Diversification of a *Salmonella* Virulence Protein Function by Ubiquitin-Dependent Differential Localization

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SUMMARY

Many bacterial pathogens and symbionts utilize type III secretion systems to deliver bacterial effector proteins into host cells. These effector proteins have the capacity to modulate a large variety of cellular functions in a highly regulated manner. Here, we report that the phosphoinositide phosphatase SopB, a *Salmonella* Typhimurium type III secreted effector protein, diversifies its function by localizing to different cellular compartments in a ubiquitin-dependent manner. We show that SopB utilizes the same enzymatic activity to modulate actin-mediated bacterial internalization and Akt activation at the plasma membrane and vesicular trafficking and intracellular bacterial replication at the phagosome. Thus, by exploiting the host cellular machinery, *Salmonella* Typhimurium has evolved the capacity to broaden the functional repertoire of a virulence factor to maximize its ability to modulate cellular functions.

INTRODUCTION

Bacterial pathogens that have coexisted with their hosts for extended periods of time have evolved complex functional interfaces involving specific bacterial adaptations to modulate cellular functions and secure the pathogen’s survival and replication. One of these adaptations is the type III secretion system (TTSS), a multiprotein nanomachine that mediates the delivery of bacterial effector proteins into target host cells (Galán and Woldt-Watz, 2006). *Salmonella enterica* serovar Typhimurium (S. Typhimurium) encodes two of these machines within its pathogenicity islands 1 and 2 (SPI-1 and SPI-2), which at different times during infection, deliver more than 60 proteins into host cells (Galán, 2001; Waterman and Holden, 2003). These effector proteins have the capacity to modulate a variety of cellular processes, including actin dynamics, vesicular trafficking, and transcriptional responses (Galán, 2001; Waterman and Holden, 2003), and many do so by mimicking the activities of host-cell proteins (Stebbins and Galán, 2001). It is thought that the activity of type III secreted effector proteins within target cells must be regulated so that their function is exerted at the appropriate time and subcellular space. However, little is known about mechanisms that control these critical aspects of bacterial effector protein function. One of the effector proteins delivered by the *S. Typhimurium* SPI-1 TTSS is the phosphoinositide phosphatase SopB (Galyov et al., 1997; Hong and Miller, 1998; Norris et al., 1998). This effector protein mediates a diverse set of responses at different times during infection. Through the activation of SGEF, an exchange factor for the Rho-family GTPase RhoG, SopB mediates actin-dependent bacterial internalization (Patel and Galán, 2006; Zhou et al., 2001). In addition, SopB modulates vesicular trafficking by altering the metabolism of phosphoinositides at the *Salmonella* phagosomal membrane (Hernandez et al., 2004; Mallo et al., 2008). Furthermore, SopB activates the serine protein kinase Akt (Steele-Mortimer et al., 2000) and stimulates the production of nitric oxide (Drecktrah et al., 2005) by poorly understood mechanisms. Remarkably, all of these activities are strictly dependent on the phosphatase activity of SopB. The mechanism by which this effector modulates such a diverse set of functions with the same enzymatic activity is not understood. We show here that SopB diversifies its function by localizing to different cellular compartments at different times during infection. Early in infection, SopB localizes to the plasma membrane to mediate bacterial entry and Akt activation. Later in infection, SopB localizes to the *Salmonella*-containing vacuole, where it is required for bacterial replication. We also show that the translocation of SopB from the plasma membrane to the *Salmonella*-containing vacuole requires its ubiquitination. Therefore by co-opting the host-cell ubiquitination machinery, SopB can modulate distinct functions during the infection process utilizing the same enzymatic activity.

RESULTS

Ubiquitinated SopB Persists during *S. Typhimurium* Infection of Cultured Epithelial Cells

The observation that SopB modulates different cellular processes at different times during infection prompted us to
examine the levels of translocated SopB throughout the infection process. We used a S. Typhimurium strain expressing carboxy terminus FLAG-tagged SopB, which had been introduced in its chromosome by allelic exchange (see Experimental Procedures). Epitope-tagged SopB was found to behave in a manner that was indistinguishable from wild-type SopB (Figure S1 available online). SopB could be detected within infected cells from as early as a few minutes to up to several hours after infection (Figure 1A), which is consistent with previous results (Drecktrah et al., 2005). Although a proportion of SopB detected late in infection represented newly synthesized protein, we found that a significant fraction represented protein delivered early in infection since it was detected even in the presence of a bacterial protein synthesis inhibitor (Figure 1B). Shortly after infection, a significant proportion of translocated SopB was rapidly modified, resulting in a change of its mobility in SDS-PAGE (Figures 1A and 1B), an observation previously attributed to its ubiquitination (Marcus et al., 2002; Rogers et al., 2008). To confirm that the change in SopB mobility was due to its ubiquitination, COS-2 cells transfected with a plasmid encoding HA-epitope-tagged ubiquitin were infected with a S. Typhimurium strain expressing FLAG epitope-tagged SopB or untagged SopB for 1 hr. The presence of ubiquitinated SopB in infected cells was analyzed by immunoprecipitation and western blot analysis.

Figure 1. SopB Is Ubiquitinated upon Bacterial Delivery and Persists during Infection

(A) SopB persists during infection. Henle-407 cells were infected with a S. Typhimurium strain expressing FLAG epitope-tagged SopB for 1 hr, and its presence in the protein-translocated fraction was examined by western immunoblot.

