induced filament formation.
A–D. HeLa cells were infected for 8 h with either wild-type *S.* Typhimurium (A) or isogenic mutants deficient in *ssaR* (B) or *sopD2* (C). Infected cells were then fixed and co-immunostained for LAMP-1 and *S.* Typhimurium and analysed by confocal microscopy. Tubular extensions of the bacterial vacuole known as *Salmonella*-induced filaments (Sifs) are indicated with an arrow in (A). Wild-type bacteria induce Sif formation whereas the *ssaR* mutant does not. Sif formation was decreased in cells infected with the *sopD2* mutant (C), which was complemented by expression of the *sopD2* gene on a plasmid (D). Bar, 10 μm.
E. HeLa cells were infected with the indicated strains and Sif formation was enumerated for at least for experiments. Values shown are the average ± the standard deviation for three experiments.

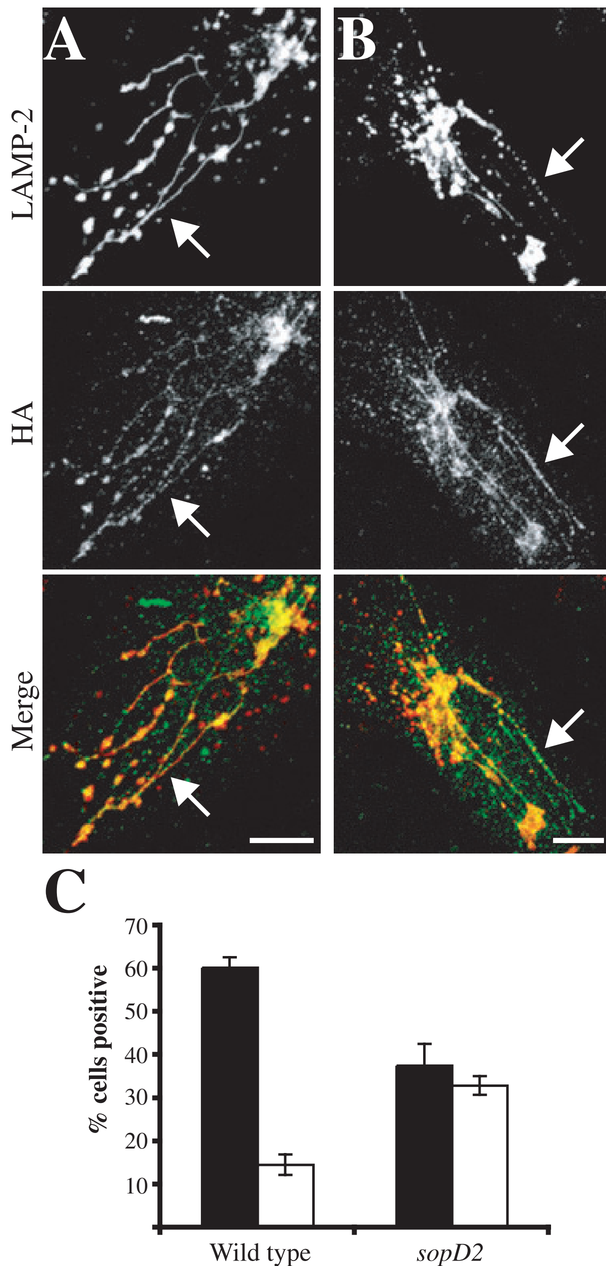

Fig. 5. Deletion of *sopD2* arrests Sif formation at an intermediate stage.
A and B. HeLa cells were infected for 8 h with either wild-type *S.* Typhimurium (A) or an isogenic mutant of this strain with a disruption in the *sopD2* gene (B). Both strains carried a plasmid expressing SifA with two internal HA epitope tags, as previously described (Brumell *et al.*, 2002). Infected cells were then fixed and co-immunostained for LAMP-2 and the HA epitope, and analysed by confocal microscopy. The HA-tagged SifA protein was localized predominantly to Sifs in wild-type infected cells (see arrows in A). SifA was also localized to filamentous structures that contained a punctate rather than continuous distribution of LAMP-1, a phenotype known as 'pseudo-Sifs' that was more common in cells infected with the *sopD2* mutant (see arrows in B). Bar, 10 μm.
C. Cells were infected as in (A) and the formation of Sifs (filled boxes) or pseudo-Sifs (open boxes) was enumerated. Values shown are the average ± the standard deviation for three experiments.

