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A *Salmonella* virulence protein that inhibits cellular trafficking

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Salmonella enterica **requires a type III secretion system, designated Spi/Ssa, to survive and proliferate within macrophages. The Spi/Ssa system is encoded within the SPI-2 pathogenicity island and appears to function intracellularly. Here, we establish that the SPI-2-encoded SpiC protein is exported by the Spi/Ssa type III secretion system into the host cell cytosol where it interferes with intracellular trafficking. In J774 macrophages, wild-type** ***Salmonella*** **inhibited fusion of** ***Salmonella*****-containing phagosomes with lysosomes and endosomes, and interfered with trafficking of vesicles devoid of the microorganism. These inhibitory activities required living** ***Salmonella*** **and a functional** ***spiC*** **gene. Purified SpiC protein inhibited endosome–endosome fusion** ***in vitro*****. A Sindbis virus expressing the SpiC protein interfered with normal trafficking of the transferrin receptor** ***in vivo*****. A** ***spiC*** **mutant was attenuated for virulence, suggesting that the ability to interfere with intracellular trafficking is essential for** ***Salmonella*** **pathogenesis.**

Keywords: endosome/intracellular trafficking/phagosome/*Salmonella*/type III secretion

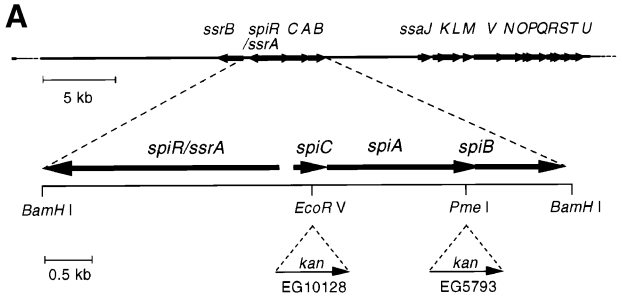
Introduction

Type III secretion systems are specialized protein export machineries that use a Sec-independent mechanism to deliver virulence proteins into the cytosol of host cells (Lee, 1997; Hueck, 1998). The Gram-negative pathogen *Salmonella enterica* harbors a type III system (designated Inv/Spa and encoded with the SPI-1 pathogenicity island) that is active extracellularly and mediates invasion of non-phagocytic cells (Galán, 1996). But *Salmonella* harbors a second type III secretion system (designated Spi/Ssa and encoded in the SPI-2 pathogenicity island) (Ochman *et al.*, 1996; Shea *et al.*, 1996; Hensel *et al.*, 1997b) which is unusual in that it appears to function intracellularly: the Spi/Ssa system is transcriptionally induced to high levels within macrophages (Valdivia and Falkow, 1997; Cirillo *et al.*, 1998) and it is essential for survival/proliferation within macrophages (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998). (These two systems are in addition to the type III system responsible for flagellar export/assembly.) In this paper, we identify the SPI-2-encoded SpiC protein as an effector of the Spi/Ssa system and show that SpiC is delivered into the cytosol of the host cell where it interferes with intracellular membrane trafficking.

Salmonella enterica can survive within host phagocytic cells and this ability is paramount to its pathogenicity since *Salmonella* mutants that are unable to replicate within macrophages *in vitro* are highly attenuated for mouse virulence *in vivo* (Fields *et al.*, 1986). A large number of macrophage survival genes have been identified including several mapping to SPI-2, a 40 kb pathogenicity island located at 31 min in the *S.enterica* serovar. Typhimurium chromosome (Ochman *et al.*, 1996; Shea *et al.*, 1996; Hensel *et al.*, 1997a). [Pathogenicity islands are chromosomal gene clusters harboring virulence genes that are absent from related, non-pathogenic species (Groisman and Ochman, 1996; Hacker *et al.*, 1997).] The 32 genes identified so far in SPI-2 encode a type III secretion system (referred to as Spi/Ssa), a two-component regulatory system and proteins of unknown function including putative effectors of the Spi/Ssa system (Ochman *et al.*, 1996; Shea *et al.*, 1996; Hensel *et al.*, 1997b, 1998, 1999; Cirillo *et al.*, 1998) (Figure 1A).

The SPI-2 island was originally implicated in survival/proliferation within host cells because a *spiA* mutant, which lacks a putative component of the Spi/Ssa type III system, was found to be defective for replication in J774 macrophages (Ochman *et al.*, 1996). This view was transiently challenged (Hensel *et al.*, 1997b), but it has now been corroborated and extended using several other SPI-2 mutants and different types of macrophages (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). Because *Salmonella* inhibits phagosome–lysosome fusion (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991) and survives within phagosomes that diverge from the normal degradative pathway of the macrophage (Garcia-del Portillo and Finlay, 1995a; Rathman *et al.*, 1997), it has been hypothesized that the Spi/Ssa system may confer intramacrophage survival by secreting proteins that target host functions required for phagosome–lysosome fusion or phagosome acidification (Ochman *et al.*, 1996).

In this paper, we identify SpiC as the first SPI-2-encoded protein secreted via the Spi/Ssa type III system into the macrophage cytosol. We establish that *Salmonella* requires a functional *spiC* gene to inhibit phagosome–lysosome fusion and to interfere with normal trafficking of vesicle compartments devoid of *Salmonella*. We show that purified SpiC protein is sufficient to inhibit endosome–endosome fusion *in vitro* and that expression of the SpiC protein from a Sindbis virus interferes with normal trafficking of transferrin. We also demonstrate that *spiC* mutants are attenuated for virulence in mice, indicating that the ability to interfere with intracellular membrane trafficking is essential for *Salmonella* pathogenesis.



B

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MLAVLKGIPL IQDIRAEGNS RSWIMTIDGH PARGEIFSEA FSISLFLNDL
ESLPKPCLAY VTLLLAAHPD VHDYAIQLTA DGGWLNGYYT TSSSSELIAI
EIEKHLALTC ILKNVIRNHH KLYSGGV
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C

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  31   PARGEIFSEA FSISLFLNDL ESLPKPCLAY VTLLLAA     67
       |* | ||**| | * |   **   * * | |* | ||| |
1922   PSSGTIFNDA FWLDLNYLEV AKVAQSCAAH FTALLYA  1958
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Fig. 1. Map of the *spi* locus, amino acid sequence of the SpiC protein and region of sequence similarity between the SpiC and ATM proteins. (**A**) Physical and genetic map of the *spi/ssa* locus in the SPI-2 pathogenicity island of *S.enterica* serovar Typhimurium chromosome. The location of *kan* cassette insertions in the *spiC* and *spiA* mutants EG10128 and EG5793 (Ochman *et al.*, 1996), respectively, is indicated by triangles. (**B**) Deduced amino acid sequence of the SpiC protein. Underlined residues correspond to synthetic peptide used to generate anti-SpiC antibodies. (**C**) Alignment of the deduced amino acid sequences of segments of the SpiC and ATM proteins. Identical and similar residues are indicated by vertical lines and asterisks, respectively.