(B) Effect of bacterial protein synthesis inhibitors on the levels of translocated SopB at different times after infection. Henle-407 cells were infected with a S. Typhimurium strain expressing FLAG epitope-tagged SopB, and chloramphenicol was added at the indicated times after infection. Infected cells were lysed 6 hr after infection, and the presence of SopB in the translocated fraction was examined by western immunoblot.

(C) SopB is ubiquitinated upon translocation into epithelial cells. COS-2 cells transfected with a plasmid encoding HA epitope-tagged ubiquitin were infected with a S. Typhimurium strain expressing FLAG epitope-tagged SopB or untagged SopB for 1 hr. The presence of ubiquitinated SopB in infected cells was analyzed by immunoprecipitation and western blot analysis.

(D) SopB ubiquitination does not require the bacterially encoded E3 ubiquitin ligases. Henle-407 cells were infected for 1 hr with S. Typhimurium strains expressing FLAG epitope-tagged SopB and lacking the bacterially encoded E3 ubiquitin ligases SlrP, SspH2, or SopA, as indicated. The presence of SopB in the protein-translocated fraction was examined by western immunoblot.

(E) SopB is conjugated to lysine-less and K63 ubiquitin. Cultured cells transfected with a plasmid encoding HA-tagged lysine-less (K0) or K63 ubiquitin were infected with a S. Typhimurium strain expressing FLAG epitope-tagged SopB. The presence of ubiquitinated SopB in infected cells was analyzed by immunoprecipitation and western blot analysis. In all panels, the asterisk denotes the predicted mobility of unmodified SopB.
S. Typhimurium encodes three E3 ubiquitin ligases, the Hect-like protein SopA (Zhang et al., 2006), as well as three other proteins, SspH1 (absent from some strains, including the one used in this study), SlrP, and SspH2, which belong to a novel family of E3 ligases (Quezada et al., 2009; Rohde et al., 2007). These effector proteins are also delivered by the SPI-1- and SPI-2-encoded TTSSs (Miao et al., 1999). We investigated the potential involvement of these three E3 ligases and found that the pattern of SopB ubiquitination after its delivery by the S. Typhimurium ΔsspH2ΔslrPΔsopA triple mutant was indistinguishable from that observed after its delivery by wild-type S. Typhimurium (Figure 1D). These results indicate that SopB ubiquitination does not require any of the known bacterially encoded E3 ubiquitin ligases.

We have previously shown that two other effectors delivered by the SPI-1 TTSS, SopE and SptP, are also ubiquitinated upon translocation (Kubori and Galán, 2003). In this case, ubiquitination serves as a degradation signal since these effectors are degraded upon translocation. In contrast, ubiquitinated SopB persisted within cells for extended periods of time even in the presence of a bacterial protein synthesis inhibitor (Figure 1B). Furthermore, ubiquitinated SopB could be readily detected in the absence of proteasome inhibitors (Figures 1A and 1B), and addition of a proteasome inhibitor did not significantly alter the levels of ubiquitinated SopB (Figure S2). These results suggest that, in the case of SopB, ubiquitination may play a role other than that of a degradation signal. Consistent with this hypothesis, the mobility shift observed in SopB subsequent to its translocation into mammalian cells indicates that monoubiquitination is the most common modification observed in this effector (Figures 1A–1D). In fact, SopB may undergo multimono-ubiquitination since in immunoprecipitation experiments conducted in cells overexpressing lysine-less ubiquitin, which cannot form ubiquitin chains although it can be conjugated to substrates, we still observed laddering in the SopB mobility (Figure 1E). We also observed the presence of ubiquitinated SopB in infected cells overexpressing a ubiquitin mutant in which its lysine 63 (K63-ubiquitin) was the only lysine available for conjugation (Figure 1E). Like monoubiquitination, K63-linked ubiquitin is not usually associated with degradation (Urbe, 2005). However, these results do not rule out the possibility that the laddering observed under these conditions may also be the result of multimono-ubiquitination by K63 ubiquitin. Together, these results further support the hypothesis that ubiquitination of SopB plays a role other than that of a degradation signal.

Identification of the SopB Ubiquitination Sites

To evaluate the role of ubiquitination in SopB function, we mapped its ubiquitination sites. We isolated translocated SopB from infected cells by affinity purification and subjected it to mass spectrometric analysis to identify ubiquitinated peptides. This analysis identified ubiquitinated SopB fragments consisting of amino acids 14–23 and 24–41 (Figure S3). These results, in conjunction with the ubiquitin laddering observed in infected cells expressing lysine-less ubiquitin (see above), suggested that several lysine residues located at the amino terminus of SopB ubiquitination does not require any of the known bacterially encoded E3 ubiquitin ligases.
Furthermore, even pairwise substitution of lysine residues within this region had a negligible effect on SopB ubiquitination (Figure 2B). Only when all of the lysine residues contained within this region were substituted was SopB ubiquitination abrogated, resulting in its migration as a predominantly single species at the predicted molecular weight of nonubiquitinated SopB (Figure 2B). Moreover, additional mutation of a lysine residue located at the carboxy terminus (K541), which has previously been shown to be ubiquitinated upon transient overexpression of SopB in cultured cells (Rogers et al., 2008), had no effect on the ubiquitination profile of SopB (Figure S4). In addition, the mutant in which all of the amino-terminal lysines had been substituted did not coimmunoprecipitate with epitope-tagged ubiquitin (data not shown). These results revealed a high degree of complexity in the pattern of SopB ubiquitination, with different lysines potentially serving as targets for ubiquitination. Similar observations have been made for the ubiquitination sites of the epidermal growth factor receptor (Haglund et al., 2003a, 2003b; Huang et al., 2006).

