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Coiled-coil domains enhance the membrane association of *Salmonella* type III effectors

Leigh A. Knodler^{1,*}, J. Antonio Ibarra^{1,†}, Ernesto Pérez-Rueda², Calvin K. Yip³, and Olivia Steele-Mortimer¹

¹Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, Montana

²Instituto de Biotecnología, UNAM, Cuernavaca, Morelos, México

³Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada

Summary

Coiled-coil domains in eukaryotic and prokaryotic proteins contribute to diverse structural and regulatory functions. Here we have used *in silico* analysis to predict which proteins in the proteome of the enteric pathogen, *Salmonella enterica* serovar Typhimurium, harbor coiled-coil domains. We found that coiled-coil domains are especially prevalent in virulence-associated proteins, including type III effectors. Using SopB as a model coiled-coil domain type III effector, we have investigated the role of this motif in various aspects of effector function including chaperone binding, secretion and translocation, protein stability, localization and biological activity. Compared to wild type SopB, SopB coiled-coil mutants were unstable, both inside bacteria and after translocation into host cells. In addition, the putative coiled-coil domain was required for the efficient membrane association of SopB in host cells. Since many other *Salmonella* effectors were predicted to contain coiled-coil domains, we also investigated the role of this motif in their intracellular targeting in mammalian cells. Mutation of the predicted coiled-coil domains in PipB2, SseJ and SopD2 also eliminated their membrane localization in mammalian cells. These findings suggest that coiled-coil domains may represent a common membrane-targeting determinant for *Salmonella* type III effectors.

Introduction

Some pathogenic Gram-negative bacteria inject proteins, known as type III effectors, into host cells to manipulate host cell processes and establish their infective niche. Specialized injection devices, type III secretion systems (T3SS), span both bacterial membranes and puncture the host cell membrane to deliver these effectors. The enteric bacterium *Salmonella enterica* serovar Typhimurium has three major T3SS virulence determinants: the flagellar apparatus provides motility and is required for the optimal infection of non-phagocytic cells; T3SS1 is essential for the invasion of non-phagocytic cells; and T3SS2 is necessary for systemic spread of bacteria. To date, 39 *Salmonella* effectors have been identified as translocated substrates of these T3SS (McGhie *et al.*, 2009, Niemann *et al.*, 2011). While the activities of these effectors are extremely diverse, a universal theme is that these bacterial proteins must be delivered to a specific intracellular location in order to execute their

*Corresponding author: Leigh A. Knodler, PhD, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840, USA, Ph: 406-363 9332, FAX: 406-363 9380, lknodler@niaid.nih.gov.

†Current address: Department of Cellular Biology, Microbiology and Molecular Biology, University of South Florida, Tampa, FL 33620

function within host cells. Host cell membranes, both the plasma membrane (Cain *et al.*, 2004) and internal membranes (Kuhle *et al.*, 2002, Knodler *et al.*, 2003, Freeman *et al.*, 2003, Brumell *et al.*, 2003), are an especially frequent target for *Salmonella* type III effector activity. Yet very little is known about how these bacterial proteins localize to host cell membranes.

Coiled-coil (CC) domains are structural elements that comprise two or more right-handed amphipathic α -helices that wind around each other into a supercoiled structure (Cohen *et al.*, 1990). Despite their apparent simplicity, these structures show great diversity in their helix orientation (left- or right-handedness), alignment (parallel or anti-parallel), and mode of complex formation (homo- or hetero-complex) (Burkhard *et al.*, 2001, Grigoryan *et al.*, 2008, Parry *et al.*, 2008). CC motifs, which provide protein-protein interaction sites, are found in a diverse array of proteins including those involved in transcription, membrane fusion and cell adherence (Burkhard *et al.*, 2001), and are widely distributed in proteins from Eukarya, Archaea and Bacteria. Based upon two prediction programs (COILS and MultiCoil), up to 5% and 10% of all proteins encoded by prokaryotic and eukaryotic genomes, respectively, contain CC domains (Liu *et al.*, 2001, Rose *et al.*, 2005). Interestingly, three studies have revealed a higher prevalence of CC-containing proteins associated with T3SS, including needle proteins, translocators and effectors, compared to the average prokaryotic proteome (Pallen *et al.*, 1997, Delahay *et al.*, 2002, Gazi *et al.*, 2009). This increased incidence has also recently been extended to T2SS and T4SS (Gazi *et al.*, 2009). It has been suggested that the presence of CC domains in type III effectors might promote their unfolding prior to translocation and re-folding in the host cell (Gazi *et al.*, 2009). Alternatively, since CC domains are often involved in protein-protein interactions, their presence may increase the likelihood of type III effector interaction(s) with host cell targets. Here we used two widely used CC prediction programs, Marcoil and Pepcoil, to evaluate the prevalence of CC domain containing proteins in the 4741 open reading frames (ORFs) of the pathogenic bacterium, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). We identified 660 proteins to potentially containing one or more CC motifs, corresponding to 13.9 % of the entire proteome. Notably, and in agreement with previous reports (Pallen *et al.*, 1997, Delahay *et al.*, 2002, Gazi *et al.*, 2009), the majority of known *Salmonella* type III effectors were predicted to contain at least one CC domain, suggesting that this motif could be a major determinant in their function or targeting.