infected with wild-type bacteria expressing SifA-2HA, the SifA-2HA associated with Sifs, and colocalized with LAMP-2. In cells infected with the *sopD2* mutant expressing SifA-2HA, the SifA-2HA was associated with smaller Sif-like structures (Fig. 5B). In addition, in the *sopD2* mutant-infected cells, we observed filamentous structures containing SifA-2HA that did not colocalize completely with LAMP-2. Instead of a continuous association with LAMP-2 as observed with Sifs, punctate vesicles containing this lysosomal glycoprotein were observed in association with the SifA-2HA filaments (see arrows). These filamentous structures of SifA-2HA resemble those described by Kuhle and Hensel (2002). In their analysis of M45 epitope-tagged SseJ, these authors observed filamentous tubules containing this SPI-2 effector, dubbed 'pseudo-Sifs', that lacked continuous LAMP-1 distribution. Consistent with the nomenclature of Kuhle and Hensel, we will refer to filamentous structures that contain SifA-2HA, but are not continuously colocalized with LAMP-2, as pseudo-Sifs.

Utilizing SifA-2HA as a marker, we enumerated the number of Sifs and pseudo-Sifs in infected HeLa cells (Fig. 5C). In wild type-infected cells, the number of pseudo-Sifs was low compared with Sifs. In contrast, cells infected with the *sopD2* mutant had an increased number of pseudo-Sifs concomitant with a decrease in Sif formation. These findings suggest that deletion of *sopD2* blocks Sif formation at an intermediate stage, before fusion of late endosome compartments with the SCV.

SopD2 and SifA act cooperatively to form Sifs in epithelial cells

In previous studies, we have demonstrated that expression of SifA as a fusion to the N-terminus of GFP in HeLa cells causes the reorganization of late endosomal compartments (Brumell *et al.*, 2001a,b). As shown in Fig. 6A, expression of SifA-GFP caused swelling and perinuclear aggregation of LAMP-1$^+$ compartments in the majority of transfected cells. Furthermore, SifA-GFP caused the formation of long LAMP-1$^+$ tubules that resemble Sifs in ≈18% of transfected cells (Fig. 6B and C). To examine the relationship of SifA and SopD2 in altering the host endosomal system, we expressed these proteins simultaneously in HeLa cells (Fig. 6B). Co-transfection of SifA-GFP with FLAG epitope-tagged SopD2 led to a dramatic increase in the number of Sif-like tubules present in transfected cells (Fig. 6B and C). Expression of SopD-GFP had no effect on the ability of SifA-GFP to form Sif-like tubules (data not shown). This suggests that SifA and SopD2 can act in a complementary fashion to promote endosome alterations that lead to Sif formation, in the absence of other bacterial factors.

