Cell, Vol. 115, 333–342, October 31, 2003, Copyright ©2003 by Cell Press

Temporal Regulation of *Salmonella* Virulence Effector Function by Proteasome-Dependent Protein Degradation

Tomoko Kubori and Jorge E. Galán*
Section of Microbial Pathogenesis
Yale University School of Medicine
New Haven, Connecticut 06536

Summary

***Salmonella enterica* invasion of host cells requires the reversible activation of the Rho-family GTPases Cdc42 and Rac1 by the bacterially encoded GEF SopE and the GAP SptP, which exert their function at different times during infection and are delivered into host cells by a type III secretion system. We found that SopE and SptP are delivered in equivalent amounts early during infection. However, SopE is rapidly degraded through a proteosome-mediated pathway, while SptP exhibits much slower degradation kinetics. The half-lives of these effector proteins are determined by their secretion and translocation domains. Chimeric protein analysis indicated that delivery of SptP into host cells by the SopE secretion and translocation domain drastically shortened its half-life. Conversely, delivery of SopE by the SptP secretion and translocation signals significantly increased its half-life, resulting in persistent actin cytoskeleton rearrangements. This regulatory mechanism constitutes a remarkable example of a pathogen's adaptation to modulate cellular functions.**

Introduction

Type III protein secretion systems (TTSSs) are specialized organelles that are essential for the virulence of many important bacterial pathogens, ranging from *Salmonella enterica*, the causative agent of food poisoning and typhoid fever, to *Yersinia pestis*, the cause of plague (Cornelis and Van Gijsegem, 2000; Galán and Collmer, 1999). The central function of this organelle is to deliver a specific set of bacterial proteins into host cells to modulate or interfere with cellular functions.

Proteins destined to travel through the TTSS pathway (effector proteins) are targeted to the secretion organelle by information contained within their first ∼120 amino acids (Cheng et al., 1997; Michiels and Cornelis, 1991; Sory et al., 1995). This secretion and translocation domain is not cleaved upon secretion, and when added to the amino terminus of reporter proteins, is capable of delivering them into host cells (Sory and Cornelis, 1994). In the bacterial cytoplasm, this domain serves as the binding site for a family of related customized chaperones, which are not secreted and are released upon secretion of the cognate effector proteins (Page and Parsot, 2002; Schesser et al., 1996; Wattiau et al., 1994; Wattiau and Cornelis, 1993). Recent crystallographic studies have shown that these chaperones maintain significant portions of the amino terminus of the effector proteins in an extended conformation that is presumably primed for rapid secretion (Birtalan et al., 2002; Stebbins and Galán, 2001). Furthermore, the crystal structure of two of these effectors, SptP and YopE, in complex with their respective chaperones, revealed that the main chain path across the chaperones is strikingly similar, despite the notable lack of overall primary sequence similarity among chaperone binding domains (Birtalan et al., 2002; Stebbins and Galán, 2001). This finding suggests that this feature may serve as recognition signal for targeting the complexes to the TTSS.

Salmonella enterica serovar Typhimurium (*S. typhimurium*) encodes two TTSSs that function at different stages of pathogenesis (Galán, 2001). One of the systems, encoded within the pathogenicity island 1 (SPI-1), mediates the initial interactions of *Salmonella* with intestinal cells leading to bacterial internalization (Galán and Curtiss, 1989). The other system, encoded within the pathogenicity island 2 (SPI-2), is required for the establishment of systemic infection (Hensel et al., 1995; Ochman et al., 1996).

Salmonella internalization into intestinal cells is the result of the coordinated modulation of the activity of the Rho-GTPase family members Cdc42 and Rac1 by a subset of SPI-1 TTSS effector proteins (Chen et al., 1996a). Thus, interaction of *Salmonella* with host cells leads to the activation of Cdc42 and Rac1 by the two highly related, bacterially encoded GEFs, SopE and SopE2, resulting in profuse membrane ruffling, actin cytoskeleton rearrangement, and subsequent bacterial uptake (Hardt et al., 1998; Stender et al., 2000). An additional bacterial effector, the phosphoinositide phosphatase SopB (Zhou et al., 2001), also contributes to this process. Following bacterial internalization, *Salmonella* is actively involved in the reversal of the cellular changes induced by the initial interaction (Fu and Galán, 1999). *Salmonella* restores the cellular architecture by injecting an additional effector protein, SptP, which in a remarkable "yin and yang" exerts its function as a GAP for Cdc42 and Rac1 (Fu and Galán, 1999). Consequently, cells infected with a wild-type strain of *S. typhimurium*, which undergo profuse membrane ruffling early (10–30 min) during infection, display a normal actin cytoskeleton ∼2–3 hr after infection despite the presence of a large number of internalized bacteria. In contrast, cells infected with an isogenic *S. typhimurium* strain deficient in SptP are unable to fully recover the normal appearance of the actin cytoskeleton even 3 hr after infection (Fu and Galán, 1999). Such coordinated activity of proteins with opposing function necessitates the existence of a mechanism to temporally regulate their activity and/or delivery into host cells. Indeed, the complexity of functions mediated by the various TTSS effector proteins of different pathogens suggests that temporal regulation must be central to the function of all these systems. However, little is known about the mechanisms that are responsible for such regulation. Here we show that the temporal regulation of the Rho GTPase GEF SopE and the GAP SptP is mediated by their differential

*Correspondence: jorge.galan@yale.edu

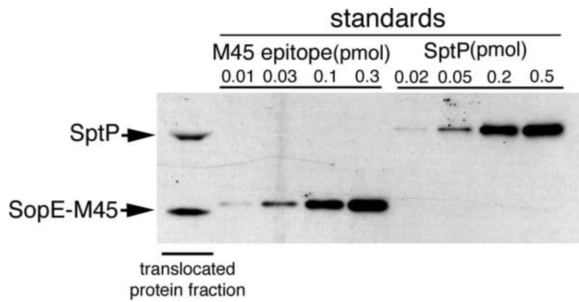


Figure 1. *S. typhimurium* Delivers Equivalent Molar Amounts of SopE and SptP into Host Cells during Initial Infection

Intestinal Henle-407 cells were infected with *S. typhimurium* strain SB1318 for 15 min, and the levels of translocated SopE and SptP (indicated as "translocated protein fraction" in the blot) were quantified by comparison with known amounts of purified SptP and CdtB-M45 as standards (see Experimental Procedures). The latter was used as a surrogate for SopE-M45, which carries the same epitope tag.

proteasome-dependent degradation within host cells in a process that is controlled by the amino terminal secretion and translocation domains of these effector proteins.

Results

Salmonella typhimurium Delivers Equivalent Molar Amounts of SopE and SptP during Initial Host Cell Infection

One possible mechanism by which *Salmonella* could temporally regulate the activities of SopE and SptP would be to inject these proteins at different time during infection. Under this hypothesis, higher levels of SopE than SptP should be present within host cells early during infection when the initial cellular responses are triggered by the bacteria. Signs of *Salmonella*-induced actin cytoskeletal rearrangements and membrane ruffling are observed within ~10 min of infection and are readily apparent after ~20 min, peaking at ~30 min following infection. Therefore, we quantified the relative amount of SopE and SptP present in host cells 15 min after infection. Using an optimized cell fractionation and infection protocol (see Experimental Procedures), we found that *S. typhimurium* delivered equivalent amounts of SopE and SptP into host cells within this time frame (SopE:SptP molar ratio = ~1.0, Figure 1). This result suggests that differential levels of SopE and SptP during the initial infection period do not account for the temporal regulation of the function of these two proteins, as differential delivery should result in significant differences in the relative levels of these two proteins. Since in vitro these proteins exhibit similar specific enzymatic activity (Fu and Galán, 1999; Hardt et al., 1998), and infected cells displayed profuse ruffling during this time frame, these results also indicate that SopE is able to exert its function in the presence of an equivalent amount of SptP.