Results

The spiC gene is required for virulence

The *spiC* gene encodes a 127 amino acid polypeptide (Figure 1B) that has no homologs in the sequence databases. To examine the role of *spiC* in *Salmonella* pathogenesis, we constructed a mutant harboring a *kan* cassette in the chromosomal copy of the *spiC* gene (Figure 1A) and investigated the virulence properties of the resulting strain. The *spiC* mutant could not survive within macrophages but displayed wild-type levels of invasion into epithelial cells (Figure 2), similar to a strain harboring a mutation in the adjacent gene *spiA* (Ochman *et al.*, 1996). The *spiC* mutant was highly attenuated for virulence in mice: the median lethal dose (LD_{50}) of the *spiC* strain was $>3.6\times10^6$ versus <10 for wild-type *Salmonella* in intraperitoneally inoculated animals. The virulence defect of the *spiC* mutant was solely due to the absence of *spiC*, shown by the fact that both macrophage survival and mouse virulence could be restored to wild-type levels by a plasmid harboring the *spiC* gene (Figure 2).

The SpiC protein is secreted into the macrophage cytosol

We hypothesized that SpiC might be an effector protein of the Spi/Ssa type III secretion system (rather than a structural component of the secretion apparatus or an effector chaperone) because it does not exhibit similarity to the type III secretion proteins described to date and does not display features typical of effector chaperones (Hueck, 1998). We explored the possibility that the SpiC protein might be secreted into the macrophage cytosol using confocal microscopy and indirect immunofluorescence. When macrophages were infected with wild-type *Salmonella*, the SpiC protein was detected in the host cell cytosol and it was also associated with intracellular bacteria (Figure 3D–F). The amount of SpiC protein in the cytosol increased during the course of infection: it was higher at 6 h than at 1 h post-infection (Figure 3M–O). In contrast, when macrophages were infected with the *spiA* mutant (Ochman *et al.*, 1996), which is defective in a putative component of the Spi/Ssa type III secretion apparatus, the SpiC protein was only associated with intracellular bacteria and could not be detected in the host cell cytosol (Figure 3G–I). Thus, in the *spiA* mutant the SpiC protein was being produced but not exported. The few SpiC-positive structures that did not stain with the anti-*Salmonella* antibody (Figure 3F) might correspond to bacteria present in a different plane of focus. As expected, no SpiC staining was detected in the bacteria or host cytosol

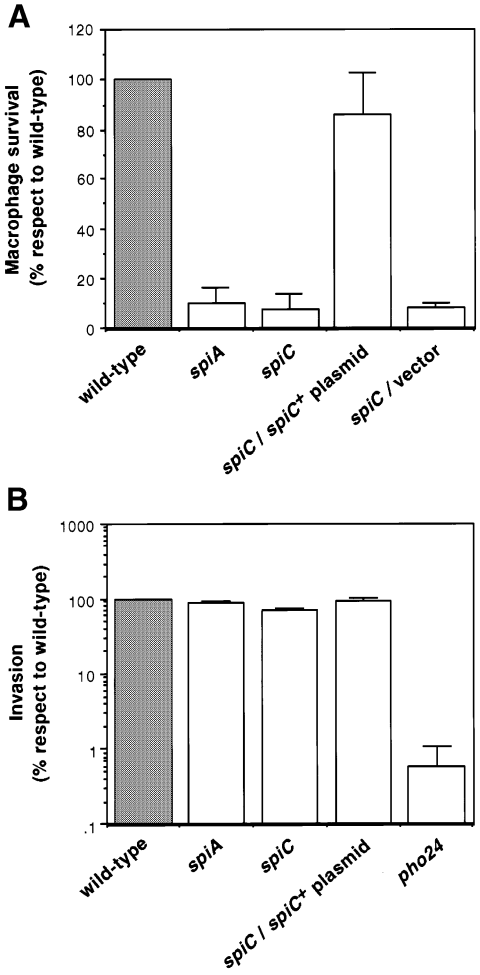

Fig. 2. A *Salmonella spiC* mutant is defective for intramacrophage survival. (**A**) Intramacrophage survival properties of wild-type, *spiA* and *spiC* mutant strains of *S.enterica* in J774 macrophages. Survival was determined 18 h after infection of the macrophage as described in the Materials and methods. For each mutant, the percentage survival relative to the wild-type strain is presented. Macrophage survival can be restored to the *spiC* mutant by the $spiC^+$-containing single copy plasmid pEG9127 but not by the plasmid vector (pBAC108L). (**B**) *Salmonella spiC* and *spiA* mutants exhibit wild-type levels of invasion into Henle-407 cells. The invasion-defective mutant *pho-24* was evaluated in the same experiments and is shown here for comparative purposes. Values represent the mean of three independent experiments ±SD.