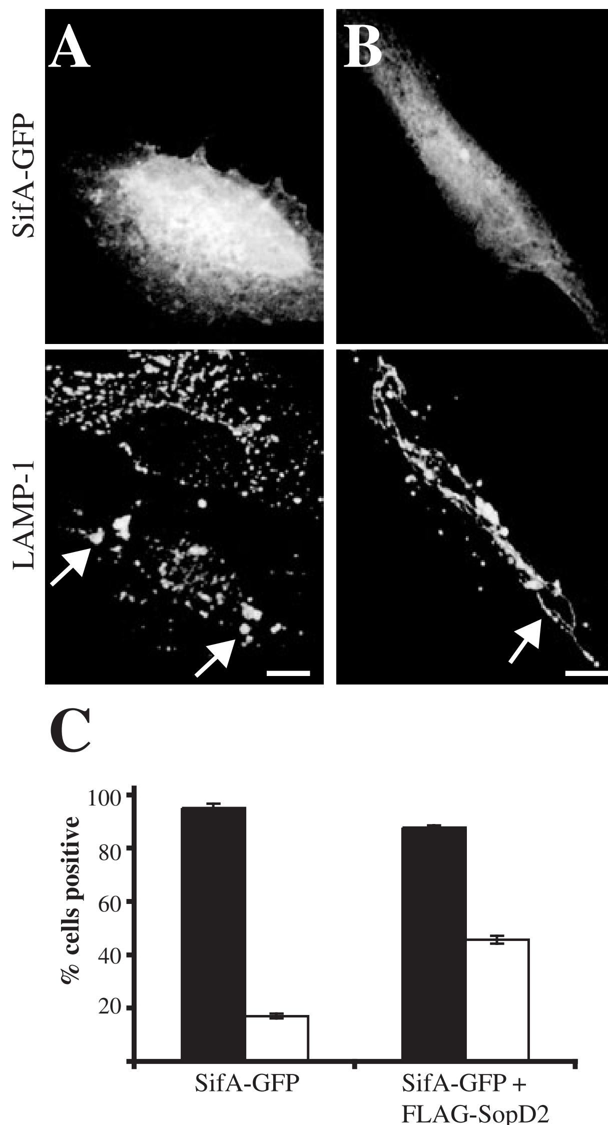

Fig. 6. SifA and SopD2 act cooperatively to promote formation of Sif-like tubules in transfected cells.
A and B. HeLa cells were transfected with either SifA-GFP alone (A) or in the presence of a plasmid encoding FLAG epitope-tagged SopD2 (B). Transfected cells were then fixed, immunostained for LAMP-1 and analysed by confocal microscopy. As shown in (A), expression of SifA-GFP led to the swelling and perinuclear aggregation of late endocytic compartments (see arrows). Coexpression of both the SifA and SopD2 fusion proteins had a similar effect, but also promoted the formation of LAMP-1$^+$ tubules that resembled Sifs (see arrow in B). Bar, 10 µm.
C. Cells were transfected as in (A) and (B) and the formation of swollen and aggregated LAMP-1$^+$ compartments (filled boxes) or Sif-like tubules (open boxes) was enumerated. Values shown are the average ± the standard deviation for three experiments.

Association of SopD2 with Salmonella*-induced filaments and pseudo-Sifs*

We next examined the localization of SopD2 during Sif formation. HeLa cells were infected for 8 h with the *sopD2* mutant of *S.* Typhimurium expressing SopD2-2HA. As shown above, SopD2-2HA was sufficient to complement loss of the chromosomal *sopD2* gene for maximal Sif formation, indicating that the epitope-tagged protein is functional (Fig. 4F). By immunostaining infected cells with antibodies to the HA epitope, we could visualize association of SopD2-2HA with Sifs in infected cells (Fig. 7A). Sifs were detected as long tubular structures containing LAMP-2, and association of SopD2-2HA with these structures was witnessed in all infected cells containing Sifs (see asterisk). As with SifA-2HA, we also observed tubules of SopD2-2HA emanating from the SCV that did not colocalize continuously with LAMP-2 (see arrow). These SopD2-2HA tubules were observed in ≈15% of infected cells, and reveal the association of this effector with pseudo-Sifs. The N-terminus of SopD2 was sufficient for association with Sifs and pseudo-Sifs, as an HA-tagged fusion to the first 200 amino acids of SopD2 was