We tested this hypothesis using SopB as a model CC domain-containing protein. *sopB* is transcribed in an operon from *Salmonella* pathogenicity island 5 (SPI5) together with its cognate chaperone, *sigE* (Hong *et al.*, 1998), and is translocated via T3SS1, and is associated with host cell membranes upon translocation (Marcus *et al.*, 2002). SopB has inositol phosphatase activity, is essential for activation of the mammalian pro-survival kinase, Akt/PKB, in epithelial cells (Steele-Mortimer *et al.*, 2000) and promotes actin rearrangements at the plasma membrane (Zhou *et al.*, 2001). A CC domain has been predicted in this type III effector between amino acid residues 118 and 142 (Marcus *et al.*, 2002). We altered this putative CC domain by either deleting it entirely (SopB Δ CC) or mutating three key hydrophobic residues at the interface of the α -helix (SopB-L³D). We assessed these mutants for chaperone binding, stability, secretion, translocation, localization, and biological activity in host cells. We discovered that the CC domain influences SopB stability inside bacteria and after translocation, and facilitates the membrane association of SopB in mammalian cells. Many *Salmonella* effectors that are membrane-associated upon translocation are predicted to contain CC domains (Table 1). We extended our analysis to other *Salmonella* type III effectors and found that these α -helical domains are necessary, but not sufficient, for subcellular targeting. Therefore CC domains function cooperatively with other domains to mediate the membrane localization of type III effectors in host cells.

Results

Prediction of CC domain-containing proteins in *Salmonella*

Previous analyses have suggested an increased prevalence of CC domain-containing proteins in the proteomes of Gram-negative bacterial species harboring T3SS and T4SS (Pallen *et al.*, 1997, Delahay *et al.*, 2002, Gazi *et al.*, 2009). In order to identify the ensemble of *S. enterica* proteins containing CC domains, the sequences of predicted open reading frames (ORFs) of *S. Typhimurium* SL1344 were obtained from the Sanger Institute (www.sanger.ac.uk/Projects/Salmonella/SL1344_web.tab) and putative functions were assigned by BLAST, based upon similarities with the *S. Typhimurium* LT2 proteome and manual searches in genomic databases (Ibarra *et al.*, 2010). 4741 annotated ORFs were analyzed for potential CC motifs by the Pepcoil and Marcoil prediction programs. The Pepcoil program calculates the probability of a CC structure for windows of n residues (14, 21 or 28) in a protein sequence using the method of Lupas *et al.* (Lupas *et al.*, 1991). Marcoil predicts CC domains based on a hidden Markov model-based algorithm (Delorenzi *et al.*, 2002). Together, these two programs predicted 660 CC-domain containing proteins, corresponding to 13.9% of the complete ORFs annotated in the SL1344 proteome (see Supplementary Table 1). These CC domain-containing proteins can be categorized into five functional classes according to their COG assignment and BLAST searches to the *S. Typhimurium* LT2 genome (Supplementary Table 2). The first group includes proteins that currently have no assigned functions (32%). One-quarter (26%) of the predicted proteins are involved in multiple metabolic pathways, transport, catabolism and biosynthesis. The third category (26%) comprises either transcriptional regulators, including various two-component system sensor proteins, such as PhoQ, SsrA, NarX, and BarA, DNA and RNA polymerase components, and/or components of the protein synthesis pathways (ribosomal proteins, tRNA synthetases, etc.). The fourth category (8.4%) includes proteins devoted to cell processes, such as motility and cell wall/membrane biogenesis. Lastly, 8.2% of the predicted proteins are involved in motility, signal transduction, trafficking and secretion and/or virulence processes. This last functional class includes structural components of the three T3SS in *S. enterica* as well as the flagellins, FlhC and FlhB, and numerous type III effectors.