SopE and SptP Exhibit Different Stability within Host Cells

Since the levels of SopE and SptP delivered into host cells were found to be equivalent during the initial period of infection when membrane ruffling and actin cytoskeleton reorganization are profuse, we examined the levels of these two proteins later during infection when actin cytoskeleton rearrangements subside. We found that the levels of translocated SopE drastically diminished shortly after infection and could not be detected after 30 min of infection (Figure 2A). In contrast, SptP could be readily detected even 3 hr after infection (Figure 2A), although its level slowly declined over time. The persistence of SptP over time was not likely due to its continuous delivery from internalized bacteria, since little SptP was detected in the fraction containing internalized bacteria later during infection (Figure 2A). To examine if the different levels of translocated SopE and SptP over time were due to their differential synthesis during infection, we examined the levels of translocated SopE and SptP in the presence of the bacterial protein synthesis inhibitor chloramphenicol. Addition of chloramphenicol 15 min after infection did not significantly change the levels of translocated SopE and SptP over time (Figure 2B), even though control experiments established that under these experimental conditions, addition of the antibiotic almost immediately blocked de novo protein synthesis (data not shown). Furthermore, addition of chloramphenicol did not alter the levels of these proteins within the internalized bacteria (Figure 2B), indicating that little if any de novo protein synthesis of these proteins occurs in internalized bacteria. This finding is consistent with the notion that expression of the SPI-1 TTSS system is shut off once bacteria are internalized (Deiwick et al., 1999; Lucas and Lee, 2000). Taken together, these results indicate that although SopE and SptP are initially delivered into host cells in roughly equivalent amounts, only SptP remains within host cells beyond 30 min of infection. In addition, the different levels of translocated SopE and SptP over time are not due to their differential synthesis and/or delivery once bacteria are internalized, but may be due to their different stability once translocated into the host cell cytosol.

Proteasome-Dependent Protein Degradation Controls the Differential Levels of Translocated SopE and SptP

The observation that the levels of translocated SopE rapidly decreased shortly after infection, while SptP remained readily detectable 3 hr after infection even in the presence of a bacterial protein synthesis inhibitor, suggested that these bacterial proteins are subject to differential protein degradation. Protein degradation in eukaryotic cells is largely the result of a coordinated process whereby proteins destined for degradation are tagged with specific signals (e.g., ubiquitination) and are subsequently targeted to the proteasome for destruction (Glickman and Ciechanover, 2002; Hochstrasser, 1996, 2000). To ascertain whether protein degradation through a proteasome-dependent pathway was responsible for the observed different levels of translocated SopE and SptP, we examined the amount of these proteins translocated after infection in the presence of the proteasome inhibitor MG132. As shown in Figure 3A, addition of the proteasome inhibitor rescued SopE from immediate degradation, and therefore its levels remained constant for the duration of the experiment (up

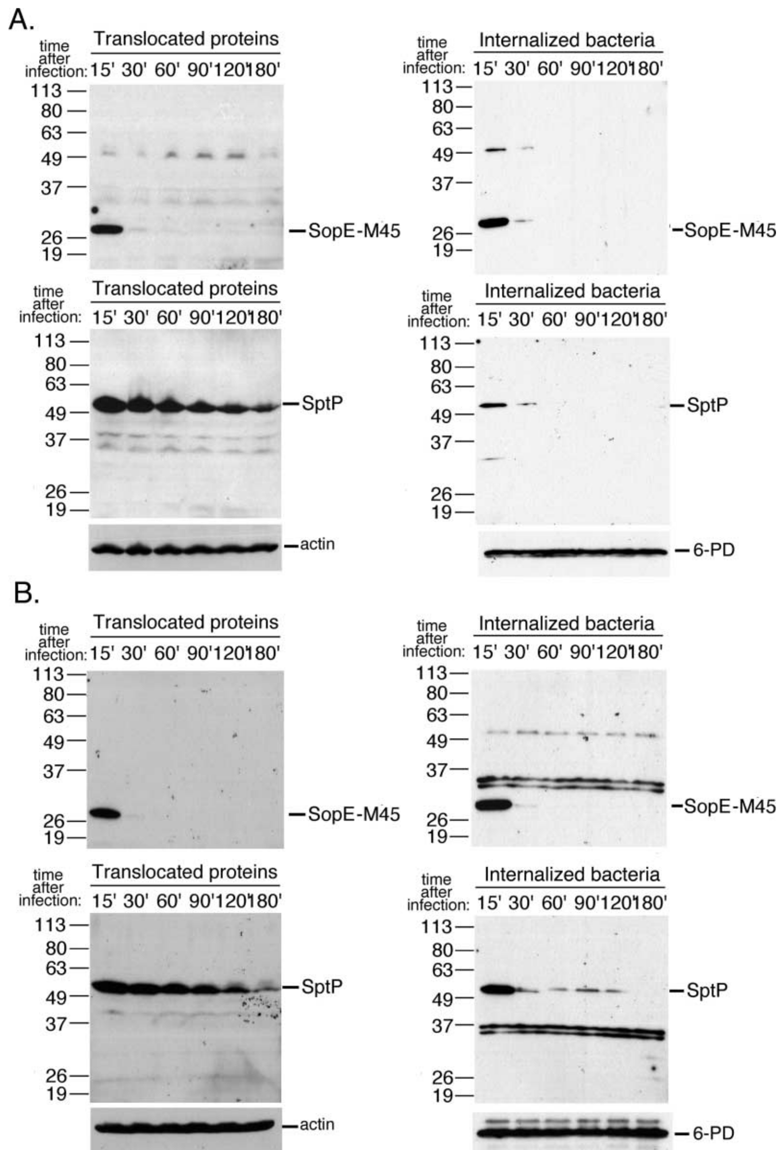


Figure 2. Translocated SopE and SptP over Time and Effect of Bacterial Protein Synthesis Inhibition on the Translocation Levels

(A) Levels of translocated SopE and SptP at different times after infection. Intestinal Henle-407 cells were infected with *S. typhimurium* strain SB1318, and at different times after infection, the levels of SopE and SptP that were delivered into host cells (indicated as "translocated proteins") or that remained within the internalized bacteria (indicated as "internalized bacteria") were examined by Western immunoblot analysis as indicated in the Experimental Procedures. Blots were sequentially probed with monoclonal antibodies directed to the M45 epitope (to detect SopE), and to SptP. To control for sample loading, blots were reprobed with polyclonal antibodies to actin (as a control for the host-cell cytosolic fraction) or to the bacterial cytoplasmic protein 6-phosphogluconate dehydrogenase (6-PD) (as a control for the internalized bacterial protein fraction).

(B) Inhibition of bacterial protein synthesis does not alter the levels of translocated SopE and SptP over time. Intestinal Henle-407 cells were infected with *S. typhimurium* strain SB1318. Fifteen minutes after infection, chloramphenicol (100 μg/ml, final concentration) was added to stop bacterial protein synthesis. At different times after infection, the levels of SopE and SptP that were either delivered into host cells (indicated as "translocated proteins") or remained within the internalized bacteria (indicated as "internalized bacteria") were examined by Western immunoblot analysis as indicated for (A).

to 120 min after infection). Addition of the proteasome inhibitor did not affect the levels of SopE within the internalized bacteria (Figure 3A), further indicating that the increased levels of translocated SopE upon addition of MG132 were the result of its effect on the cellular degradation machinery. The proteasome inhibitor also rescued the levels of translocated SptP, although only 180 min after infection since the degradation of this protein occurs with much slower kinetics (Figure 3B). The levels of SptP within the internalized bacteria were not changed by the addition of the proteasome inhibitor (Figure 3B). Equivalent results were obtained when experiments were conducted in the presence of lactacystin, another proteasome inhibitor (data not shown).