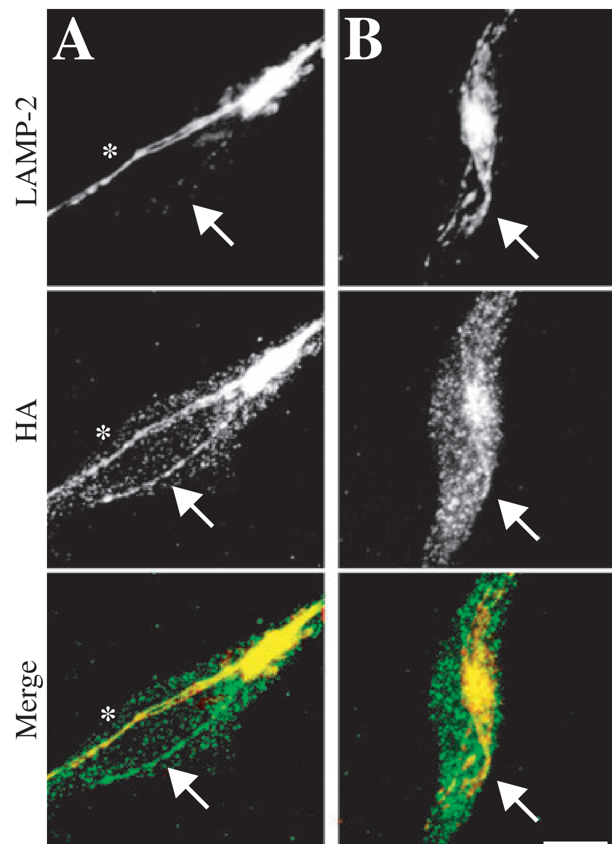

Fig. 7. SopD2 associates with *Salmonella*-induced filaments and pseudo-Sifs. HeLa cells were infected for 8 h with a *sopD2* mutant of *S.* Typhimurium expressing either HA-tagged full-length SopD2 (A) or the first 200 amino acids of this effector (B). Infected cells were then fixed and co-immunostained for LAMP-2 and the HA epitope, and analysed by confocal microscopy. The SopD2-2HA protein localized to Sifs (see asterisk in A) and pseudo-Sifs (see arrow in A). The first 200 amino acids of SopD2 were sufficient to target this protein to Sifs and pseudo-Sifs (see arrow in B). Bar, 10 µm.

observed in assocation with these membrane structures (Fig. 7B). These observations demonstrate that SopD2 and SifA act cooperatively and in a localized fashion to promote late endosomal alterations that lead to Sif formation.

Discussion

In this report, we examined the role of *sopD* and *sopD2* in pathogenesis by *S.* Typhimurium. *SopD2* was found to be broadly conserved among a wide variety of *Salmonella* serovars, including clinical and environmental isolates. A notable exception was *S. bongori*, which lacks SPI-2 and does not contain the *sopD2* gene (http://www.sanger.ac.uk). Our results are in accord with the findings of Porwollik *et al*. (2002) who used microarray analysis to determine the presence of homologues of *S.* Typhimurium LT2 genes in all seven subspecies of *Salmonella*. Their analysis revealed the *sopD2* gene is present in at least one serovar from each of the six *S. enterica* subspecies but not *S. bongori*. Altogether, we conclude that *sopD2* was acquired shortly after acquisition of SPI-2 and divergence of contemporary *S. enterica*. However, it is not clear whether *sopD2* is functional in all *S. enterica* strains. Genomic sequence analysis has revealed that *sopD2* is a pseudogene in *S.* Typhi CT18 and Ty2 (Parkhill *et al*., 2001; Deng *et al*., 2003). As *S*. Typhi evolved as recently as 50 000 years ago (Kidgell *et al.*, 2002), inactivation of *sopD2* may represent a host-specific adaptation.

Based on the high degree of similarity between *sopD* and *sopD2*, it is possible that a gene duplication event occurred early in the evolution of *Salmonella* and that *sopD2* evolved to perform a specialized role in colonization of the host. Other members of the STE family of effectors may have evolved in a similar manner. For example, SifA and SifB are 27% identical (44% similarity) and yet only the former is thought to be involved in Sif formation (Brumell *et al*., 2002). SspH2, SseI, SspH1 and SlrP are also highly similar and contain multiple leucine-rich repeats, yet only the former two effectors interact with actin modulatory proteins in the host cell (Miao *et al*., 2003). Indeed, effector duplication could be the result of ongoing evolution of host–pathogen interactions for *S. enterica.* Similarly, the presence of effector pseudogenes may represent 'fine tuning' of the these interactions.

This study reveals several novel aspects of the role of *sopD* in pathogenesis by *S.* Typhimurium. This protein was first identified as an SPI-1 effector (Jones *et al*., 1998) and plays a major role in promoting enteropathogenesis (Jones *et al*., 1998; Wallis *et al*., 1999; Zhang *et al*., 2002). We show here that *sopD* is also involved in systemic disease in mice. We also show that *sopD* is required for optimal replication in mouse macrophages, the host cell niche exploited during systemic disease in mice (Richter-Dahlfors *et al*., 1997; Salcedo *et al*., 2001). Thus, SopD appears to be acting as a 'dual effector' of both the SPI-1 and SPI-2 TTSS. While the mechanism(s) by which *sopD* contribute to early and late stages of disease are not known, our data provide new insight into the important role of this effector.