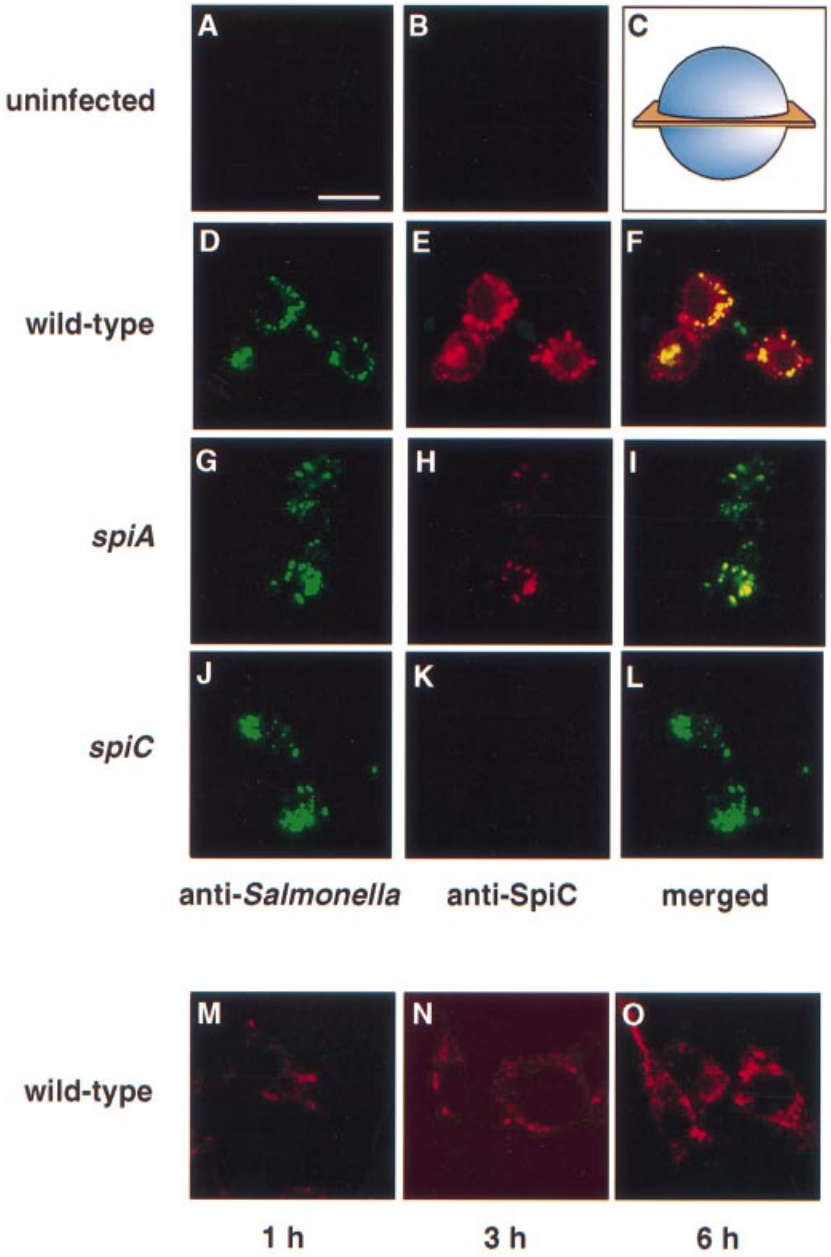

Fig. 3. The *Salmonella* SpiC protein is secreted into the macrophage cytosol in a Spi/Ssa-dependent manner. Confocal immunofluorescence analysis of SpiC protein location in J774-macrophages: non-infected (**A**, **B**), or infected with wild-type (**D**–**F**), *spiA* (**G**–**I**) and *spiC* (**J**–**L**) *Salmonella*. Images correspond to cells harvested 6 h after bacterial internalization. (**C**) The plane of focus where pictures were taken. (**M**–**O**) Cells infected with wild-type *Salmonella* 1, 3 and 6 h after bacterial internalization. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100 as described in Materials and methods prior to incubation with antibodies. The primary antibodies were purified polyclonal anti-SpiC peptide (B, E, H, K) and monoclonal anti-*Salmonella* (A, D, G, J). The secondary antibodies were FITC-labeled donkey anti-rabbit and rhodamine-labeled donkey anti-mouse IgG antibodies. (F, I, L) A superimposition of the images in (D, E), (G, H) and (J, K): yellow indicates areas of colocalization between the internalized FITC-labeled-*Salmonella* and rhodamine-labeled-SpiC protein. The SpiC protein is present in the cytosol of macrophages infected with wild-type *Salmonella* but not in the cytosol of *spiA* or *spiC* mutants. Intracellular wild-type and *spiA Salmonella* harbor SpiC, whereas microorganisms located extracellularly do not express SpiC and are stained only with the the anti-*Salmonella* monoclonal antibody. Increasing amounts of SpiC protein are detected in the cytosol of infected cells as the infection progresses from 1 to 6 h. Bar corresponds to 8 μm.

in macrophages infected with the *spiC* mutant (Figure 3J–L) or in mock-infected macrophages (Figure 3A and B).

SpiA-dependent secretion of the SpiC protein was further examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis. A band corresponding to SpiC was present in cytosolic extracts of macrophages infected with wild-type *Salmonella* (Figure 4). This band was absent from the cytosol of mock-infected macrophages and the cytosol of macrophages infected with the *Salmonella spiC* mutant (Figure 4). Consistent with the immunofluorescence results, when macrophages were infected with the *spiA* mutant, the SpiC protein was detected in the bacteria-containing pellet fraction but not in the cytosol (Figure 4). Control experiments argue against the possibility that the SpiC protein detected in the macrophage cytosol originated

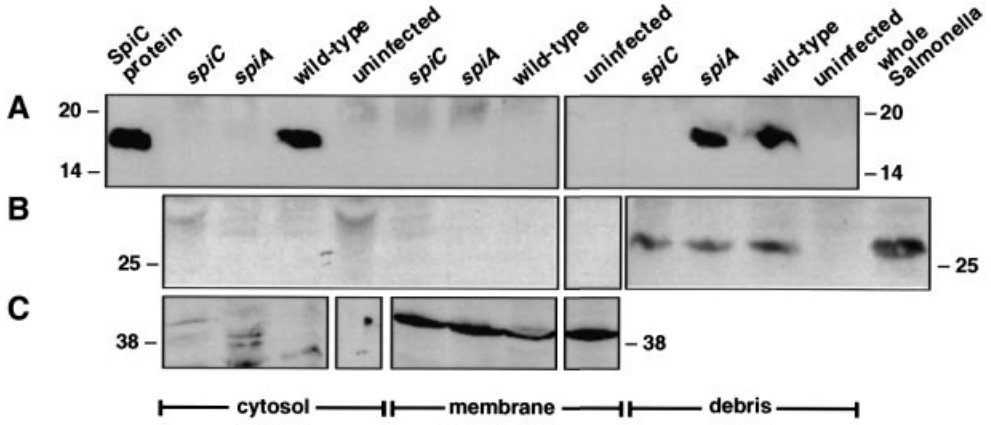

Fig. 4. The *Salmonella* SpiC protein is secreted into the macrophage cytosol in a Spi/Ssa-dependent manner. Western blot analysis of macrophage cytosolic extracts of mock-infected or infected with wild-type, *spiC*, or *spiA Salmonella*, harvested 6 h post infection. Cytosolic extracts were prepared as described in the Materials and methods, run on a 12.5% SDS–polyacrylamide gel, transferred to nitrocellulose membranes and developed using: (**A**) polyclonal antibodies against a 19 amino acid SpiC-derived peptide (Figure 1B). Purified SpiC protein (1 μg) was run as a control. The SpiC protein is detected in the cytosol of macrophages infected with wild-type *Salmonella* but not in the cytosol of *spiC* and *spiA* mutants or in mock-infected macrophages, or in any membrane fraction. The SpiC protein is present in the debris (bacteria-containing fraction) corresponding to macrophages infected with wild-type or *spiA Salmonella*. (**B**) The anti-*Salmonella* monoclonal antibodies reacted with material in the debris fraction of macrophages infected with wild-type, *spiC* or *spiA Salmonella*. However, there was no reactivity with the cytosolic or membrane fractions, indicating that the SpiC protein secreted into the macrophage cytosol did not originate from lysed bacterial cells. (**C**) The anti-Fc receptor antibodies gave a positive reaction in the membrane fraction of *Salmonella*-infected and mock-infected cells but not in the cytosolic fraction, arguing against membrane contamination of the cytosolic fraction.

from lysed bacterial cells: there was no reactivity with an anti-*Salmonella* monoclonal antibody in the cytosolic or membrane fractions, whereas the bacteria-containing pellet fraction was positive (Figure 4). Moreover, reprobing the blots with anti-Fc receptor antibodies detected a band in the membrane but not in the cytosolic fraction (Figure 4), indicating that the cytosolic preparations were not contaminated with host cell membranes. It is noteworthy that the SpiC protein was detected in the macrophage cytosol shortly after infection, when there are no differences in bacterial viability between wild-type *Salmonella*, and the *spiC* and *spiA* mutants. Cumulatively, these results indicate that *Salmonella* delivers the SpiC protein into the host cell cytosol using the Spi/Ssa type III secretion system.

Salmonella inhibits phagosome–lysosome in a spiC-dependent manner

Salmonella inhibits phagosome–lysosome fusion in a process that requires bacterial viability and protein synthesis (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991). To examine whether the *spiC* gene product was required for this inhibitory activity, we investigated the co-localization of bacteria and BSA-gold particles within membrane-bound compartments in macrophages infected with either wild-type or *spiC Salmonella*. We found that wild-type *Salmonella* inhibited phagosome–lysosome fusion (Table I), in agreement with previous reports that used other microscopy techniques (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991; Garcia-del Portillo and Finlay, 1995a; Rathman *et al.*, 1997). In contrast, the *spiC* mutant was defective in this inhibitory activity, exhibiting phagosome–lysosome fusion levels (Table I) similar to those displayed by heat-killed *Salmonella* or by an avirulent strain of *Escherichia coli* (Buchmeier and Heffron, 1991). Wild-type *Salmonella* also inhibited fusion of phagosomes with endosomes in a *spiC*-dependent manner (Table I), indicating that the inhibitory activity of the *spiC* gene extends to endocytic compartments. The inability of *spiC Salmonella* to inhibit fusion of phagosomes with lysosomes and endosomes is specifically associated with the absence of *spiC* and is not due to an incapacity to survive within macrophages, which is shown by the fact that a *purB* mutant (which is defective for intramacrophage survival owing to a nutritional defect) exhibited wild-type levels of phagosome–lysosome and phagosome–endosome fusion (Table I). Furthermore, inhibition of vesicle fusion could be restored to wild-type levels by introducing a plasmid carrying the wild-type *spiC* gene into the *spiC* mutant (Table I).