To gain further evidence that the effector protein SopE was degraded by the proteasome machinery, we investigated whether this protein was ubiquitinated when translocated into host cells. Cos-2 cells transiently expressing Myc-tagged ubiquitin were infected with *S. typhimurium* in the presence of the proteasome inhibitor MG132, and 60 min after infection, SopE was immunoprecipitated with a specific monoclonal antibody. Immunoprecipitates were separated on SDS-PAGE and the presence of ubiquitin conjugated to the bacterial proteins probed by Western immunoblotting using a specific antibody to the Myc epitope tag. As shown in Figure 4, ubiquitinated SopE was readily detected in infected, but not in uninfected, cells. Taken together, these results indicate that the different levels of translocated SopE and SptP over time are likely due to their different kinetics of degradation by the ubiquitin-mediated proteasome degradation pathway.

SopE Is Rapidly Degraded when Transiently Expressed in Culture Mammalian Cells

To investigate whether the instability of SopE within host cells was the result of intrinsic properties of this protein and not the consequence of the activity of other bacterial factors, we examined the level of full-length SopE when transiently expressed in cultured COS-2 cells. Consis-

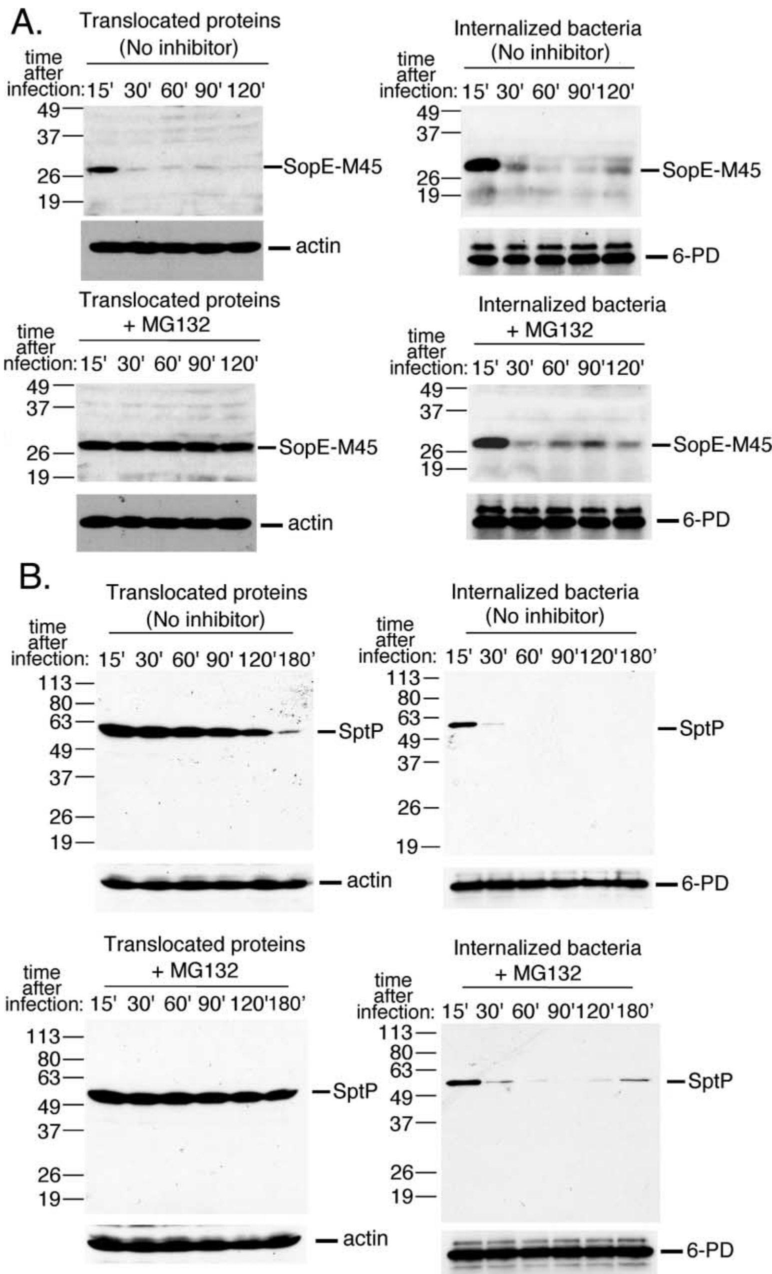


Figure 3. Proteasome-Dependent Protein Degradation Controls the Differential Levels of Translocated SopE and SptP

Intestinal Henle-407 cells were infected with *S. typhimurium* strain SB1318 in the absence or presence of the proteasome inhibitor MG132 (10 μM final concentration added 15 min prior to infection). At different times after infection, the levels of SopE (A) and SptP (B) that were either delivered into host cells (indicated as "translocated proteins") or remained within the internalized bacteria (indicated as "internalized bacteria") were examined by Western immunoblot analysis as indicated in the Experimental Procedures. Blots were probed with a monoclonal antibody directed to the M45 epitope (to detect SopE) (A), or a monoclonal antibody to SptP (B). Blots were reprobed with polyclonal antibodies to actin or the bacterial cytoplasmic protein 6-phosphogluconate dehydrogenase (6-PD) (to verify equal loading of the different samples).

tent with its activity as an exchange factor for Cdc42 and Rac1, and as previously shown (Hardt et al., 1998), SopE-transfected cells exhibited profuse membrane ruffling (data not shown). However, we were unable to detect the presence of SopE by Western immunoblot analysis of whole-cell lysates of transfected cells (Figure 5). Since we observed actin cytoskeleton rearrangements in a significant proportion of the transfected cells, we reasoned that our failure to detect SopE was due to its rapid degradation rather than the lack of its expression. To test this hypothesis, we transiently expressed SopE in Cos-2 cells in the presence of the proteasome inhibitor MG132. As observed in cells transfected in the absence of the inhibitor, profuse membrane ruffling and actin cytoskeleton rearrangements were apparent in transfected cells. However, in the presence of the inhibitor, SopE was readily detected in cell lysates of the transfected cells (Figure 5). These results indicate that properties intrinsic to the SopE protein determine its poor stability in host cells and that no other bacterial factors are required to determine its short half-life.

Crystallographic and functional studies have established that the effector domain of SopE is located from amino acids 78 to 240 (Buchwald et al., 2002; Hardt et al., 1998). This domain alone is capable of recapitulating all the known activities of SopE when delivered into host cells by microinjection or transfection (Hardt et al., 1998). We hypothesized that the first 78 amino acids of SopE, which contain the secretion and translocation signals, may be important in determining the half-life of this protein within host cells. To test this hypothesis, we transiently expressed a deletion mutant of this protein lack-