Despite the fact that *sopD* and *sopD2* are highly similar in terms of their primary sequence, our studies suggest that these two effectors are not redundant: deletion of either effector had a significant impact on virulence in mice and replication in mouse macrophages. Surprisingly, our studies also suggest a functional relationship between *sopD* and *sopD2*. This is supported by several observations: (i) the *sopD,sopD2* double mutant is not less attenuated for replication in the spleens of infected mice than either of the single effector mutants, (ii) the double mutant competes equally with either single mutant *in vivo* and (iii) the double mutant is not more attenuated than either single mutant for replication in mouse macrophages *in vitro*. As discussed by Beuzon and Holden (2001), two possible scenarios might explain our findings. First, *sopD* and *sopD2* may contribute to the same virulence function in the host. Such a relationship between SopD and SopD2 may seem unlikely as these two effectors are localized to different compartments in the host cell, and as only SopD2 was found to play a role in Sif formation. However, effectors can have more than one function and it remains possible that SopD and SopD2 both contribute to an undetermined phenotype in the host cell. Another possible explanation for our findings is that the functions of SopD and SopD2 are linked because their actions are interdependent. Further study of the function of these two effectors is required to shed light on their possible relationship during infection of the host.

Our understanding of how *S.* Typhimurium initiates Sif formation continues to progress. We propose a model in which the first essential step is delivery of SifA into host cells by the SPI-2 TTSS and its association with SCV/Sifs (Brumell *et al*., 2002), anchored to these membranes by the C-terminus of the protein (Boucrot *et al*., 2003). Next, SifA maintains integrity of the SCV by competing with the actions of SseJ, which is targeted to this compartment and is thought to be involved in its destabilization (Ruiz-Albert *et al*., 2002). Presumably SifA stabilizes the SCV with the recruitment of endosomal membrane from late endocytic compartments, and leads to Sif formation. While pseudo-Sifs appear to be an intermediate in the progression of Sif formation, the nature of these structures is not known. In particular, it remains unclear as to whether these are membranous structures emanating from the SCV or whether they represent filaments of SPI-2 effector proteins attached to microtubules. How SopD2 contributes to Sif formation is not known, but our observation that co-transfection of SopD2 with SifA promotes formation of Sif-

like tubules suggests that they act by complementary biochemical mechanisms. Deletion of either SopD2 (this work) or SseF/SseG (Kuhle and Hensel, 2002; Kuhle *et al.*, 2004) interrupts Sif formation at an intermediate stage, where pseudo-Sifs are observed. Thus, at least four SPI-2 effectors are required for normal Sif formation, providing an interesting parallel with the SPI-1 TTSS in which four effectors are required for invasion (Galan and Zhou, 2000). Deletion of SopD2 or SseF/SseG blocks Sif formation but does not appear to affect stability of the SCV (Fig. 4; Kuhle and Hensel, 2002). Based on these observations, we propose that these three effectors define a second class of SPI-2 effectors that contribute to Sif formation but play little or no role in maintenance of the SCV.

In summary, we have demonstrated that the related effectors *sopD* and *sopD2* play an important role in the mouse model of *S.* Typhimurium infection. We also provide a phenotype for the recently identified SPI-2 effector SopD2. This effector collaborates with at least 3 other SPI-2 effectors to promote endosome transport alterations in the host cell that lead to Sif formation. The mechanisms of how these effectors contribute to Sifs and how their activities are co-ordinated are not known, but the fact that four SPI-2 effectors are required for Sif formation highlights the importance of this phenotype in pathogenesis by *S. enterica*.

Experimental procedures

Cell culture

HeLa (human epithelial cell line) and RAW 264.7 (mouse macrophage cell line) cells were obtained from ATCC. Cells were maintained in DMEM (HyClone) supplemented with 10% FBS (Wisent) at 37°C in 5% CO_2 without antibiotics. Cultures were used between passage numbers 5–25.