The Nonubiquitinated SopB Mutant Accumulates at the Plasma Membrane of Infected Cells after Its TTSS-Mediated Delivery

To investigate the role of SopB and its ubiquitination during infection, we compared the localization of the nonubiquitinated (K1-9 substituted, see Figure 2) SopB mutant (from here forth referred to as SopBub) with that of wild-type SopB. Cultured epithelial cells were infected with S. Typhimurium strains expressing a chromosomally encoded FLAG-tagged wild-type SopB or its SopBub mutant derivative constructed by allelic exchange, and the localization of these effector proteins was examined by confocal microscopy. Ten minutes after infection, wild-type SopB was observed localized at the plasma membrane, tightly around entering bacteria (Figure S5). After internalization, wild-type SopB was seen decorating the Salmonella-containing vacuoles, and its association with this compartment was maintained for up to 3 hr (Figure 3A; Movie S1; data not shown). In contrast, the SopBub mutant revealed a strikingly different localization at the plasma membrane in the form of surface puncta radiating from the site of infection (Figure 3B; Movie S2; data not shown). Within 30 min of infection, diffuse SopBub staining over the entire plasma membrane was readily evident in x-z and y-z projections after reconstruction of confocal z-stacks (Movie S2). Despite the significant differences in localization, biochemical fractionation of lysates of host cells infected with bacteria expressing the different constructs indicated that the levels of translocated SopBub mutant protein were equivalent to those of wild-type SopB (Figure 3C). The half-life within host cells of the nonubiquitinated SopB mutant was equivalent to that of wild-type SopB (data not shown), further demonstrating that, in the case of this effector, ubiquitination does not serve as a degradation signal. Consistent with the role of ubiquitination in SopB localization, mutants in which lysine substitutions (individual or in combination) did not result in changes in its ubiquitination pattern (Figure 2B) localized in a manner indistinguishable from wild-type SopB (Figure S6).

To further explore the role of ubiquitination in the localization of SopB, we examined the distribution of the SopBub mutant upon delivery by bacteria competent for TTSS-mediated protein translocation but defective for internalization. We reasoned that this would allow us to examine the distribution of this effector protein without the potential influence of actin remodeling and bacterial entry. To render the bacteria unable to induce its own internalization, we constructed catalytic mutant derivatives of the wild-type (SopB(C460S)) and its nonubiquitinated mutant (SopBub(C460S)), and we expressed them in a strain lacking the effectors SopE and SopE2, which are functionally redundant with SopB in mediating bacterial entry. When delivered in the context of this noninvasive strain, SopB(C460S) was seen in small intracellular vesicle-like compartments that could be labeled with endocytic tracers (Figure 3D). In addition, mixed infection experiments showed that SopB delivered by noninvasive bacteria could be observed surrounding phagosomes of invasive bacteria, demonstrating that this delivery pathway can result in the transfer of translocated SopB to Salmonella-containing phagosomes (Movie S3). SopB(C460S) was readily ubiquitinated upon delivery into cells by noninvasive bacteria, indicating that its phosphatase activity is dispensable for this modification (Figure 3E). In contrast, SopBub(C460S) delivered by noninvasive bacteria was seen localized exclusively at the plasma membrane, did not colocalize with endocytic tracers (Figure 3D) or bacterial-containing phagosomes after mixed infections, and, as expected, was not ubiquitinated upon its delivery to infected cells (Figure 3E). These results indicate that the localization of SopB to an intracellular vesicular compartment such as the Salmonella-containing vacuole is not the consequence of its passive trapping in the phagosomal membrane during bacterial uptake. Rather, these results indicate that ubiquitination may serve as a signal necessary for the removal of SopB from the host-cell plasma membrane and its delivery to intracellular vesicular compartments such as the Salmonella-containing vacuole. This mechanism is reminiscent of the ubiquitination-dependent internalization mechanisms of growth factor receptors after stimulation by their ligands (Dikic, 2003). However, our results cannot rule out the possibility that, during bacterial internalization, ubiquitination may also serve as a signal to localize SopB to the nascent phagosome and contribute to its targeting to the bacterial phagosome.

Identification of a Domain Required for SopB Association with the Host-Cell Membrane

We found that after translocation into host cells, SopB partitioned primarily to a membrane fraction and could not be extracted by high salt concentrations (Figure 4A). Similar observations have been previously made after transient overexpression of SopB in epithelial cells (Marcus et al., 2002). SopBub also partitioned to the membrane fraction and was equally resistant to extraction by salt, which indicates that ubiquitination is not required for SopB to associate with host-cell membranes after TTSS-mediated translocation (Figure 4A). The association of SopB with the host-cell membrane in a manner resistant to extraction with high-salt treatment suggests a strong interaction equivalent to that of a hydrophobic domain that may be intimately associated with the membrane. We examined the SopB sequence for the presence of such a domain and identified a region between amino acids 288 and 309 whose features
would be consistent with those of a transmembrane domain as indicated by the TMpred prediction algorithm (data not shown). However, this domain does not appear to be a bona fide transmembrane domain since other prediction algorithms did not detect it (data not shown). To investigate the potential contribution of this domain to the membrane localization of SopB, we constructed an S. Typhimurium strain expressing a SopB mutant lacking this hydrophobic region (SopB\textsuperscript{D288–309}) and examined its phenotype. SopB\textsuperscript{D288–309} was secreted from the bacteria and delivered into host cells by the SPI-1 TTSS, albeit at slightly lower levels than those of the wild-type protein (Figure 4B). Importantly, translocated SopB\textsuperscript{D288–309} migrated as a single species on SDS-PAGE with the predicted molecular weight of the unmodified protein, which indicates that, unlike wild-type SopB, this mutant form cannot be ubiquitinated (Figure 4B). Furthermore, translocated SopB\textsuperscript{D288–309} exhibited cytoplasmic localization (Figure 4C), indicating that the hydrophobic domain is essential for the localization of SopB to host-cell membranes. A S. Typhimurium \textit{ΔsopE ΔsopE2 ΔsopB} mutant strain expressing SopB\textsuperscript{D288–309} and cells were probed for the presence of SopB in the translocated fraction by western immunoblot 1 hr after infection. The asterisk denotes the predicted mobility of unmodified SopB.