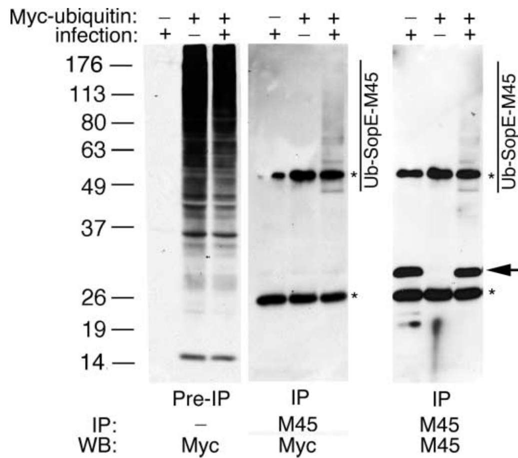


Figure 4. SopE Is Ubiquitinated upon Translocation into Host Cells

COS-2 cells transfected with a plasmid expressing Myc-epitope-tagged ubiquitin were infected with *S. typhimurium* strain SB1318 in the presence of the proteasome inhibitor MG132 (10 μM final concentration, added 60 min prior to infection). One hour after infection, cell lysates from infected as well as uninfected cells were subjected to immunoprecipitation with a monoclonal antibody directed to the M45 epitope present in SopE-M45, run on SDS-PAGE, and analyzed by Western immunobloting with an anti-Myc or anti-M45 monoclonal antibodies as indicated in the Experimental Procedures. Samples from the lysates prior to immunoprecipitation (indicated as Pre-IP) were examined to evaluate levels of expression of Myc-ubiquitin. Asterisks denote the position of the heavy and light chains of the M45 monoclonal antibody. Arrow denotes the position of nonconjugated SopE-M45.

ing this domain and compared its stability in host cells with that of the full-length protein. As previously observed (Hardt et al., 1998), expression of this domain of SopE resulted in profuse membrane ruffling (data not shown). However, contrary to what was observed with full-length SopE, the expression of $SopE_{78–240}$ was readily detected in cell lysates of transfected cells even in the absence of the proteasome inhibitor MG132 (Figure 5).

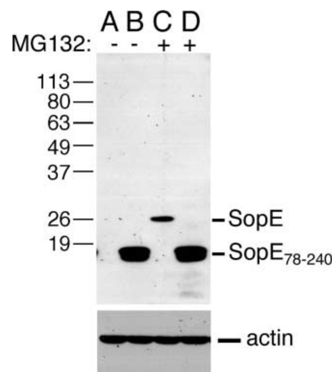


Figure 5. Differential Stability of SopE and $SopE_{78–240}$ when Transiently Expressed in Cultured Mammalian Cells

M45-epitope tagged full-length SopE (lanes A and C) or a mutant derivative lacking its secretion and translocation amino terminal domain (lanes B and D) were transiently expressed in COS-2 cells in the absence (lanes A and B) or presence (lanes C and D) of the proteasome inhibitor MG132 (10 μM final concentration). The levels of these proteins in total cell lysates were examined by Western immunoblot analysis as indicated in the Experimental Procedures.

Taken together, these results suggest that the half-life of SopE within host cells is largely determined by intrinsic properties of this molecule, and more specifically by intrinsic features of its amino terminal secretion and translocation domain, since its removal resulted in a significant stabilization of this effector protein.

The Differential Stability of SopE and SptP Is Controlled by their Secretion and Translocation Domains

The observation that the presence or absence of the secretion and translocation domain of SopE has a major effect on its stability within host cells suggested that the differential levels of translocated SopE and SptP observed after bacterial infection might be dependent on these domains. We reasoned that if this was the case, it should be possible to alter the stability of translocated SopE and SptP by exchanging their respective secretion and translocation domains. We therefore constructed chimeric proteins where the effector domains of SopE (amino acids 78–240) and SptP (amino acids 101–543) were fused to the translocation domains of SptP (amino acids 1–160) and SopE (amino acids 1–100), respectively. The design of the chimeric proteins was guided by the crystallographic and functional studies that have precisely defined these domains (Buchwald et al., 2002; Fu and Galán, 1998a, 1998b; Hardt et al., 1998; Stebbins and Galán, 2000, 2001). To maintain the native control of gene expression, the resulting chimeric genes were introduced by allelic exchange into the chromosome at the respective locus encoding the secretion and translocation domains. Cultured intestinal Henle-407 cells were then infected with a *S. typhimurium* strain encoding $SopE_{1–100}$-$SptP_{101–543}$ and $SptP_{1–160}$-$SopE_{78–240}$ chimeric proteins (instead of the wild-type alleles), and the levels of translocated proteins over time were examined by Western immunoblot analysis. As shown in Figure 6, the levels of translocated chimeric proteins strictly correlated with the identity of the amino terminal domain: the $SopE_{1–100}$ translocation domain destabilized the SptP effector domain, while the $SptP_{1–160}$ translocation domain increased the half-life of the SopE effector domain. As in the case of the wild-type proteins, addition of the proteasome inhibitor MG132 effectively stabilized both translocated chimeric proteins (Figure 6). These results demonstrate that the temporal regulation of the levels of SopE and SptP within host cells is controlled by intrinsic properties of their secretion and translocation domains, which ultimately determine their half-life.

Stabilization of SopE Prevents Cellular Recovery after Bacterial Infection

We have previously shown that the marked actin remodeling and membrane ruffling that follow the delivery of the Rho-family GTPase activators SopE, SopE2, and SopB by *S. typhimurium* are reversed within 2 or 3 hr of infection (Fu and Galán, 1999). We have also demonstrated that the GAP activity of SptP is directly involved in the recovery process and that cells infected with a *S. typhimurium* strain lacking this effector significantly delayed their recovery (Fu and Galán, 1999). We hypothesized that if the balance between the activation and downmodulation of Cdc42 and Rac1 by SopE and SptP