Table I. Inhibition of phagosome–lysosome and phagosome–endosome fusion by wild-type and mutant *Salmonella*

Strains	Time (h)	Phagosome–lysosome fusion	Phagosome–endosome fusion
wild-type	4	21.3 ± 2.3	28.9 ± 4.2
	14	44.8 ± 3.2	52.0 ± 0.7
spiC	4	45.2 ± 2.0	56.2 ± 6.4
	14	74.7 ± 0.5	87.3 ± 0.3
spiC/*spiC*$^+$ plasmid	4	17.2 ± 1.0	26.5 ± 4.6
	14	35.9 ± 10.0	47.8 ± 16.3
purB	4	21.5 ± 0.8	34.6 ± 7.7
	14	n.t.	n.t.

J774-E clone macrophages were first incubated with BSA-gold (30 nm) for 24 h to label lysosomal compartments and then incubated with bacteria for 4 or 14 h. After bacterial internalization, macrophages were incubated with BSA-gold (10 nm) for 30 min to label endosomal compartments, and then incubated for 1.5 h before analysis. Cells were fixed with glutaraldehyde and examined by electron microscopy. Phagosome–lysosome fusion was defined as the number of phagosomes positive for 30 nm BSA-gold divided by the total number of phagosomes multiplied by 100. Phagosome–endosome fusion was defined as the number of phagosomes positive for 10 nm BSA-gold divided by the total number of phagosomes multiplied by 100. The data represent the mean ± SD of counts from at least 100 cells on each of three electron microscopy grids. n.t., not tested.

The SpiC protein interferes with normal cellular trafficking

Because the SpiC protein is secreted into the macrophage cytosol (Figures 3 and 4), we hypothesized that SpiC might interfere with different membrane fusion events in addition to those involving the phagosome harboring *Salmonella*. Trafficking, as reflected by proteolysis of an internalized ligand, [^{125}I]Man-BSA, was strongly inhibited in macrophages infected by wild-type *Salmonella* (Figure 5). This inhibitory effect required living bacteria; heat-killed *Salmonella* did not alter trafficking (Figure 5). Moreover, it was shown to be dependent on a functional *spiC* gene; macrophages infected with a *spiC* mutant behaved like those infected with heat-killed *Salmonella* and the *spiC*-containing plasmid restored trafficking inhibition to the *spiC* mutant (Figure 5). In this assay, the magnitude of the inhibitory effect displayed by wild-type *Salmonella* was similar to that observed upon treatment of macrophages with wortmannin (K.Funato and P.D.Stahl, unpublished results), an inhibitor of phosphoinositol-3 kinase that affects transport to lysosomes (Brown *et al.*, 1995). These results indicate that the *spiC* gene is required to interfere with normal trafficking of vesicles devoid of the microorganism.

The SpiC protein is sufficient to alter normal trafficking of the transferrin receptor in vivo

To examine whether the SpiC protein was sufficient to interfere with membrane fusion events *in vivo*, we investigated whether expression of the *spiC* gene from a Sindbis virus vector affected trafficking of the transferrin receptor. Endocytosis of [^{125}I]transferrin was inhibited in cells infected with a SpiC-expressing Sindbis virus relative to cells infected with the Sindbis virus vector (Figure 6A). The degree of inhibition was virtually identical to that displayed by a Sindbis virus expressing a dominant negative form of the GTPase Rab5 (S34N), which was previously shown to inhibit this process (Stenmark *et al.*, 1994). The SpiC protein also inhibited the release of [^{125}I]transferrin (Figure 6B), whereas cells expressing the dominant negative form of the Rab5 protein (S34N) behaved similarly to vector-infected cells, consistent with Rab5 not being involved in this process.

Purified SpiC protein inhibits endosome–endosome fusion in a cell-free system

The process of vesicle fusion has been reconstituted *in vitro* (Diaz *et al.*, 1988) and shown to require ATP and cytosol (Diaz *et al.*, 1989; Mayorga *et al.*, 1993; Rodriguez *et al.*, 1994). We found that extracts prepared from macrophages infected with wild-type *Salmonella* were defective in promoting endosome–endosome fusion (Figure 7A), whereas cytosol from macrophages infected with the *spiC* mutant exhibited the stimulatory activity typical of uninfected macrophages (Figure 7A). These results suggest that wild-type *Salmonella* interferes with