**Figure 3. Subcellular Localization of Wild-Type SopB and Its Ubiquitination-Deficient Mutant**

(A and B) Henle-407 cells were infected with a S. Typhimurium strain expressing (A) wild-type epitope-tagged SopB or (B) its ubiquitination-deficient SopB\textsuperscript{Δub} mutant and were examined by fluorescence confocal microscopy after staining with antibodies directed to the epitope tag (green) or to S. Typhimurium (red). The scale bar represents 10 μm.

(C) Levels of SopB or SopB\textsuperscript{Δub} in infected cells. Henle-407 cells were infected with S. Typhimurium strains expressing FLAG epitope-tagged wild-type SopB or the SopB\textsuperscript{Δub} mutant. At the indicated times after infection, cells were probed for the presence of SopB in the translocated fraction by western immunoblot. Blots were reprobed with antibodies directed to SipB (another SPI-1 TTSS effector protein) or actin to control for loading. The asterisk denotes the predicted mobility of unmodified SopB.

(D) Localization of wild-type SopB or its ubiquitination-deficient mutant derivative after their delivery by a noninvasive S. Typhimurium mutant. Cultured epithelial cells preloaded with Alexa 594-dextran to label endosomal compartments were infected for 1 hr with the invasion-defective \textit{ΔsopE ΔsopE2 ΔsopB} S. Typhimurium mutant strain expressing FLAG epitope-tagged catalytically inactive SopB\textsuperscript{C460S} or its ubiquitination-defective derivative SopB\textsuperscript{C460S Δub}. Cells were fixed, stained for SopB (green) and DAPI (blue), and examined by fluorescence microscopy. Insets show enlarged detail area. The scale bar represents 10 μm.

(E) Levels of SopB\textsuperscript{C460S} or its derivative SopB\textsuperscript{C460S Δub} after their TTS-mediated delivery by the noninvasive S. Typhimurium mutant strain. Henle-407 cells were infected with \textit{ΔsopE ΔsopE2 ΔsopB} S. Typhimurium mutant strains expressing FLAG epitope-tagged wild-type SopB or its nonubiquitatable mutant (SopB\textsuperscript{Δub}) and cells were probed for the presence of SopB in the translocated fraction by western immunoblot 1 hr after infection. The asterisk denotes the predicted mobility of unmodified SopB.
Figure 4. Identification of a SopB Domain Required for Its Membrane Localization

(A) Membrane association of SopB after type III secretion-mediated translocation. Henle-407 cells were infected with a S. Typhimurium strain expressing FLAG epitope-tagged wild-type SopB (upper panel) or its mutant SopB\(^{D288-309}\) (lower panel), and their presence in the membrane-insoluble (P) or -soluble (S) fractions of infected cells before and after extraction with NaCl (1 M), Na\(_2\)CO\(_3\) (pH 11), or Triton X-100 was examined by western immunoblot. The asterisk denotes the predicted mobility of unmodified SopB.

(B) TTSS-mediated secretion and translocation into host cells of the SopB\(^{D288-309}\) mutant. Whole-cell bacterial lysates, bacterial culture supernatants, or the TTS-protein-translocated fraction of cultured epithelial cells infected with the indicated strains of S. Typhimurium expressing the FLAG epitope-tagged SopB\(^{D288-309}\) mutant or wild-type SopB (as control) were analyzed by western immunoblot. The asterisk denotes the predicted mobility of unmodified SopB.

(C) Subcellular localization of the SopB\(^{D288-309}\) mutant. Henle-407 cells were infected with a S. Typhimurium strain expressing an epitope-tagged SopB\(^{D288-309}\) mutant and were examined by immunofluorescence confocal microscopy with an antibody directed to the epitope tag (green) or actin (red). The scale bar represents 10 \(\mu\)m.

(D) Ability of wild-type SopB and the SopB\(^{D288-309}\) mutant to mediate bacterial entry into cultured epithelial cells. Henle-407 cells were infected with a S. Typhimurium \(\Delta\)sopE \(\Delta\)sopE2 \(\Delta\)sopB mutant strain (as control, indicated “\(\Delta\)sopB” in the panel) or a S. Typhimurium \(\Delta\)sopE \(\Delta\)sopE2 mutant expressing wild-type SopB or SopB\(^{D288-309}\) (as indicated), and the levels of internalized bacteria were determined as indicated in Experimental Procedures. Values are the mean ± standard deviation of three independent experiments and represent the percentage of the original bacterial inoculum that survived the antibiotic treatment due to internalization.
hydrophobic domain since purified SopB^ub exhibited almost wild-type phosphatase activity in vitro (Figure 4F). However, this experiment cannot rule out the possibility that loss of function of this SopB mutant may be due to localized conformational changes induced by the introduction of the small deletion. Taken together, however, these results indicate that the association of SopB with host-cell membranes requires a discrete hydrophobic domain and is critical for its ubiquitination and function.