Bacterial strains

Wild-type *S.* Typhimurium SL 1344 was used for these studies (Hoiseth and Stocker, 1981). The *ssaR* (Brumell *et al.*, 2001a), *sifA* (Stein *et al.*, 1996) and *sopD2* (Brumell *et al.*, 2003) mutants of this strain have been previously described. To construct the *sopD* mutant with an in frame deletion of codons 6–313 (318 total), we first cloned two 1 kb fragments of flanking DNA from *S.* Typhimurium SL 1344 by polymerase chain reaction (PCR) amplification using Pfx DNA Polymerase (Invitrogen). The primers used to obtain the upstream flanking sequences were sopDKOUSF (5′-ATG C**TC TAG A**TA CAA CCG CAG CGG AAA TAA ACC A-3′) and sopDKOUSR (5′-CGA GGA TCC TAA AGT GAC TGG CAT AAT ATT TT-3′). The downstream flanking sequences were obtained with sopDKODSF2 (5′-CGA GGA TCC ATA TTA CTG ACA TAA AGA TAG TCA-3′) and sopDKODSR2 (5′-ATG C**GC ATG** CCT GGT CGT TAT AAC CTG C-3′). These primers overlap the first and last five codons of the *sopD* open reading frame, respectively, and introduce a *Bam*HI site (underlined in sopDKOUSR and sopDKODSF2) to replace the intervening coding sequence. Both PCR products were cloned into pCR-Blunt II TOPO vector (Invitrogen), and confirmed by sequencing. The DNA fragments flanking the *sopD* gene were released from pCRTOPO2.1 by digestion with *Bam*HI and either *Xba*I (bold in sopDKOUSF for upstream fragment) or *Sph*I (bold in sopDKODSR2 for downstream fragment) and ligated in a trimolecular ligation into the corresponding sites of the positive selection suicide vector pRE112 (Cm^R) (Edwards *et al.*, 1998). The product of this ligation was then transformed into *E. coli* SM10 λpir (Miller and Mekalanos, 1988) and the *S.* Typhimurium SL1344 *sopD* mutant was then constructed by allelic exchange as described (Edwards *et al.*, 1998). A transformant was conjugated with either wild-type SL1344 or the *sopD2* mutant of SL 1344 for generation of the *sopD,sopD2* double mutant and Sm^R/Cm^R colonies were selected. These were grown for 4 h in LB without antibiotic selection, plated on LB agar containing 5% sucrose and incubated overnight at 30°C. Sucrose-resistant colonies were chosen and the in frame deletion of *sopD* coding sequence was confirmed by PCR analysis and sequencing.

The mouse studies were conducted with a separate set of mutants that were generated according to the scheme described above. The upstream flanking sequence of *sopD* was generated with primers sopD1-5′EXT (CGG AAT TCC TCC AAT GCC TTC TGC) and 3′-IGsopD1 (GGA GAT GCA CGG ATG CAT CAT GCG TTC ACC ACG GCC CTG CCC GAT G), which add an *Eco*RI site (underlined) at the 5′ end. The downstream flanking sequence was generated with primers 5′-IGsopD1 (CTA CGG GCA GGG CCG TGG TGA ACG CAT GAT GCA TCC GTG CAT CTC C) and sopD1-3′EXT (GCT CTA GAT GGC GTC TGT GTA GGC), which add an *Xba*I site (underlined) at the 3′ end. The internal primers are entirely homologous to one another to allow for deletion of the intervening sequences. To make the deletion construct, the two PCR products from above were used as the template for another PCR reaction, using primers sopD1-5′EXT and sopD1-3′EXT. The deletion fragment was cloned into the suicide vector at *Eco*RI/*Xba*I, and selection continued as above. The upstream flanking sequence of *sopD2* was generated with primers sopD2-5′EXT (GAA GAT CTT CTG GTG GAT CTC ATC ATT C) and sopD2-3′INT (CTT CTA ATA ATT CTG GAT AGC GGC CCG GAA TGT GTC CAT AAA C), which add a *Bgl*II site (underlined) at the 5′ end. The downstream flanking sequence was generated with primers sopD25′-INT (GTT TAT GGA CAC ATT CCG GGC CGC TAT CCA GAA TTA TTA GAA G) and sopD2-3′EXT (GCT CTA GAG CAG GAC GTT ATT ACT CG), which add an *Xba*I site (underlined) at the 3′ end. These two PCR products were used as the template for another PCR reaction, using sopD2-5′EXT and sopD2-3′EXT. The deletion fragment was cloned into the suicide vector at *Bgl*II/*Xba*I, and selection continued as above. The *sopD,sopD2* double mutant was constructed by the sequential introduction of the *sopD* and *sopD2* deletion constructs.