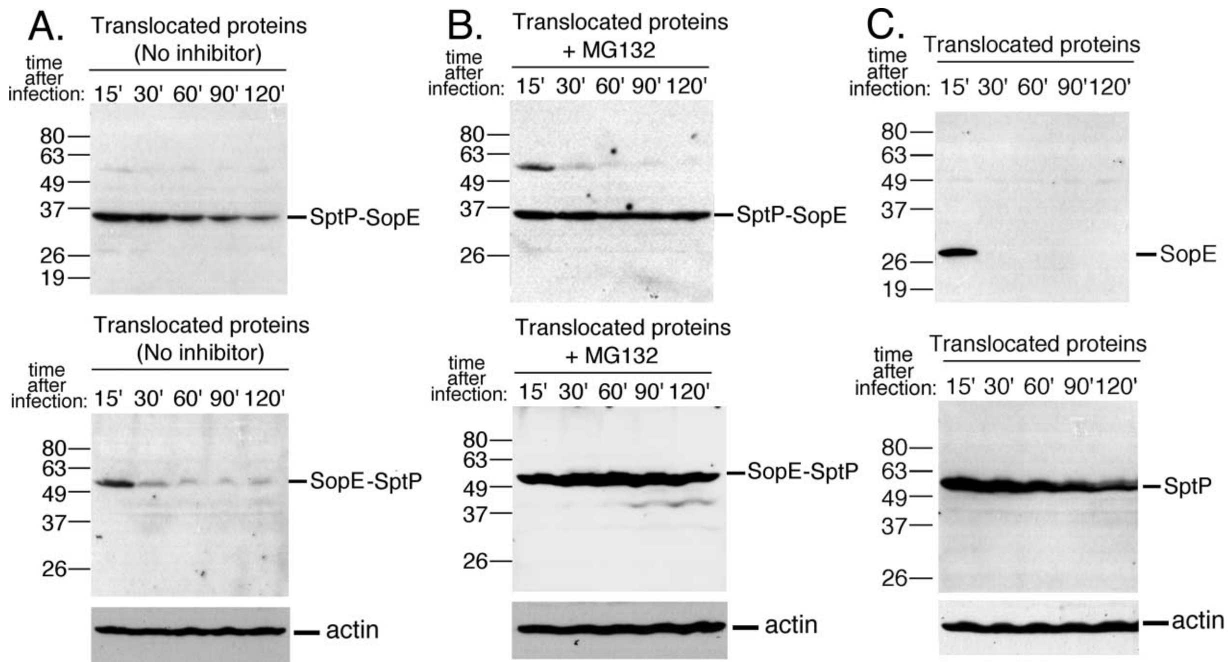


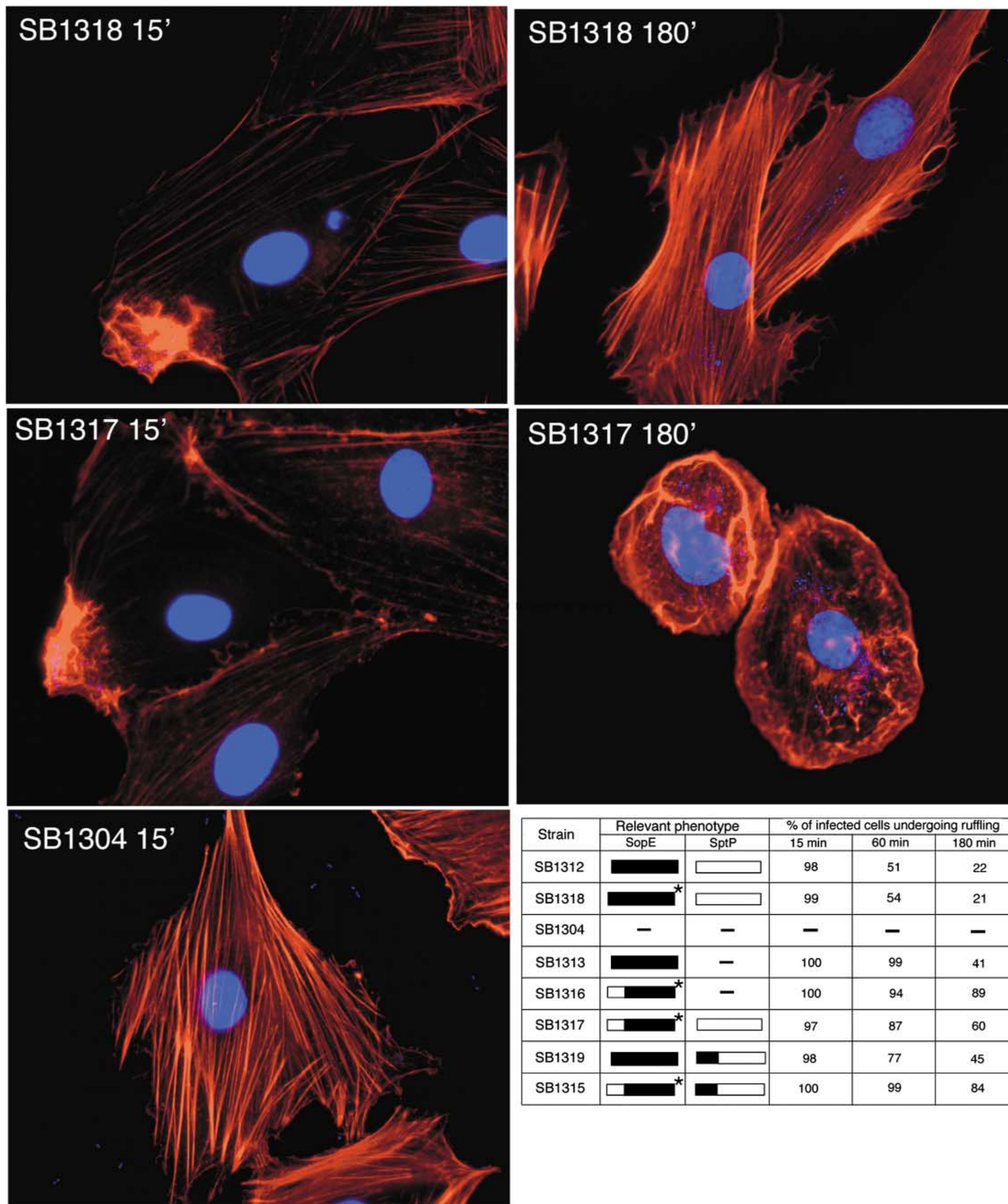
Figure 6. The Differential Stability of SopE and SptP Is Controlled by their Secretion and Translocation Domains

Intestinal Henle-407 cells were infected with a *S. typhimurium* strain SB1315 expressing chimeric SopE and SptP proteins in which their respective secretion and translocation signals had been exchanged (A and B), or with a *S. typhimurium* control strain SB1318, which expresses wild-type forms of these proteins for comparison (C). The chimeric proteins expressed by strain SB1315 were $SptP_{1-160}$-$SopE_{78-240}$ (referred to as SptP-SopE) and $SopE_{1-100}$-$SptP_{101-543}$ (referred to as SopE-SptP). The levels of translocation of these proteins over time in the presence (B) or absence (A and C) of the proteasome inhibitor MG132 (10 μM final concentration) were examined by Western immunoblot analysis as indicated in Experimental Procedures. Blots were sequentially probed with a monoclonal antibody to the M45 epitope (to detect the SptP-SopE chimera), a monoclonal antibody directed to the carboxy terminus of SptP (to detect the SopE-SptP chimera), and a polyclonal antibody to actin (to verify equal loading of the different samples).

is dependent on their different half-life, changes in their degradation kinetics of SopE and SptP should result in alteration in the dynamics and kinetics of the cellular responses. We therefore compared the interaction with host cells of *S. typhimurium* strains expressing either wild-type SopE (SB1318) or the chimeric protein $SptP_{1-160}$-$SopE_{78-240}$ (SB1317) by time-lapse video microscopy. A roughly equivalent amount of membrane ruffling was observed shortly after infection with either bacterial strain (Figure 7 and Supplemental Movie S1 at http://www.cell.com/cgi/content/full/115/3/333/DC1). In cells infected with a strain expressing wild-type SopE, the ruffling activity peaked at about 20 min after infection and had diminished drastically by ~60 min after infection. In contrast, the ruffling activity in cells infected with a *S. typhimurium* strain expressing $SptP_{1-160}$-$SopE_{78-240}$ did not subside for the duration of the experiment (180 min) (Figure 7 and Supplemental Movie S1). We quantified the degree of persistence of the actin cytoskeletal rearrangements after infection with strains expressing chimeric mutants of SopE and SptP that exhibit different half-life when translocated host cells. Cells infected with strains expressing $SptP_{1-160\text{-}SopE78-240}$, which exhibits longer half-life than wild-type SopE, showed persistent actin cytoskeleton rearrangements, regardless of whether the strains expressed wild-type SptP or not (Figure 7). In addition, cells infected with *S. typhimurium* expressing $SopE_{1-100}$-$SptP_{101-543}$, which exhibits a much shorter half-life than wild-type SptP, also displayed persistent actin cytoskeleton rearrangements in a manner similar to cells infected with a *S. typhimurium* strain lacking SptP. Taken together, these results demonstrate that differential protein stability is central to the coordination of the activity of the bacterial effectors that modulate cellular responses.