S. Typhimurium Expressing SopB^ub Promotes Increased Actin Remodeling, Macropinocytosis, and Akt Activation

To investigate the functional significance of SopB ubiquitination, we examined the SopB-dependent phenotypes of a S. Typhimurium strain expressing the ubiquitination-deficient SopB^ub mutant. Since SopB is functionally redundant with the Rho-family GTPase exchange factors SopE and SopE2 in mediating bacterial internalization (Zhou et al., 2001) (Patel and Galán, 2006), we expressed the SopB^ub mutant in a strain lacking these exchange factors so that signaling for entry was exclusively delivered through SopB^ub. We found that a S. Typhimurium strain expressing SopB^ub was able to enter cells in a manner indistinguishable from that of a strain expressing wild-type SopB (Figure 5A). Interestingly, the S. Typhimurium sopB^ub mutant strain induced more profuse actin cytoskeletal rearrangements than the strain expressing wild-type SopB. Whereas the strain expressing wild-type SopB induced localized actin remodeling, the strain expressing SopB^ub induced diffuse membrane ruffling along the cell periphery that coincided with a loss in cellular stress fibers (Figures 5B and 5C). Moreover, in contrast with the transient actin remodeling induced by wild-type SopB, the actin rearrangements stimulated by the S. Typhimurium sopB^ub mutant strain persisted for up to 3 hr after infection (data not shown). These results suggest that SopB triggers signaling for entry when it is localized at the plasma membrane, and that its persistence at this location results in increased signaling to the actin cytoskeleton. These results also suggest that the removal of SopB from the plasma membrane by a ubiquitination-mediated mechanism may contribute to the downregulation of this signaling event after bacterial entry.

We investigated whether ubiquitination of SopB influences the magnitude and temporal kinetics of macropinocytosis, a phenotype also dependent on SopB (Hernandez et al., 2004; Mallo et al., 2008). Henle-407 cells infected with S. Typhimurium expressing wild-type SopB or the SopB^ub mutant were analyzed by live microscopy for their ability to induce macropinocytosis. In comparison to a wild-type strain, cells infected with the S. Typhimurium sopB^ub mutant exhibited increased and more prolonged macropinocytic activity (Figures 6A and 6B). These results suggest that the persistence of SopB^ub at the plasma membrane enhances its ability to stimulate macropinocytosis.

We also compared the activation of Akt over time in cells infected with S. Typhimurium expressing wild-type SopB or the SopB^ub mutant. Although at 30 min after infection the levels of Akt activation were equivalent in cells infected with either bacterial strain, cells infected with the S. Typhimurium sopB^ub mutant showed more sustained kinase activation (Figure 6C). Three hours after infection, the levels of phosphorylated AKT in infected cells were compared with those in uninfected cells.

(A) Henle-407 cells were infected with a S. Typhimurium ΔsopE ΔsopE2 mutant strain expressing wild-type SopB or the SopBΔub mutant (as indicated), and the levels of internalized bacteria were determined by the gentamicin-resistance assay. Values are the mean ± standard deviation of three independent experiments and represent the percentage of the original bacterial inoculum that survived the antibiotic treatment due to internalization. (B and C) Ref-52 cells were infected with a ΔsopE ΔsopE2 S. Typhimurium strain expressing either wild-type SopB or its ubiquitination-deficient mutant derivative (SopB^ub). Cells were stained with rhodamine-phalloidin to visualize the actin cytoskeleton, with a FITC-labeled antibody to visualize S. Typhimurium, and were examined by fluorescence microscopy. Cells exhibiting either localized or peripheral actin cytoskeletal changes were quantified. Values shown in (B) represent the percentage of infected cells showing the indicated changes and are the mean ± standard deviation of three experiments in which a minimum of 200 cells were examined for each category. The values obtained in cells infected with the SopBΔub mutant were statistically significant (p < 0.02) when compared to those of wild-type with the Student’s t test.

Figure 5. The Ubiquitination-Deficient SopB Mutant Induces Efficient Entry and Distinct Actin Reorganization

(E) Ability of wild-type SopB and the SopBΔub mutant to activate Akt. Henle-407 cells were infected with a S. Typhimurium expressing FLAG epitope-tagged wild-type SopB or with the SopBΔub mutant, and the levels of phosphorylated (activated) or total Akt were examined by western immunoblot analysis 1 hr after infection. The levels of translocated epitope-tagged SopB or SopBΔub were determined by western immunoblot analysis of the fraction of infected cells containing the translocated protein (upper panel).

(F) Comparison of the in vitro phosphoinositide phosphatase activity of purified wild-type SopB, the SopBΔub mutant, and the phosphatase-inactive SopBΔ505 mutant. The phosphatase activity was calculated based on the standard curve (shown in inset) for inorganic phosphate standards by using a malaehite green chromogenic assay. Values are the mean ± standard deviation of three independent experiments.
In summary, persistence of SopB at the plasma membranes resulted in an enhanced ability to stimulate actin remodeling, macropinocytosis, and Akt activation, suggesting that these activities require the presence of SopB at this location.