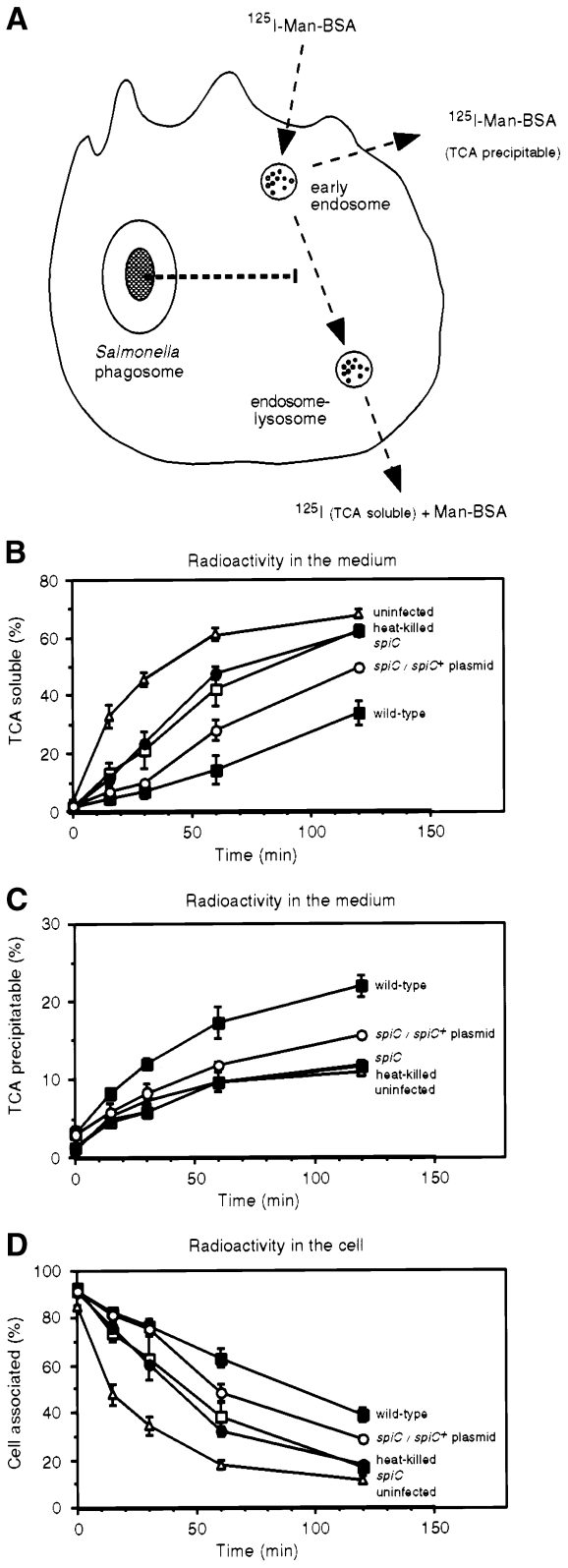

Fig. 5. *Salmonella* interferes with normal cellular trafficking. (**A**) Experimental strategy to examine the ability of *Salmonella* to interfere with degradation of a mannosylated ligand along the endocytic pathway. Macrophages plated into 24-well culture dishes at 5×10^5 cells/well were infected with 1×10^7 bacteria for 14 h. [^{125}I]Man-BSA was added to infected macrophages and the plates incubated and processed as described in the Materials and methods. (**B**) Trichloroacetic acid (TCA)-soluble radioactivity in the medium, which reflects the fraction of [^{125}I]Man-BSA that was processed to free ^{125}I. (**C**) TCA-precipitable radioactivity in the medium, which reflects the fraction of [^{125}I]Man-BSA that remained as [^{125}I]Man-BSA. (**D**) Cell-associated radioactivity, reflecting the fraction of [^{125}I]Man-BSA that remained in intracellular vesicles. Experiments were performed in triplicate.

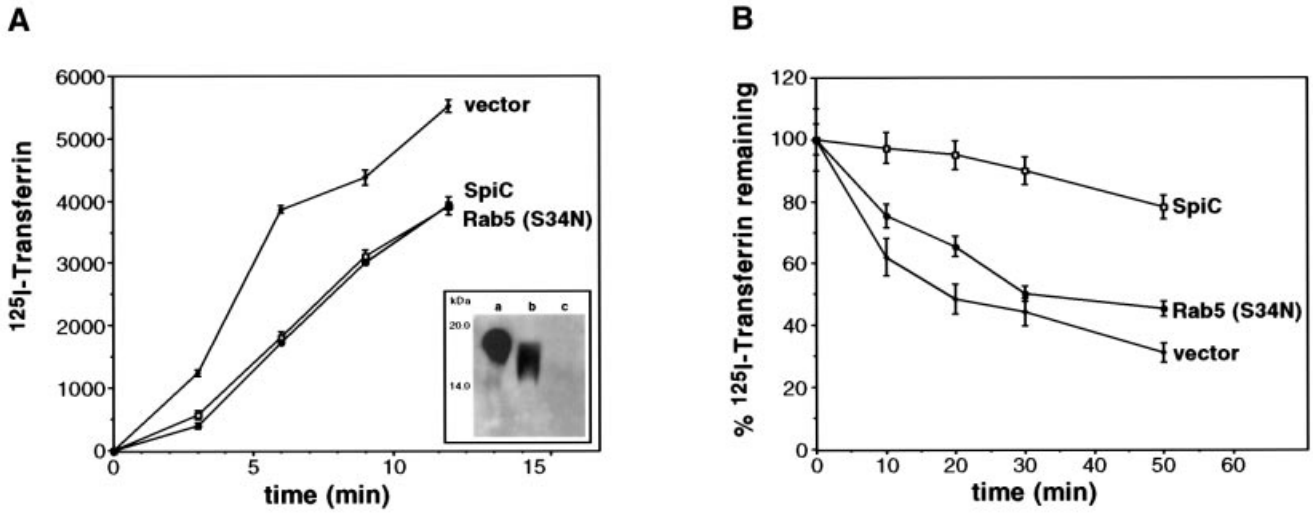

Fig. 6. SpiC interferes with transferrin uptake and recycling *in vivo*. (**A**) ^{125}I-labeled transferrin was incubated with TRVb-1 cells transfected with the Sindbis virus vector, or with virus containing SpiC or Rab5 (S34N), warmed as described in the Materials and methods and washed to remove surface-bound transferrin. Cells were solubilized to determine the amount of internalized transferrin at different times. SpiC- and Rab5 (S34N)-expressing cells inhibit transferrin uptake relative to the Sindbis virus control. Inset shows Western blot analysis of TRVb-1 cells infected with SpiC-expressing Sindbis virus (lane b) or the Sindbis virus vector (lane c) using anti-SpiC peptide antibodies. Lane a corresponds to purified SpiC protein. (**B**) To measure recycling, TRVb-1 cells (transfected with the Sindbis virus vector, or with virus containing SpiC or Rab5 [S34N]) were incubated with ^{125}I-labeled transferrin for 2 h at 37°C. The cells were washed to remove surface-bound transferrin as described in the Materials and methods and incubated in 600 μg/ml unlabeled transferrin. At various times, the medium and one wash were collected, and the cells were solubilized to determine recycled and cell-associated radioactivity. SpiC-expressing cells inhibited transferrin recycling relative to vector or Rab5 (S34N)-expressing cells. Data correspond to mean values of two independent experiments done in duplicate ±SD.

normal cellular trafficking by secreting SpiC (and potentially other proteins) into the macrophage cytosol.

Recombinant His-tagged SpiC protein purified from *E.coli* K-12 inhibited endosome–endosome fusion in a dose-dependent manner (Figure 7B) with an IC_{50} of 25 μM. Boiling (Figure 7B) or trypsinization (data not shown) of SpiC destroyed its inhibitory activity. The inhibitory effect of the SpiC protein was not due to the histidine tag or to trace amounts of bacterial contaminants in the preparation, shown by the fact that His-tagged PmrA, a *Salmonella* DNA-binding protein purified in a similar fashion to the His-tagged SpiC protein, did not inhibit fusion (Figure 7B). The degree of inhibition exhibited by the SpiC protein is similar to that displayed by a dominant-negative mutant of Rab5 (S34N) (Barbieri *et al.*, 1994). These results demonstrate that the SpiC protein is sufficient to inhibit vesicle fusion in a defined *in vitro* system. Moreover, they are in agreement with the *in vivo* phenotype of *spiC* mutants, which cannot inhibit fusion of the *Salmonella*-containing phagosome and either lysosomes or endosomes (Table I).

Discussion

Several facultative intracellular pathogens can inhibit phagosome–lysosome fusion (Garcia-del Portillo and Finlay, 1995b). However, our experiments provide a singular example of an intracellular pathogen—*S.enterica* serovar. Typhimurium—that interferes with fusion events involving vesicular compartments devoid of the microorganism. We have identified a mutant that cannot inhibit vesicle fusion and established that this mutant is missing a protein (SpiC) that is exported into the macrophage cytosol by a type III secretion system that functions intracellularly. We also demonstrate that the SpiC protein is sufficient to inhibit membrane fusion events both *in vitro* and *in vivo*.