Discussion

A central function of TTSSs is the delivery of a diverse array of bacterially encoded effector proteins that modulate complex cellular functions (Cornelis and Van Gijsegem, 2000; Galán and Collmer, 1999). These effector proteins sometimes exhibit opposing activities (Fu and Galán, 1999; Hardt et al., 1998), which suggests the existence of mechanisms to temporally regulate their delivery and/or their function. It has been proposed that in *Yersinia* spp., the temporal regulation of effector protein activity might be controlled by differential gene expression or differential delivery into host cells (Boyd et al., 2000; Francis et al., 2001; Wulff-Strobel et al., 2002). Although differential gene expression may indeed schedule the activity of different type III secretion systems encoded within the same bacterium (e.g., the TTSSs encoded with *Salmonella enterica* SPI-1 and SPI-2 pathogenicity islands) (Galán, 2001), different patterns of gene expression may not explain the temporal regulation of the function of some TTSS effector proteins delivered by the same TTSS. Furthermore, little is known about potential mechanisms that could establish a hierarchy in the secretion process itself as a way of estab-



Strain	Relevant phenotype		% of infected cells undergoing ruffling		
	SopE	SptP	15 min	60 min	180 min
SB1312	▬	▭	98	51	22
SB1318	▬*	▭	99	54	21
SB1304	–	–	–	–	–
SB1313	▬	–	100	99	41
SB1316	▭▬*	–	100	94	89
SB1317	▭▬*	▭	97	87	60
SB1319	▬	▬▭	98	77	45
SB1315	▭▬*	▬▭	100	99	84

Figure 7. Stabilization of SopE Prevents Cellular Recovery after Bacterial Infection

Cultured Ref52 cells were infected for 15 min with different strains of *S. typhimurium* as indicated. At different times after infection, cells were stained with TRITC-labeled phalloidin to visualize polymerized actin and DAPI to visualize bacteria. The percentage of cells that had been infected by the different strains of *S. typhimurium* and that exhibited actin cytoskeleton rearrangement are indicated in the lower right panel. At least 100 cells were examined for each strain and each time point. Equivalent results were obtained in several repetitions of this experiment. In addition to the indicated relevant genotypes, all strains listed in the lower right panel also carry deletions in the *sopB* and *sopE2* genes to ensure that the observed actin-cytoskeletal responses were exclusively due to the proteins under study (i.e. SopE and/or SptP and corresponding chimeras). The presence of an asterisk denotes the presence of an M45-epitope tag at the C terminus of SopE. Also shown are representative photomicrographs of cells infected with the indicated strains for the indicated amount of time and subsequently stained with TRITC-labeled phalloidin (red) to stain polymerized actin, and DAPI (blue) to visualized bacteria. The strain SB1304 carries loss-of-function mutations in *sptP, sopE*, *sopE2*, and *sopB*, and therefore is unable to induce actin cytoskeleton rearrangements (negative control). A time-lapse microvideo of cultured intestinal Henle-407 cells infected with *S. typhimurium* strains expressing either wild-type SopE (SB1318) or the chimeric mutant $SptP_{1-160}$-$SopE_{78-240}$ (SB1317) can be seen in the Supplemental Data at http://www.cell.com/cgi/content/full/115/3/333/DC1.

lishing a temporal regulation of the delivery of TTSS effector proteins.

The temporal difference in the activities of the GEF SopE and the GAP SptP, which are engaged in the reversible activation of the Rho-family GTPases Cdc42 and Rac1, afforded us the opportunity to closely examine potential mechanisms that may be responsible for such a regulation. We found comparable levels of trans-

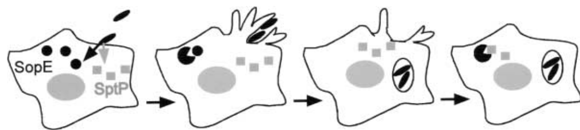


Figure 8. Model for the Temporal Regulation of the Activities of the *Salmonella* GEF SopE and the GAP SptP

Upon infection of host cells, *Salmonella* delivers roughly equivalent amounts of SopE and SptP. Shortly after infection (~15–20 min), SopE is rapidly degraded by a mechanism that is dependent on the host-cell proteasome and the amino terminal secretion and translocation domain of the bacterial protein. SptP is eventually also degraded by the proteasome, but with a half-life much longer than that of SopE.

located SopE and SptP shortly after infection, indicating that differential delivery of these effectors is not the mechanism by which their activity is temporally regulated. However, we found that the levels of the GEF SopE drastically decreased over time and were below the level of detection ~30 min after infection. In contrast, the levels of the GAP SptP decreased very slowly after infection. The different levels of SopE and SptP were not due to their differential synthesis after infection, since the same pattern was observed when infections were carried out in the presence of a bacterial protein synthesis inhibitor. These results also argue that the delivery of SopE and SptP must occur very early during infection and must cease shortly after bacteria internalization.

Our results indicate that the different levels of translocated SopE and SptP over time were due to their differential degradation by the proteasome-dependent pathway. This was supported by at least two lines of evidence: (1) addition of a proteasome inhibitor effectively prevented the degradation of SopE, resulting in sustained levels of this effector, and (2) ubiquitination of translocated SopE was readily detected after infection. Although SptP was also subject to degradation by the proteasome pathway, the kinetics of this process were much slower than those of SopE. These results indicate that the differential stability of the two-effector proteins ultimately determines their temporal regulation (Figure 8).

We found that the short half-life of SopE was due to intrinsic properties of this effector protein. This was demonstrated in transient expression experiments, where we observed that, despite the ability of SopE to stimulate membrane ruffling, its expression could not be detected by Western immunoblot analysis. However, SopE was readily detected in the presence of a proteasome inhibitor. Furthermore, the half-life of SopE in transfected cells was drastically increased by the removal of its secretion and translocation signal, implicating this domain in the establishment of its half-life. To further investigate the hypothesis that the secretion and translocation domains of TTSS effector proteins ultimately determine their half-life within host cells, we constructed chimeric proteins consisting of the secretion and translocation signals of SopE or SptP fused to the respective heterologous effector domains. Consistent with this hypothesis, the chimeric proteins exhibited equivalent half-lives to those of the effector proteins contributing the secretion and translocation domains. Thus, the half-life of SopE was markedly increased when fused to the secretion and translocation domain of SptP, and conversely, the half-life of SptP was drastically decreased when its secretion and translocation domain was placed under the control of the SopE signal. These experiments also rule out the possibility that the epitope tag present at the C terminus of SopE could have influenced its half-life, which is also supported by the observation that the epitope tag did not affect the function of SopE in vivo (Figure 7, compare strains SB1312 and SB1318).

The interaction of *Salmonella* with cultured cells results in membrane ruffling (mediated by the Rho GTPase activators), which rapidly subsides largely due to the GAP activity of SptP (Chen et al., 1996a; Fu and Galán, 1999; Hardt et al., 1998). Altering the half-lives of SopE and SptP by either adding a proteasome inhibitor (data not shown) or by exchanging their secretion and translocation signals resulted in persistent membrane ruffling, demonstrating that the differential degradation of effector proteins is central to the temporal regulation of their activities within host cells.

It is not clear what the bases are for the differential stability of SopE and SptP. Close inspection of the primary amino acid sequence of the secretion and translocation domains of these proteins did not reveal obvious features such as the presence of known degradation signals that could account for such a difference. Many lysine residues that could potentially serve as ubiquitination sites are found distributed throughout the secretion and translocation domains of both SopE and SptP. However, more studies will be necessary to ascertain which if any of these sites may play a role in determining the half-lives of these proteins.

There is little primary amino acid sequence similarity between the secretion and translocation domains of different effector proteins (Cornelis and Van Gijsegem, 2000; Page and Parsot, 2002). However, comparison of the crystal structure of the *Yersinia* spp. effector YopE bound to its chaperone SycE with that of SptP bound to its chaperone SicP revealed that despite the lack of primary amino acid sequence similarity between the chaperone binding domains, the main chain path that they followed across the chaperones is highly conserved (Birtalan et al., 2002). In addition, there is conservation of key residues that are exposed for inspection by the secretion machinery. This design may allow for the different chaperone binding domains to be recognized by the secretion machinery through common structural features while retaining significant primary amino acid sequence diversity that could potentially confer specific properties to each effector, such as their differential stability once in the host cell. This design would also provide selection pressure for the evolution of diversity among chaperone binding domains of effectors that are delivered by the same TTSS. Although it is unknown whether the activity of other effector proteins is regulated by differential protein stability, it is intriguing that the *S. typhimurium* effector protein SigD (SopB) has been recently shown to be ubiquitinated within host cells (Marcus et al., 2002).