**S. Typhimurium Expressing SopB^{ub} Is Defective for Intracellular Replication**

It has been previously shown that SopB is required for the efficient intracellular replication of S. Typhimurium by modulating the vesicular trafficking of the *Salmonella*-containing vacuole (SCV) (Hernandez et al., 2004). It has also been recently shown that recruitment of Rab5 to the SCV is dependent on the presence of SopB, and it has been postulated that this recruitment is necessary for efficient S. Typhimurium intracellular replication (Mallo et al., 2008). We reasoned that these phenotypes must depend on the presence of SopB at the SCV, in which case a S. Typhimurium expressing SopB^{ub}, which is retained at the plasma membrane, may exhibit a defect in these phenotypes.

To test this hypothesis, we first compared the ability of S. Typhimurium expressing either wild-type SopB or the SopB^{ub} mutant with a ΔsopB strain for their ability to recruit Rab5 to the SCV. Both the ΔsopB and the strain expressing the SopB^{ub} mutant were equally defective in their ability to recruit Rab5 to the SCV (Figures 7A and S7). To further evaluate the importance of ubiquitin-mediated SopB localization to the SCV, we compared the ability of a S. Typhimurium ΔsopE ΔsopE2 mutant strain expressing either wild-type SopB or the SopB^{ub} mutant to grow within epithelial cells. Although both strains were internalized at equivalent levels, 24 hr after infection a significantly lower number of colony-forming units were recovered from cells infected with the strain expressing the SopB^{ub} mutant (Figure 7B). We also compared the S. *typhimurium* ΔsopB mutant with wild-type S. Typhimurium or an isogenic mutant strain expressing SopB^{ub} for their ability to survive within primary bone-marrow-derived macrophages. As shown in Figure 7C, when compared to wild-type S. Typhimurium, the ΔsopB and the strain expressing SopB^{ub} were equally defective in their ability to survive within macrophages. Introduction of a plasmid encoding wild-type sopB readily complemented the S. Typhimurium strain expressing the SopB^{ub} mutant for its ability to survive within macrophages. Combined, these results are consistent with the hypothesis that SopB modulation of bacterial intracellular replication requires the localization of SopB at the SCV. In addition, together with the results presented above, these findings indicate that the differential localization of SopB at different sites allows it to modulate different cellular processes and hence diversify its function.

**DISCUSSION**

TTSSs have the capacity to mediate a very diverse array of cellular functions. They do so by delivering a variety of effector proteins, which in many cases function as “mimics” of eukaryotic cell proteins (Galán, 2007; Stebbins and Galán, 2001). In order to exert their function, the activity of these effector proteins must be precisely coordinated and regulated in time and space. For example, in S. *typhimurium*, two effector proteins, SopE and SptP, modulate Rho-family GTPases through opposing
biochemical activities. SopE activates Rho-family members by functioning as a guanine nucleotide exchange factor (GEF) for these GTPases (Hardt et al., 1998), whereas SptP downmodulates the activity of the same GTPases by functioning as a GTPase-activating protein (GAP) (Fu and Galán, 1999). These effectors ensure activation of the GTPases to allow bacterial internalization while preserving cellular homeostasis, which is transiently altered during bacterial entry. The opposing activities of these two effectors are coordinately regulated by their ubiquitin-dependent differential degradation (Kubori and Galán, 2003). We have shown here another example of ubiquitin-mediated regulation of effector function. Rather than degradation, the mechanism described here implicates ubiquitination in the differential localization of the effector protein SopB during the infection cycle, resulting in the diversification of its function. Upon translocation from the bacteria, SopB localizes to the plasma membrane of infected cells, from where it mediates actin cytoskeleton reorganization, macropinocytosis, bacterial entry, and Akt activation by unknown mechanisms. Membrane-localized SopB is ubiquitinated (monoubiquitinated or linked via K63), which results in its entrapment in the incoming phagosome or its removal from the plasma membrane and its subsequent translocation to the Salmonella-containing vacuole, where it modulates vesicle trafficking by altering phosphoinositide metabolism at that site, thus avoiding the delivery of S. Typhimurium to lysosomes. See the text for further details.