Southern blotting

The following serovars of *S. enterica* were examined by dot blot analysis for the presence of *sopD* and *sopD2*: *S. typhimurium*, *S. agona*, *S. branderup*, *S. brandenburg*, *S. glos-*

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trup, *S. give*, *S. london*, *S. montevideo*, *S. nierstedten*, *S. othmarschen*, *S. panama*, *S. schwarzengrund*, *S. vejle*, *S. virchow*, *S. hadar*, *S. infantis*, *S. anatum*, *S. bovismorbificans*, *S. java*, *S. tennessee*, *S. ohio*, *S. newport*, *S. manhattan*, *S. heidelberg*, *S. arizonae*, *S. enteriditis*, *S. typhi*, *S. dublin*, *S. gallinarum*, *S. cholerasuis*, *S. senftenberg*, *S. litchfield*. Genomic DNA (5–10 μg) from each strain was spotted onto GeneScreen membrane and allowed to dry for 1 h at room temperature. As a control, ≈10 μg of genomic DNA from *E. coli* was also spotted on the membrane. The membrane was then probed overnight at 65°C and processed according to the manufacturer's instructions for ECL random prime labelling and detection kits (Amersham Life Sciences). The *sopD* probe was generated from a PCR product encoding the full-length open reading frame of *sopD*: primers sopD.FOR (GCT ACC ATG GCT ATG CCA GTC ACT TTA AGC) and sopD1 3Nco (CAT GCC ATG GCT GTC AGT AAT ATA TTA CG). The *sopD2* probe was generated from a PCR product encoding the full-length open reading frame of *sopD2*: primers sopD2.FOR (GCT AAC ATG TCT ATG CCA GTT ACG TTA AGT) and sopD2.REV (CAT GAC ATG TCT ATA AGC ATA TTG CGA CA). The probes were labelled according to the protocol for random prime labelling in the ECL kit. For Southern blots, 10 μl of genomic DNA was digested overnight with either *Hin*dIII or *Eco*RI. As a control, digested *E. coli* genomic DNA was included, as well as undigested plasmid DNA containing the open reading frames of either *sopD* or *sopD2*. The DNA was run on a 1.2% agarose gel and transferred to GeneScreen membrane. The membrane was probed and processed as above.

Bacterial infection of mice

For the competition assays, 6- to 8-week-old female BALB/c mice were inoculated by intraperitoneal injection with 100 μl of inoculum containing a total of 10^5 bacteria, comprised of equal numbers of the two strains to be tested. After 48 h, mice were euthanized and their spleens removed. Spleens were mechanically homogenized in 5 ml of PBS. Dilutions were plated onto LB plates containing the appropriate antibiotics and incubated overnight at 37°C. The different strains used were differentiated on the basis of antibiotic sensitivity. Colonies were counted and the number of each strain recovered was calculated.

Plasmids and transfection

The low-copy plasmid pACYC184 (NCBI Accession No. X06403) was used to express SifA (Brumell *et al*., 2002), SopD or SopD2 (Brumell *et al*., 2003) in *S.* Typhimurium with two HA epitope tags, as previously described. Plasmids were transformed into *S.* Typhimurium by electroporation and expression of epitope-tagged SPI-2 effectors was confirmed by immunoblotting bacterial cell lysates and by immunostaining infected cells with antibodies to the HA epitope.

Generation of GFP fusions to the C-termini of SifA (Brumell *et al*., 2001a) and SopD2 (Brumell *et al*., 2003) has been previously described. For expression of SopD2 in HeLa cells, C-terminal fusion to the FLAG epitope tag was accomplished by PCR amplification of the *sopD2* gene from a genomic library of *S.* Typhimurium, using the following primers: JBO119 (5′-CGC GGA TCC ATG CCA GTC ACT TTA AGC TTC GGT AAT-3′) and JBO120 (5′-TGC GGT CGA CTT ATG TCA GTA ATA TAT TAC GAC TGC A-3′). After digestion with *Bam*HI and *Sal*I (sites underlined above), the PCR product was ligated into the multiple cloning region of the pCMV-Tag2B vector from Stratagene. Plasmid DNA was purified using the Midiprep kit from Qiagen and used for transfection of cells with the GeneJuice transfection reagent (Oncogene Research Products) according to the manufacturer's instructions.