We have described here a mechanism by which bacterial TTSS effector protein activities are temporally regulated within host cells through differential protein stabil-

ity. This remarkable adaptation to precisely modulate host cell functions is another example of the refined nature of the interactions of microbial pathogens and their hosts, the product of evolution through their long-standing close association.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Condition

All bacterial strains were grown under conditions that stimulate the invasion-associated type III secretion system (Chen et al., 1996b). All *S. typhimurium* strains were derived from the wild-type strain SL1344 (Hoiseth and Stocker, 1981). The isogenic strain SB1304 carrying deletions in *sopE, sopE2, sopB*, and *sptP*, and therefore completely defective in the ability to stimulate actin cytoskeleton rearrangements and membrane ruffling, has been previously described (Zhou et al., 2001). The strains SB1312, which carries deletion mutations in *sopE2* and *sopB*, SB1318, which carries deletions in *sopE2* and *sopB* and encodes M-45 epitope-tagged SopE in the chromosome, and SB1313, with deletion mutations in *sopE2, sopB*, and *sptP*, were constructed by allelic exchange as previously described (Kaniga et al., 1994). These strains were used to construct isogenic derivatives expressing SopE-SptP chimeras ($SptP_{1-160}$-$SopE_{78-240}$ and/or $SopE_{1-100}$-$sptP_{101-543}$) as indicated in Figure 7. In all cases, the chimeric constructs were introduced in the chromosomal locus corresponding to the gene encoding the secretion and translocation signals of the chimeric protein to ensure native levels of expression. It should be noted that all these strains have deletions in *sopE2*, *sopB*, and, when indicated, *sptP*, to ensure that the modulation of the actin cytoskeleton was solely due to the activity of the chimeric proteins (Zhou et al., 2001). All mutant strains were analyzed by PCR to ascertain the correct allelic replacement, and by Western immunoblot to verify the expression and type III dependent secretion of the different constructs.

The eukaryotic expression plasmids expressing full-length M45-epitope tagged SopE and truncated $SopE_{78-240}$ have been previously described (Hardt et al., 1998). A plasmid expressing Myc-tagged ubiquitin was generously provided by Eric Hughes, Yale University.

Bacterial Protein Translocation Assay

TTSS-mediated effector protein translocation into host cells was examined by biochemical fractionation with a modification of a previously described protocol (Collazo and Galán, 1997). Briefly, semiconfluent (70%–80% confluence) cultured intestinal Henle-407 cells grown in 100 mm tissue culture dishes were washed twice with HBSS prewarmed at 37°C and infected with *S. typhimurium* strains at a multiplicity of infection (moi) of 100. After 15 min of infection, the cells were washed with HBSS prewarmed at 37°C, and at this point gentamicin (100 μg/ml) was added to kill extra cellular bacteria. In bacterial protein synthesis inhibition experiments, chloramphenicol (100 μg/ml) was added together with gentamicin 15 min after infection. At various time points after infection, the cells were washed twice with cold HBSS and lysed with 1.0 ml of HBSS containing 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). This procedure lyses the infected cells but does not affect the integrity of the bacterial membrane (Collazo and Galán, 1997). Cell lysates were scrapped off, collected in chilled microfuge tubes, and centrifuged at 13,000 rpm for 15 min at 4°C to separate the soluble fraction, containing bacterial proteins that have been injected into the host cell cytosol, from the insoluble fraction, which contains the internalized bacteria. The soluble fraction was filtered through a 0.22 μm pore-size filter and subjected to trichloroacetic acid (TCA) precipitation. Both fractions were analyzed by Western immunoblotting using monoclonal antibodies against the M45 epitope (to detect SopE-M45) (Obert et al., 1994) and the C terminus of SptP (Fu and Galán, 1998b). When indicated, the proteasome inhibitors MG132 (Calbiochem) (10 μM), lactacystin (10 μM), or their solvent DMSO were added to cells 15 min prior to infection. The reversible proteasome inhibitor MG132 was also included in all washing solutions and lysis buffer.

The quantification of the levels of SopE and SptP translocated into cultured Henle-407 cells 15 min after infection with *S. typhimurium* strain SB1318 was carried out by Western immunoblotting with monoclonal antibodies against the M45 epitope tag and SptP. Blots were scanned, and the intensity of the signals was determined using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) and compared to the signals generated by serial dilutions of known amounts of purified SptP and CdtB-M45 (Lara-Tejero and Galán, 2000) (to calibrate the sensitivity of the monoclonal antibody directed to the epitope tag).

Transfection of Cultured Cells, Fluorescence, and Video Microscopy

Transfection of COS-2 cells and Western blot analysis of protein expression was carried out as previously described (Hardt et al., 1998). When appropriate, the proteasome inhibitor MG132 (10 μM) was added 6 hr prior to cell harvesting. The recovery of the actin cytoskeleton architecture after bacterial infection was evaluated by fluorescence microscopy as previously described (Fu and Galán, 1999). Briefly, semiconfluent Ref52 cells were infected with different *S. typhimurium* strains for various time periods with an moi of 50. At 15 min after infection, the cells were washed with HBSS prewarmed at 37°C and 100 μg/ml gentamicin was added to the cells. At different times after infection, cells were fixed with 1% paraformaldehyde for 15 min and permeabilzed with 0.1% Triton X-100 for 15 min. The cells were then labeled with DAPI to detect bacteria and with TRITC-conjugated phalloidin to stain the host actin cytoskeleton. Normal cytoskeletal structure for Ref52 cells is characterized by well-developed stress fibers when visualized by fluorescent microscopy in the presence of TRITC-labeled phalloidin. Those structures are significantly altered upon induction of membrane ruffling shortly after wild-type *Salmonella* infection and recover their normal appearance of the cytoskeleton ~3 hr after infection. Because recovery of the cytoskeleton is a dynamic process involving the reassembly of stress fibers and membrane changes, subjectivity was minimized by insuring that the cells examined were infected with *Salmonella* and by counting recovered cells blind to the bacterial strain with which they were infected. Time-lapse video microscopy of intestinal Henle-407 cells infected with different strains of *S. typhimurium* with an moi of 20 was carried out in a Nikon TE2000-U Eclipse inverted microscope fitted with a Micromax Princeton digital camera and controlled by the Metamorph software package (Universal Imaging).

Immunoprecipitation Analysis to Detect Ubiquitinated Proteins

Semiconfluent (60% confluence) cultured Cos-2 cells grown in 6-well tissue culture dishes were transfected with 1 μg per well of a plasmid expressing Myc-tagged ubiquitin. Forty-eight hours after transfection, cells were infected with *S. typhimurium* strain SB1318 in the presence of the proteasome inhibitor MG132 as described for the bacterial protein translocation assay. After 60 min infection, the cells were lysed with 0.25 ml per well of lysis buffer (50 mM Tris-Cl [pH 7.5], 1% NonidetP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μM MG132, 10 mM N-ethylmileimide (NEM) (Sigma, St. Louis, MO), protease inhibitors (Complete, Roche Diagnostics GmbH, Mannheim, Germany), and scraped off the dishes, and the lysates from all wells were combined and placed on ice. The cell lysates were centrifuged to remove insoluble materials and preabsorbed to protein A sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) for 1 hr at 4°C. Monoclonal antibody directed to M45 was added to the preabsorbed lysates and incubated for 15 hr at 4°C under gentle rocking. Immunocomplexes were then recovered with protein A sepharose for 3 hr at 4°C, and washed 5 times with 1 ml of the lysis buffer by centrifugation at 4,000 rpm for 5 min in a microcentrifuge. The samples were finally washed with 50 mM Tris-Cl (pH 7.5), boiled in SDS loading buffer to dissociate proteins from the beads, loaded on 10% SDS-PAGE, and analyzed by Western blotting using an anti-Myc monoclonal antibody (clone 9E10, Santa Cruz Biotechnology) or with a monoclonal antibody against the M45 epitope to detect SopE-M45, as appropriate.