SpiC secretion by the Spi/SsA type III system: export from within

Type III secretion systems typically deliver virulence proteins into host cells by extracellularly located microorganisms (Mecsas and Strauss, 1996; Lee, 1997; Hueck, 1998). These virulence proteins are often found in the culture supernatants of bacteria grown under laboratory conditions. The identity of the effector proteins exported by the Spi/Ssa type III system of *Salmonella* has remained unknown, perhaps because this system appears to be expressed only within host cells (Valdivia and Falkow, 1997; Cirillo *et al.*, 1998). Several SPI-2 genes have been proposed to encode effectors of the Spi/Ssa system because they exhibit sequence similarity to effector proteins of other microorganisms (Hensel *et al.*, 1998); however, export of these proteins has not been demonstrated. Our experiments identify the SpiC protein as the first effector protein of the Spi/Ssa type III system: the levels of SpiC increased between 1 and 6 h post-infection in the cytosol of macrophages infected with wild-type *Salmonella* (Figures 3 and 4). SpiC was also detected in intracellular bacteria but not in extracellularly located microorganisms (Figure 3). On the other hand, in macrophages infected with a *spiA* mutant the SpiC protein remained associated with the bacterium (Figures 3 and 4). The *spiA* gene product is a predicted outer membrane component of the Spi/Ssa type III system which exhibits sequence identity to several type III secretion proteins (e.g. *Salmonella* InvG, *Yersinia* YscC) that are required for the export of their respective effector proteins (Hueck, 1998).

As *Salmonella* does not escape from host cell phagosomes (Garcia-del Portillo and Finlay, 1995b), our

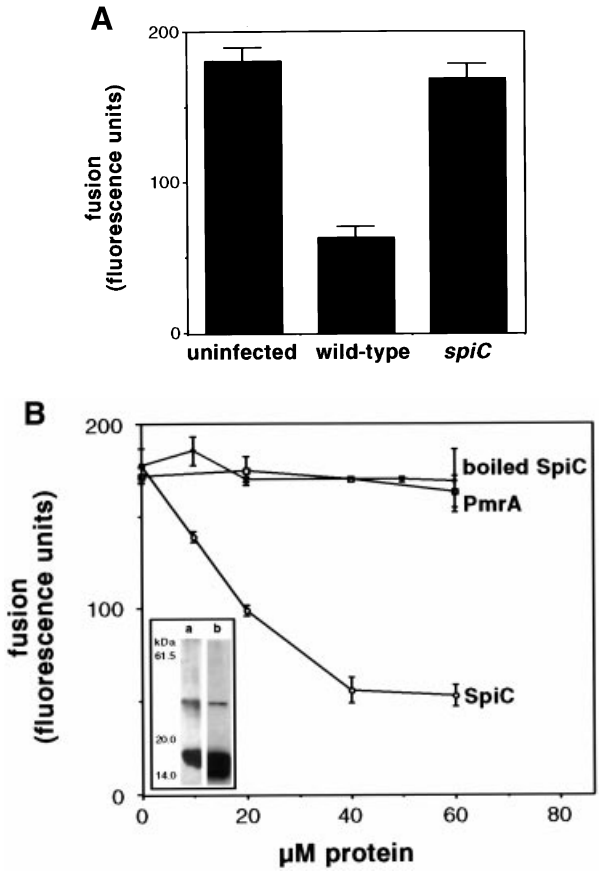

Fig. 7. The SpiC protein inhibits endosome–endosome fusion in a cell-free system. (**A**) Cytosol of macrophages infected with wild-type *Salmonella* (wild-type) is defective in promoting endosome fusion relative to cytosol of uninfected macrophages (uninfected) or cytosol of macrophages infected with *spiC Salmonella* (*spiC*). (**B**) Purified SpiC protein inhibits endosome fusion in a dose-dependent manner (SpiC) and boiling destroys this inhibitory activity (boiled SpiC). Inset shows purity of the SpiC protein used in these assays as determined by silver staining of purified SpiC (lane a) and Western blot analysis with anti-SpiC peptide antibody (lane b). The *Salmonella* PmrA protein, purified from the same strain as SpiC, does not interfere with vesicle fusion.

experiments suggest that the Spi/Ssa system functions intracellularly to deliver SpiC (and possibly other virulence proteins) across the phagosomal membrane and into the macrophage cytosol. *Salmonella* may not be the only bacterial species with a type III system that functions intracellularly: the identification of type III secretion genes in the genome of *Chlamydia psittaci* (Hsia *et al.*, 1997) suggests this pathogen also harbors a type III apparatus that functions intracellularly, shown by the fact that *Chlamydia* is metabolically inactive extracellularly and only expresses proteins several hours after infection.

Salmonella interferes with host vesicle trafficking

Facultative intracellular pathogens use different strategies to survive and replicate within host phagocytic cells (Garcia-del Portillo and Finlay, 1995b). A few intracellular pathogens (e.g. *Listeria monocytogenes*) lyse the phagosomal membrane and escape into the permissive environment of the cytoplasm (Portnoy and Jones, 1994; Sheehan *et al.*, 1994), but the vast majority remains within membrane-lined vacuoles and have evolved mechanisms that either prevent phagosome–lysosome fusion [e.g. *Legionella pneumophila*, *Toxoplasma gondii* and *Chlamydia trachomatis* (Horwitz, 1983; Joiner *et al.*, 1990; Heinzen *et al.*, 1996)] or withstand killing by host antimicrobial agents and replicate within phagolysosomal compartments [e.g. *Coxiella burnetti*, *Leishmania mexicana* and *Francisella tularensis* (Alexander and Vickerman, 1975; Russell *et al.*, 1992; Fortier *et al.*, 1995; Heinzen *et al.*, 1996)].

Salmonella's survival strategy within host cells appears to involve secretion of SpiC (and possibly other proteins), which interferes with normal cellular trafficking and thereby hinders maturation of both *Salmonella*-containing phagosomes as well as vesicle compartments devoid of the microorganism. Trafficking, as reflected by proteolysis of an internalized ligand, was strongly inhibited in macrophages infected by wild-type *Salmonella*, required living microorganisms and a functional *spiC* gene (Figure 5). The fact that the SpiC protein may be largely responsible for this inhibitory activity *in vivo* is suggested by its ability to hinder normal endocytosis and release of transferrin when expressed from a Sindbis virus vector (Figure 6).

Salmonella survives in macrophages within a distinct phagosomal compartment that diverges from the normal degradative pathway of the host cell (Rathman *et al.*, 1997). *Salmonella* inhibition of phagosome–lysosome fusion was known to require bacterial viability and protein synthesis (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991), indicative of a bacterial protein(s) being responsible for this process. We suggest that SpiC may be such a protein because: (i) a *spiC* mutant displayed levels of phagosome–lysosome fusion that were much higher than those displayed by wild-type *Salmonella* (Table I) and similar to those exhibited by heat-killed *Salmonella* or by an avirulent strain of *E.coli* (Ishibashi and Arai, 1990; Buchmeier and Heffron, 1991). The inability of the *spiC* mutant to inhibit phagosome–lysosome fusion is specifically due to the absence of *spiC* and not simply a macrophage survival defect, as shown by the fact that a *purB* mutant, which cannot survive within macrophages due to a nutritional defect, exhibited wild-type levels of phagosome–lysosome fusion (Table I). (ii) The SpiC protein localizes to the cytosol of infected macrophages (Figures 3 and 4), where it can exert its inhibitory effect. (iii) Purified SpiC protein inhibited endosome–endosome fusion *in vitro* (Figure 7B) to the same extent as a dominant-negative mutant of the GTPase Rab5 (S34N) (Barbieri *et al.*, 1994).