Bacterial infection of cell cultures

HeLa epithelial cells were seeded at 5×10^4 cells per well in 24-well tissue culture plates 16–24 h before use. Late-log bacterial cultures were used for infecting HeLa cells and prepared using a method optimized for bacterial invasion (Steele-Mortimer *et al*., 1999). In brief, bacteria were grown for 16 h at 37°C with shaking and then subcultured (1:33) in LB broth for 3 h. Bacterial inocula were prepared by pelleting at 10 000 *g* for 2 min and then directly resuspending in PBS, pH 7.2. The inoculum was diluted and added to cells at a multiplicity of infection (moi) of ≈100:1 at 37°C for 10 min. After infection, extracellular bacteria were removed by extensive washing with PBS and addition of growth medium containing gentamicin (50 μg ml^{-1}). For experiments in which cells were incubated for more than 2 h after bacterial infection, the gentamicin concentration was subsequently decreased to 5 μg ml^{-1}.

To enumerate Sif formation, HeLa cells were fixed and immunostained for LAMP-1 and intracellular bacteria (see below). The number of infected cells that contain Sifs was determined for at least 100 infected cells. Each time point was performed in duplicate, and each individual experiment was performed at least three times. The average ± the standard deviation for these experiments is presented.

For enumeration of intracellular bacteria, cells were washed three times with PBS and lysed in Lysis buffer (1.0% Triton X-100, 0.1% SDS in PBS, pH 7.2) and a dilutions series was plated onto LB agar plates with 50 μg ml^{-1} streptomycin. Plates were incubated overnight at 37°C and colonies were counted. Each time point was performed in duplicate, and each individual experiment was performed at least three times. Fold replication of intracellular bacteria was determined by dividing the number of bacteria recovered at each time point by the number recovered 1.5 h post infection. The average ± the standard deviation for these experiments is presented.

RAW macrophages were seeded at 2.5×10^5 cells per well in 24-well tissue culture plates 16–24 h before use. Stationary-phase bacterial cultures were used for infecting RAW cells. Bacteria were cultured overnight at 37°C with shaking and then opsonized in PBS containing 20% human serum for 20 min at 37°C. Bacteria were then washed three times with PBS, resuspended in DMEM containing 10% FBS and added to cells at an moi of ≈10:1. Cultures were centrifuged at 1000 *g* for 5 min at 4°C to promote bacterial contact with macrophages. Infected cultures were then incubated at 37°C for 20 min, washed three times with PBS and then incubated in growth medium containing gentamicin (100 μg ml^{-1}). The

gentamicin concentration was subsequently decreased to 10 μg ml^{-1} after 2 h. Intracellular bacteria were enumerated as above for Hela cells. Fold replication of intracellular bacteria was determined by dividing the number of bacteria recovered at 21 h by the number recovered 2 h post infection. The average ± the standard deviation for these experiments is presented.

Immunofluorescence

Cells were fixed in 2.5% paraformaldehyde in PBS pH 7.2 for 10 min at 37°C. Fixed cells were washed twice with PBS and permeabilized/blocked by treatment with 0.2% saponin (Calbiochem) in PBS containing 10% normal goat serum (SS-PBS) for 1–16 h. Primary and secondary antibodies were overlaid on coverslips in SS-PBS for 1–2 h, followed by three washes with PBS. Coverslips were mounted onto 1 mm glass sides using Mowiol (Aldrich). Samples were analysed using a Zeiss Axiovert microscope (63× objective). Confocal sections were assembled into flat projections using Zeiss LSM software, imported into Adobe Photoshop in RGB format and assembled in Adobe Illustrator for labelling.

Antibodies

Rabbit polyclonal antibodies to human LAMP-2 were generously provided by Dr M. Fukuda, La Jolla Cancer Research Foundation, USA (Carlsson *et al*., 1988). Murine monoclonal anti-human LAMP-1 antibodies (clone H4A3) developed by T. August were obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Rabbit polyclonal antibodies to *S.* Typhimurium were obtained from Difco. Mouse monoclonal antibodies to the HA epitope tag (MMS-101R; Covance) were used at a final dilution of 1:1000 for immunofluorescence, after removal of non-specific cross reactivity by incubation with an acetone powder from HeLa cells. Secondary antibodies used were: Alexa 488-conjugated donkey anti-rabbit IgG, Alexa 488-conjugated goat anti-murine IgG, Alexa 568-conjugated donkey anti-rabbit IgG and Alexa 568-conjugated goat anti-murine IgG, all from Molecular Probes. Cy5-conjugated donkey anti-rabbit IgG was from Jackson Immunoresearch Laboratories.

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