Acknowledgments

We thank Eric Hughes for the plasmid expressing Myc-tagged ubiqutin, and María Lara-Tejero and Sang-Ho Lee for purified prepara-

tions of CdtB-M45 and SptP, respectively. We also thank members of the Galán laboratory for critical review of this manuscript. This work was supported by Public Health Service Grants #AI30492 and #GM52543 from the National Institutes of Health to J.E.G.

Received: July 23, 2003
Revised: October 3, 2003
Accepted: October 10, 2003
Published: October 30, 2003

References

Birtalan, S.C., Phillips, R.M., and Ghosh, P. (2002). Three-dimensional secretion signals in chaperone-effector complexes of bacterial pathogens. Mol. Cell *9*, 971–980.

Boyd, A., Lambermont, I., and Cornelis, G. (2000). Competition between the Yops of *Yersinia enterocolitica* for delivery into eukaryotic cells: role of the SycE chaperone binding domain of YopE. J. Bacteriol. *182*, 4811–4821.

Buchwald, G., Friebel, A., Galán, J.E., Hardt, W.D., Wittinghofer, A., and Scheffzek, K. (2002). Structural basis for the reversible activation of a Rho protein by the bacterial toxin SopE. EMBO J. *21*, 3286–3295.

Chen, L.M., Hobbie, S., and Galan, J.E. (1996a). Requirement of CDC42 for Salmonella-induced cytoskeletal and nuclear responses. Science *274*, 2115–2118.

Chen, L.M., Kaniga, K., and Galán, J.E. (1996b). *Salmonella* spp. are cytotoxic for cultured macrophages. Mol. Microbiol. *21*, 1101–1115.

Cheng, L.W., Anderson, D.M., and Schneewind, O. (1997). Two independent type III secretion mechanisms for YopE in *Yersinia enterocolitica*. Mol. Microbiol. *24*, 757–765.

Collazo, C., and Galán, J.E. (1997). The invasion-associated type III system of *Salmonella typhimurium* directs the translocation of Sip proteins into the host cell. Mol. Microbiol. *24*, 747–756.

Cornelis, G.R., and Van Gijsegem, F. (2000). Assembly and function of type III secretory systems. Annu. Rev. Microbiol. *54*, 735–774.

Deiwick, J., Nikolaus, T., Erdogan, S., and Hensel, M. (1999). Environmental regulation of Salmonella pathogenicity island 2 gene expression. Mol. Microbiol. *31*, 1759–1773.

Francis, M., Lloyd, S., and Wolf-Watz, H. (2001). The type III secretion chaperone LcrH co-operates with YopD to establish a negative, regulatory loop for control of Yop synthesis in *Yersinia pseudotuberculosis*. Mol. Microbiol. *42*, 1075–1093.

Fu, Y., and Galán, J.E. (1998a). Identification of a specific chaperone for SptP, a substrate of the centisome 63 type III secretion system of *Salmonella typhimurium*. J. Bacteriol. *180*, 3393–3399.

Fu, Y., and Galán, J.E. (1998b). The *Salmonella* spp. protein tyrosine phosphatase SptP is translocated into host cells and disrupts the host-cell cytoskeleton. Mol. Microbiol. *27*, 359–368.

Fu, Y., and Galán, J.E. (1999). A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature *401*, 293–297.

Galán, J.E. (2001). Salmonella interaction with host cells: Type III secretion at work. Annu. Rev. Cell Dev. Biol. *17*, 53–86.

Galán, J.E., and Curtiss, R., III. (1989). Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA *86*, 6383–6387.

Galán, J.E., and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. Science *284*, 1322–1328.

Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. *82*, 373–428.

Hardt, W.-D., Chen, L.-M., Schuebel, K.E., Bustelo, X.R., and Galán, J.E. (1998). *Salmonella typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell *93*, 815–826.

Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E., and Holden, D.W. (1995). Simultaneous identification of bacterial virulence genes by negative selection. Science *269*, 400–403.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. Annu. Rev. Genet. *30*, 405–439.

Hochstrasser, M. (2000). Evolution and function of ubiquitin-like protein-conjugation systems. Nat. Cell Biol. *2*, 153–157.

Hoiseth, S.K., and Stocker, B.A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature *291*, 238–239.

Kaniga, K., Bossio, J.C., and Galán, J.E. (1994). The *Salmonella typhimurium* invasion genes *invF* and *invG* encode homologues to the PulD and AraC family of proteins. Mol. Microbiol. *13*, 555–568.

Lara-Tejero, M., and Galán, J.E. (2000). A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science *290*, 354–357.

Lucas, R.L., and Lee, C.A. (2000). Unravelling the mysteries of virulence gene regulation in *Salmonella typhimurium*. Mol. Microbiol. *36*, 1024–1033.

Marcus, S., Knodler, L., and Finlay, B. (2002). *Salmonella enterica* serovar Typhimurium effector SigD/SopB is membrane-associated and ubiquitinated inside host cells. Cell. Microbiol. *4*, 435–446.

Michiels, T., and Cornelis, G.R. (1991). Secretion of hybrid proteins by the *Yersinia* Yop export system. J. Bacteriol. *173*, 1677–1685.

Obert, S., O'Connor, R.J., Schmid, S., and Hearing, P. (1994). The adenovirus E4–6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. Mol. Cell. Biol. *14*, 1333–1346.

Ochman, H., Soncini, F.C., Solomon, F., and Groisman, E.A. (1996). Identification Of a pathogenicity island required for Salmonella survival in host cells. Proc. Natl. Acad. Sci. USA *93*, 7800–7804.

Page, A.L., and Parsot, C. (2002). Chaperones of the type III secretion pathway: Jacks of all trades. Mol. Microbiol. *46*, 1–11.

Schesser, K., Frithz-Lindsten, E., and Wolf-Watz, H. (1996). Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. J. Bacteriol. *178*, 7227–7233.

Sory, M.-P., and Cornelis, G.R. (1994). Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. Mol. Microbiol. *14*, 583–594.

Sory, M.-P., Boland, A., Lambermount, I., and Cornelis, G. (1995). Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. Proc. Natl. Acad. Sci. USA *92*, 11998–12002.

Stebbins, C.E., and Galán, J.E. (2000). Modulation of host signaling by a bacterial mimic: Structure of the Salmonella effector SptP bound to Rac1. Mol. Cell *6*, 1449–1460.

Stebbins, C.E., and Galán, J.E. (2001). Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion. Nature *414*, 77–81.

Stender, S., Friebel, A., Linder, S., Rohde, M., Mirold, S., and Hardt, W.D. (2000). Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Mol. Microbiol. *36*, 1206–1211.

Wattiau, P., and Cornelis, G.R. (1993). SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE. Mol. Microbiol. *8*, 123–131.

Wattiau, P., Bernier, B., Deslée, P., Michiels, T., and Cornelis, G.R. (1994). Individual chaperones required for Yop secretion by *Yersinia*. Proc. Natl. Acad. Sci. USA *91*, 10493–10497.

Wulff-Strobel, C., Williams, A., and Straley, S. (2002). LcrQ and SycH function together at the Ysc type III secretion system in *Yersinia pestis* to impose a hierarchy of secretion. Mol. Microbiol. *43*, 411–423.

Zhou, D., Chen, L.M., Hernandez, L., Shears, S.B., and Galán, J.E. (2001). A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Mol. Microbiol. *39*, 248–259.