Figure 7. The Ubiquitination-Deficient SopB Mutant Is Defective for Rab5 Recruitment and Intracellular Growth
(A) Rab5 recruitment by different strains of S. Typhimurium. Henle-407 cells that had been transfected with a plasmid expressing Rab5-GFP were infected with a ΔsopB S. Typhimurium mutant strain (ΔsopB) or with a strain expressing either wild-type SopB (WT SopB) or its ubiquitination-deficient mutant derivative (SopBΔub). At the indicated times, the percentages of the different S. Typhimurium strains showing colocalization with Rab5-GFP were determined. Values are mean ± standard deviation of three independent determinations in which the indicated number of bacteria (n) were examined. The asterisk indicates statistically significant (p < 0.05) differences compared to the values of cells infected with wild-type bacteria by using the Student’s t test.
(B) S. Typhimurium expressing SopBΔub is defective for intracellular growth in cultured epithelial cells. Henle-407 cells were infected with a ΔsopE ΔsopE2 S. Typhimurium strain expressing either wild-type SopB or SopBΔub. At the indicated times after infection, the levels of intracellular colony-forming units (c.f.u.) of the different strains were determined. Values represent the mean ± standard deviation of three independent experiments. The asterisk indicates statistically significant (p < 0.05) differences compared to the values of cells infected with wild-type bacteria by using the Student’s t test.
(C) S. Typhimurium expressing sopBΔub is defective for survival within primary macrophages. Primary bone-marrow-derived macrophages were infected with wild-type S. Typhimurium (WT), a ΔsopB mutant (ΔsopB), a strain expressing sopBΔub (sopBΔub), or a derivative of the same strain complemented with a plasmid expressing wild-type sopB (sopBΔub + WT sopB). At the indicated times after infection, the levels of intracellular c.f.u. of the different strains were determined. Values are normalized to those of wild-type and represent the mean ± standard deviation of three independent experiments. An asterisk indicates statistically significant (p < 0.05) differences when compared to the values of cells infected with wild-type bacteria by using the Student’s t test.
(D) Model for ubiquitin-mediated diversification of SopB function. (I) Upon delivery by the S. Typhimurium TTSS, SopB localizes to the plasma membrane through its membrane localization domain. (II) While at the plasma membrane, SopB mediates entry and macropinocytosis by activating SGEF, a RhoG exchange factor, and Akt activation by unknown mechanisms. (III) Membrane-localized SopB is ubiquitinated (monoubiquitinated or linked via K63), which results in its entrapment in the incoming phagosome or its removal from the plasma membrane and its subsequent translocation to the Salmonella-containing vacuole, where it modulates vesicle trafficking by altering phosphoinositide metabolism at that site, thus avoiding the delivery of S. Typhimurium to lysosomes. See the text for further details.
translocation, SopB is multimonoubiquitinated. This modification acts as a signal required for its removal from the host-cell plasma membrane and its subsequent delivery to internal vesicular compartments and/or for its “trapping” on the nascent bacterial phagosome. At this location, SopB modulates vesicle traffic and facilitates intracellular bacterial replication. Consistent with this model, a ubiquitination-defective SopB mutant remained localized to the plasma membrane throughout bacterial infection, and S. Typhimurium strain expressing this mutant exhibited an increased and prolonged ability to stimulate actin cytoskeleton rearrangements and Akt activation. In contrast, this strain was defective for intracellular survival, which is dependent on the appropriate vesicle trafficking of the SCV. Therefore, ubiquitination is central not only for the downmodulation of the early activities of SopB, but also for the diversification of its function. In this context, the parallels with the role of ubiquitination in the modulation and diversification of growth factor receptor function are striking (Dikic, 2003; Haglund et al., 2003a). Upon stimulation by its ligand, the EGFR is monoubiquitinated and removed from the plasma membrane to be delivered to an endocytic compartment where it can further stimulate downstream signaling, or it can be sorted for recycling or degradation. Like SopB, in the absence of ubiquitination, EGFR remains at the plasma membrane, resulting in constitutive signaling. Furthermore, the similarities extend to the highly redundant nature of the potential sites for ubiquitination. We found that several lysines within the amino terminus of SopB can be targets for monoubiquitination. Likewise, several lysines clustered around the kinase domain of EGFR can be alternatively monoubiquitinated (Haglund et al., 2003a, 2003b; Huang et al., 2006). However, despite these striking similarities, there are significant differences in the cellular machinery utilized for the removal and transport of SopB and EGFR from the plasma membrane to endocytic compartments. The ubiquitination-dependent removal of the EGFR from the plasma membrane is mediated by a complex mechanism that involves proteins belonging to the endosomal sorting complexes required for transport (ESCRT) (Saksena et al., 2007), as well as dynamin and Epsin (Bache et al., 2006; Chen et al., 1998; Damke et al., 1994; Dikic, 2003; Malerod et al., 2007; Raborg et al., 2008). In contrast, depletion of or interference with dynamin, clathrin, Epsin, or members of the ESCRT complex did not affect the location of SopB (Figure S8), implying that an as yet unidentified but potentially different cellular machinery is involved in SopB’s removal from the plasma membrane and translocation to endocytic compartments.

In summary, we have described here a remarkable mechanism by which, through differential localization, a virulence factor diversifies its function to modulate rather distinct cellular processes with the same biochemical activity. This is another example of the extraordinary complexity of the pathogen/host-cell functional interface sculpted by evolutionary forces directed at securing pathogen and host survival.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Bacterial Strains, and Cell Lines**

Full-length wild-type SopB and its derivatives (including truncations and deletions), tagged at the C terminus by a FLAG epitope, were cloned into the arabinose-inducible vector pBAD24 (Guzman et al., 1995) by standard recombinant DNA procedures. SopB mutants harboring single or combinatorial Lys (K) to Glu (Q) substitutions at positions 13, 19, 23, 37, 41, 67, 93, 109, and 111 were generated by site-directed mutagenesis and were verified by DNA sequencing. Eukaryotic expression vectors encoding Epsin U1M and K44A Dynamin were provided by Pietro de Camilli (Yale University), YFP-tagged Vps4a derivatives (pEYFP-Vps4a and pEYFP-E228QVps4a) were provided by W. Sandquist (University of Utah), and HA-tagged wild-type, K63, and K0 ubiquitin were provided by Yihong Ye (National Institutes of Health), siGENOME SMARTpool (M-016835-00, Dharmacon) at a final concentration of 50 nM was used to knock down expression of endogenous HRS. RNAi efficacy was verified by qRT-PCR, by using HRS primers as previously described (Patel and Galán, 2006). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

The wild-type strain of S. Typhimurium SL1344 (Hoiseth and Stocker, 1981) and its isogenic derivatives used in this study, JsegB (SB1120) (Hernandez et al., 2004), JsegE JsegE2 (SB1301), and JsegE JsegE2 JsegB (SB1302) (Zhou et al., 2001), have been previously described. Strains expressing chromosomally encoded FLAG-tagged wild-type SopB and its derivatives were constructed by using standard recombinant DNA and allelic exchange procedures as previously described (Kaniga et al., 1994). All bacterial strains were cultured under conditions that stimulate the expression of the Salmonella pathogenicity island-1 encoded type III secretion system (Galán and Curtiss, 1990). Where appropriate, 0.05% L-arabinose was added to cultures at the early logarithmic phase of growth (OD600 of 0.4) to induce the expression of genes under the control of the paraBAD promoter.