The tetanus and botulinum neurotoxins are metalloproteases that target the vesicular membrane fusion machinery (Boquet *et al.*, 1998; Montecucco, 1998). On the other hand, it is presently unclear how the 127 residue SpiC protein inhibits membrane fusion, because it does not harbor motifs that one can ascribe particular biochemical functions. While the SpiC protein has no homologs in the sequence data bases, a 37 amino acid region of SpiC exhibits 35% identity (59% similarity) to a segment of the 3056 amino acid ATM protein (Figure 1C). The ATM protein is defective in individuals suffering from ataxia telangiectasia, a rare genetic disorder with a complex clinical phenotype that includes neuronal degeneration, cancer predisposition, and defects in the immune system (Lavin and Shiloh, 1997). Recently, it has been suggested that the ATM protein may play a role in vesicle trafficking

because it associates with β-adaptin, one of the components involved in clathrin-mediated endocytosis (Lim *et al.*, 1998). The inhibitory activity of the SpiC protein can be reversed upon addition of cytosol from uninfected macrophages to the *in vitro* fusion reaction, but rescue is eliminated upon trypsin-treatment of the cytosol (our unpublished results). This suggests that SpiC may act by inactivating a factor normally present in the host cell cytosol or by competing with a cytosolic factor for a target in the endosomal membrane. In summary, the small size of the SpiC protein argues against SpiC being an enzyme and suggests it may constitute a new type of bacterial toxin.

Role of the SPI-2 island in Salmonella pathogenesis

While the role of SPI-2 has not been fully elucidated, this pathogenicity island is essential for systemic infection in mice (Ochman *et al.*, 1996; Shea *et al.*, 1996, 1999). Thus, the macrophage survival defect of *spiC* (this work) and other SPI-2 mutants (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998) may account for their inability to cause systemic disease, because *in vivo*, wild-type *Salmonella* replicates intracellularly within liver macrophages (Richter-Dahlfors *et al.*, 1997). Finally, although we cannot rule out additional virulence functions for the SpiC protein, our data suggest that the ability to interfere with intracellular membrane trafficking is essential for *Salmonella* pathogenesis.

Materials and methods

Bacterial strains, genetic molecular techniques and growth conditions

Bacterial strains used in this study are derived from the wild-type *S.enterica* serovar Typhimurium strain 14028s. The *spiA*::*kan* derivative EG5793 was described in Ochman *et al.* (1996), and the *purB877*::Tn*10* strain EG9652 was described in Blanc-Potard and Groisman (1997). To construct the *spiC*::*kan* mutant EG10128, we introduced a 1.3 kb *kan* fragment from plasmid pUC4-KIXX into the single *Eco*RV site of plasmid pMS333 (Ochman *et al.*, 1996). The resulting plasmid, pEG9126, harbors the *kan* gene within the *spiC* coding region in the same transcriptional orientation as that of the *spiC* and *spiA* genes. The *spiC*::*kan* mutation in the plasmid was transferred into the chromosome as previously described (Groisman *et al.*, 1993) and the structure of the *spiC*::*kan* mutant was verified by Southern hybridization analysis using both *spiC*- and *kan*-specific probes (data not shown). The *spiC*::*kan* mutation is not polar on *spiA* and *spiB* because the *spiC* mutant can be rescued by the *spiC*-containing plasmid pEG9127. Plasmid pEG9127 is a single-copy plasmid harboring the *spiC* gene and was constructed as follows: the *spiC* gene was amplified by the polymerase chain reaction (PCR) using primers #535 (5′-CGGGATCCCGATGTCTGAGGAGGGATTCAT-3′) and #495 (5′-CGGGATCCCGTTATACCCCACCCGAATAAAG-3′) and plasmid pMS333 DNA (Ochman *et al.*, 1996) as template. The resulting product was digested with *Bam*HI and cloned into the single *Bam*HI site of the single-copy vector pBAC108L (Shizuya *et al.*, 1992).

Phage P22-mediated transductions were performed as described previously (Davis *et al.*, 1980). Plasmid DNA was introduced into bacterial strains by electroporation using a Bio-Rad apparatus following the recommendations of the manufacturer. Bacteria were grown at 37°C in Luria broth (LB). Ampicillin was used at 50 μg/ml, kanamycin at 40 μg/ml and chloramphenicol at 12.5 μg/ml. Recombinant DNA techniques were performed according to standard protocols.

Purification of SpiC protein

A derivative of the *spiC* gene harboring six histidine codons at the N-terminus of the *spiC* coding region was constructed as follows: the *spiC* gene was amplified by PCR using primers #687 (5′-CGGGATCCCGATGCTGGCAGTTTTAAAAGG-3′) and #495 (see above) and plasmid pMS333 DNA (Ochman *et al.*, 1996) as template. The resulting product was digested with *Bam*HI and cloned into the single *Bam*HI site of plasmid pQE-32 (Qiagen) to generate plasmid pEG9128. The absence of mutations in the *spiC* gene in pEG9128 was verified by DNA sequencing. Plasmid pEG9128 was electroporated into *E.coli* K-12 DH5α cells and the recombinant SpiC protein was overproduced and purified using a Ni-NTA resin according to protocols supplied by the manufacturer. The purity of the SpiC preparation was checked by SDS–PAGE, silver staining and Western blot analysis (Figure 6B, inset).

Macrophage survival, invasion and mouse virulence assays

J774 E clone cells, a mannose-receptor positive macrophage cell line, were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS) and 60 μM 6-thioguanin. Macrophage survival and invasion of Henle-407 cell assays were conducted as described previously (Blanc-Potard and Groisman, 1997). Virulence assays were performed by inoculating 7- to 8-week-old female BALB/c mice intraperitoneally with 100 μl of bacteria diluted in phosphate-buffered saline (PBS). Five mice were used at each dose per mutant strain. The number of microorganisms injected into mice was determined by plating the diluted bacteria onto LB agar plates and incubating overnight at 37°C to determine the number of colony forming units (c.f.u.). Mouse viability was recorded for at least 5 weeks.

Determination of phagosome–lysosome and phagosome–endosome fusion

J774 E clone cells were plated in 6-well tissue culture dishes (1.0×10^6 cells/well) and incubated overnight at 37°C in a humidified CO_2 incubator with 5% CO_2 and 95% air. For the phagosome–lysosome fusion assay, colloidal gold particles (30 nm particle size) were coated with mannosylated bovine serum albumin (Man-BSA) using standard techniques (Roth, 1983), and added to macrophages in 6-well plates. Coated-gold particles (BSA-gold) were internalized by macrophages via the mannose receptor. The plates were incubated at 37°C for 24 h to label lysosomal compartments, and infected with 1×10^7 bacteria for either 4 or 14 h. For the phagosome–endosome fusion assay, cells were further incubated with BSA-gold (10 nm particle size) to label endosomal compartment for 30 min, and then incubated for an additional 1.5 h before analysis. After incubation to allow fusion to occur, the cells were washed twice with HB-EGTA buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES–KOH pH 7.0, and fixed with 2% glutaralaldehyde in 0.1 M cacodylate buffer, pH 7.0). The cells were embedded in plastic and processed for electron microscopy. The number of BSA-gold particles of both 10 and 30 nm in 100–150 *Salmonella*-containing vacuoles and the percentage of bacteria in fused phagosomes was calculated.