**Type III Protein Secretion and Translocation Assay**

Bacterial protein secretion and translocation into host cells were examined by biochemical fractionation and western immunoblot as previously described (Collazo and Galán, 1997; Kubori and Galán, 2003). Where appropriate, the proteasome inhibitor MG132 (5 μM, Calbiochem) or its vehicle solvent DMSO was added to cells 30 min prior to infection and maintained throughout the infection and lysis procedure. In bacterial protein synthesis inhibition experiments, chloramphenicol was added at a final concentration of 100 μg/ml at different time points during infection.

**SopB Immunoprecipitation**

COS-2 cells seeded in 10 cm dishes and transfected with a plasmid encoding HA-tagged ubiquitin were infected with S. Typhimurium expressing chromosomally encoded SopB FLAG (multiplicity of infection [ moi] 30) at 37°C. At different time points after infection, cells were harvested by scraping into 1 ml modified RIPPA lysis buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM NEM, 1x protease cocktail [Roche], 1 mM PMFS) per well. Lysates were centrifuged at 20,000 × g at 4°C for 20 min to remove insoluble material. An aliquot of the supernatant was saved (−100 μl) to monitor levels of SopB FLAG translocation and HA-ubiquitin expression, whereas the remainder (−900 μl) was incubated with 15 μl prewashed anti-FLAG M2 agarose (50% slurry, Sigma), with rotation for 1 h at 4°C to precipitate translocated SopB. Immune complexes were collected by centrifugation at 1000 × g for 5 min at 4°C, washed twice with 1 ml cold lysis buffer, and boiled in SDS loading buffer to dissociate proteins from beads. Samples were analyzed by SDS-PAGE (8% gel) and western blot with antibodies directed against HA and FLAG.

**Subcellular Fractionation and Extraction**

Hertle-407 cells (10 cm plate) were infected ( moi 60) for 1 hr as described above, washed, and harvested by scraping gently into homogenization buffer (20 mM HEPES [pH 7.2], 200 mM sucrose, 0.5 mM EGTA, 1 mM PMFS, 1x protease inhibitor cocktail). Cells were disrupted by mechanical lysis with a doucen homogenizer (25x passage), and lysates were centrifuged twice at 20,000 × g at 4°C for 30 min to remove bacteria and debris. The resulting supernatant was centrifuged at 100,000 × g at 4°C to separate the membrane (pellet) from the cytoplasmic (supernatant) fractions. Extraction of SopB from the pellet fraction was performed by resuspending the pellet in buffer containing 1 M NaCl, 100 mM sodium bicarbonate (pH 11), or 1% Triton X-100 and incubating it on ice for 30 min. Samples were centrifuged at 100,000 × g to
S. Typhimurium-Induced Ruffling, Macropinocytosis Assays, and Rab5 Recruitment

S. Typhimurium-induced ruffling and macropinocytosis assays were performed in Ref-52 and Henle-407 cells as previously described (Chen et al., 1996; Patel and Galán, 2006, 2008). Rab5 recruitment was examined in Henle-407 cells after transfection of GFP-Rab5 as previously described (Mallo et al., 2008).

Extracellular Delivery of SopB

To monitor the fate of SopB delivered into cells from extracellular bacteria, we infected Henle-407 cells with a J sopE J sopE2 J sopB S. Typhimurium mutant strain expressing DsRED as a marker and a noninvasive SopB delivered from extracellular bacteria could associate with phagosomes. Typhimurium-induced ruffling and macropinocytosis assays were performed in Henle-407 cells and measured by using a malachite green chromogenic assay, as previously described (Hernandez et al., 2004).

S. Typhimurium-Induced Akt Activation

S. Typhimurium-induced Akt activation after infection was monitored in soluble cell lysates by probing with antibodies directed to phosphospecific Ser473Akt and Akt (Cell Signaling). Cells were lysed in 50 mM Tris (pH 7.6), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 2 mM MgCl₂, 100 mM NaF, 200 µM NaVO₄, 1 mM PMSF, 1 x protease inhibitor cocktail.

S. Typhimurium Replication Assay

S. Typhimurium replication assays were performed in Henle-407 cells and bone-marrow-derived macrophages from caspase-1-deficient mice (which are resistant to rapid S. Typhimurium-induced killing) as previously described (Hernandez et al., 2004).

SopB In Vitro Phosphatase Assay

GST-tagged derivatives of SopB and SopB288-309 were expressed and purified by standard affinity purification procedures by using glutathione-coupled Sepharose 4B beads (Pharmacia). SopB protein phosphatase activity was measured by using a malachite green chromogenic assay, as previously described (Lee et al., 2004).

SUPPLEMENTAL DATA

Supplemental Data include eight figures, Supplemental Experimental Procedures, and three movies and are available with this article online at http://www.cell.com/supplemental/S0092-8674(09)00157-3.

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