Detection of the SpiC protein in the cytosol of infected macrophages

J774 E-clone murine macrophages were grown to confluency overnight in DMEM + 10% FBS + 10 μg/ml penicillin/streptomycin on 6-well tissue culture plates at 37°C and 5% CO_2 (~10^6 cells/well). Cells were washed with DMEM and resuspended in 2 ml DMEM/well prior to infection. Bacterial strains were grown overnight in 5 ml LB containing the necessary antibiotics at 37°C with agitation and then diluted 1:4 in DMEM. Fifty microliters of the bacterial dilution (~5×10^7 c.f.u.) were added to each well, plates were centrifuged for 2 min at 1200 r.p.m., and incubated for 1 h at 37°C. Plates were then washed twice with PBS, 2 ml of DMEM containing gentamicin (120 μg/ml) was added to each well and cells were incubated for 1 h in order to kill extracellular bacteria. The media was aspirated and replaced by 2 ml of DMEM containing gentamicin (20 μg/ml) and plates were then incubated for different times.

Macrophages were harvested from six 6-well plates, washed once with PBS, and resuspended in 1 ml homogenization buffer (250 mM sucrose, 1 mm $MgCl_2$, 0.5 mM EGTA, 20 mM MES, pH 6.5). The suspension was homogenized by 50 passes through a Waring Blender, and then centrifuged at 14 000 r.p.m. for 10 min in an Eppendorf (model 5417c) microcentrifuge. The pellet fraction, designated debris, contained bacteria and was resuspended in 1 ml of homogenization buffer. The supernatant was then subjected to centrifugation at 50 000 *g* for 30 min: the pellet, designated membrane, was resuspended in 1 ml of homogenization buffer, whereas the supernatant was designated cytosolic fraction. Extracts were run on a 12.5% SDS–polyacrylamide gel, transferred to nitrocellulose membranes by semi-dry blotting and analyzed by Western blot using either anti-SpiC peptide polyclonal antibody, anti-*Salmonella* specific monoclonal antibody (C65336M, Biodesign International) or

anti-Fc receptor polyclonal antibodies. The Benchmark Prestained Protein Ladder (Gibco-BRL) was used as protein size standard. Western blots were developed using anti-rabbit Ig, horseradish peroxidase-linked antibodies and the ECL detection system (Amersham/Life Science).

For examination of SpiC secretion by confocal microscopy, macrophages were grown to 90% confluency on 10 mm round coverslips in 24-well plates. Macrophages were infected with the corresponding *Salmonella* strains as described above except that 15 instead of 50 µl of bacteria were used. At different time points, samples were fixed directly in 4% paraformaldehyde for 30 min and washed three times in PBS. Samples were then permeabilized in 0.1% Triton X-100 to allow entry of the various primary antibodies: purified polyclonal anti-SpiC peptide antibodies and anti-*Salmonella* monoclonal antibodies. The secondary antibodies were FITC-labeled donkey anti-rabbit and rhodamine-labeled donkey anti-mouse IgG antibodies. Confocal microscopy was carried out on a Bio-Rad MRC1024 confocal microscope using a 63×, 1.4 numerical aperture bright-field objective and fluorescence and rhodamine filter sets. Confocal sequences were collected as Bio-Rad Pic files and were converted to bitmaps for use with Photoshop 4.0.

Measurement of [^{125}I]Man-BSA degradation

Macrophages plated into 24-well culture dishes at 5×10^5/well were infected with 1×10^7 bacteria for 14 h. Following infection, Man-BSA (1 µg/well) labeled with ^{125}I by the chloramine T method (Allen *et al.*, 1984) was added at 4°C to infected macrophages in 24-well plates, and the plates were incubated at 4°C for 1 h. The plates were then warmed in a water bath at 37°C for 10 min to allow endocytosis of [^{125}I]Man-BSA, and the cells were washed with cold PBS, 5 mM EDTA to remove unbound [^{125}I]Man-BSA. After further incubation at 37°C for varying periods of time, the medium was removed and the cells were lysed by solubilization buffer (1% Triton X-100, 0.2% methylbenzethorium chloride, 1 mM EDTA, 0.1% BSA, 0.15 M NaCl, 10 mM Tris–HCl pH 7.4). Proteins were immediately precipitated from the medium or solubilized cells in the presence of 10% trichloroacetic acid (TCA) on ice. Experiments were performed in triplicate.

In vitro endosome–endosome fusion assay

Early endosomes were prepared from J774 E-clone macrophages as described previously (Mayorga *et al.*, 1989) After uptake, cells were washed and homogenized in 250 mM sucrose, 20 mM HEPES–KOH, 0.5 mM EGTA (homogenization buffer) as described previously (Diaz *et al.*, 1988). Post-nuclear supernatants were stored in liquid nitrogen. To obtain endosome-enriched fractions, post-nuclear fractions were diluted 15-fold in homogenization buffer and centrifuged sequentially at 35 000 *g* for 1 min and at 50 000 *g* for 5 min. The second pellet was used for fusion reactions which were carried out as described previously (Diaz *et al.*, 1989; Mayorga *et al.*, 1989). Briefly, endosomes that had internalized either mannose-IgG or DNP-β-glucuronidase via the mannose receptor were mixed to allow fusion. Fused endosomes were solubilized and the mannose-IgG-DNP-β-glucuronidase precipitated using protein A of *Staphylococcus aureus*. The degree of fusion was evaluated by measuring the β-glucuronidase activity associated with the complex. Experiments were performed at least three times.

Construction of a SpiC-expressing Sindbis virus

The *spiC* gene was amplified by PCR and cloned into the unique *Xba*I site of the Sindbis vector Toto1000:32J (Li and Stahl, 1993). The plasmid was then linearized with *Xho*I digestion and used as a template for *in vitro* transcription with SP6 RNA polymerase. The resulting RNA transcripts were used for transfection of confluent BHK-21 cell monolayers using a Lipofectin-mediated procedure (Life Technologies, Inc.). Cells were maintained at 37°C, and the media containing released viruses were harvested 40 h after transfection. Virus titers were generally between 10^8 and 10^9 p.f.u./ml. Virus stocks were aliquoted and kept frozen at –80°C before use.

Measurement of transferrin uptake and recycling

Iron-saturated human transferrin (Sigma) was labeled with ^{125}I to a specific activity of 25 000 c.p.m./ng as described previously (Fraker and Speck, 1978). To investigate uptake, the labeled transferrin was incubated with TRVb-1 cells in 12-mm wells on ice for 1 h in DMEM containing 20 mM HEPES pH 7.4, and 1 mg/ml BSA. Cells transfected with the Sindbis virus vector, or with virus containing SpiC or Rab5 [S34N] were warmed up in 500 µl of the DMEM with 20 mM HEPES–BSA for several minutes and washed with 50 mM MES pH 5.0 and 150 mM NaCl to remove surface-bound transferrin. Cells were solubilized to determine internalized transferrin. To measure recycling, cells transfected with the Sindbis virus vector, or with virus containing SpiC or Rab5 [S34N] were incubated with ^{125}I-labeled transferrin for 2 h at 37°C. The cells were washed with 50 mM MES pH 5.0 and 150 mM NaCl to remove surface-bound transferrin and incubated in 600 µg/ml unlabeled transferrin. At various times, the medium and one wash were collected, and the cells were solubilized to determine recycled and cell-associated radioactivity. Experiments were performed at least three times.

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