Prediction of CC domains in *Salmonella* type III effectors

In order to investigate further the prevalence of CC domains associated with T3SS substrates, we applied Marcoil and Pepcoil analyses on all known *S. Typhimurium* type III effectors (McGhie *et al.*, 2009, Niemann *et al.*, 2011)(Table 1), instead of restricting the target proteins to *S. Typhimurium* SL1344 as we had done in the initial predictions. Overall, 49% of the *Salmonella* effectors were predicted by Pepcoil and/or Marcoil to possess at least one CC domain, including all but one of the T3SS1-associated effectors, SopE2, and over one-third (36%) of the T3SS2-associated effectors. Seven effectors can be translocated by T3SS1 and T3SS2 and both programs predicted SspH1 and SlrP to harbor multiple CC domains. Pathogens use either T3SS or type IV secretion systems (T4SS) to deliver proteins into host cells. By comparison, the prevalence of CC domains in *Pseudomonas aeruginosa* type III effectors and *Legionella pneumophila* type IV effectors has been reported to be 20% and 24%, respectively (Gazi *et al.*, 2009). Therefore, of the proteomes that have been analyzed to date, there is a much higher CC content in *Salmonella* effectors than those from other pathogenic bacteria.

Of the *Salmonella* type III effectors predicted to contain CC domains, there are currently four crystal structures available; SipA(48-264) (Lilic *et al.*, 2006), SopA (163–782) (Diao *et al.*, 2008), SptP(161-543) (Stebbins *et al.*, 2000) and SspH2(166–783) (Quezada *et al.*, 2009). These structures revealed that all of the regions predicted by both Pepcoil and Marcoil to harbor CC domains (Table 1) adopt α -helical structures (Supplementary Figure

S1). Furthermore, these α -helices are localized to a solvent accessible area on the surface of their respective proteins, indicating that there is a strong potential for their association with other α -helices to form a CC structure. Overall, these structural observations reinforce our sequence predictions that *Salmonella* type III effectors contain domains capable of forming CC structures.

SopB as a model CC-domain containing type III effector

We chose SopB as a model protein to study the functional role of CC domains in *Salmonella* type III effectors. SopB has a cognate chaperone, SigE, and is translocated into host cells by T3SS1. A short region in the N-terminus of SopB is predicted by Pepcoil (amino acid residues 117–138) and Marcoil (residues 100–169) to form a CC domain (Table 1). In Figure 1A, a ribbon model of the predicted three-dimensional structure of SopB is shown. The corresponding CC motif identified in α -helix 10 is indicated and corresponds to amino acid residues 118–138 of SopB (residues 100–120 in the modeled sequence).

Typically, CC domains consist of one or more heptad repeats, denoted as $(a-b-c-d-e-f-g)_n$. The “a” and “d” residues are usually small, hydrophobic amino acids such as leucine, valine and isoleucine. CC dimerization is driven by hydrogen bonding between these residues, shielding them in a hydrophobic core at the helix interface. Positions “b”, “c”, “e”, “f” and “g” are normally occupied by hydrophilic residues and form the solvent-exposed part of the CC. The COILS program (Lupas *et al.*, 1991) (http://www.ch.embnet.org/software/COILS_form.html) predicted that five hydrophobic residues of SopB; Ala124, Leu127, Leu131, Leu134 and Leu145, occupy the “a” and “d” positions of their consecutive heptad repeats (Figure 1B). Using this prediction framework, we constructed two SopB CC mutants; an in-frame deletion of amino acids 118–142 (SopB Δ CC) and a triple leucine-to-aspartate mutant, L127D L131D L134D (SopB-L³D). These mutants were compared against wild-type SopB in a number of different assays for type III effector function.

SopB binds to its cognate chaperone, SigE, independently of its CC domain

To determine whether the SopB CC domain is required for effector-chaperone interaction, we used a pulldown assay based on AviTag™ technology. This 15 amino acid peptide tag is biotinylated *in vivo* and *in vitro* by the biotin ligase, BirA (Schatz, 1993), which is widely distributed in eubacteria and archaea, including *S. enterica* (Rodionov *et al.*, 2002). Biotinylated proteins can then be detected or purified using streptavidin derivative products. Δ sopB-sigE bacteria were complemented with plasmids encoding SopB and SigE (pWSKDE), SopB tagged at the C-terminus with AviTag™ and SigE (pWSKDE-AviTag) or SopB Δ CC tagged at the C-terminus with AviTag™ and SigE (pWSKDE Δ CC-AviTag). The SopB-AviTag™ fusions were biotinylated *in vivo* and competent for secretion and translocation (data not shown). SopB binding to SigE was analyzed by streptavidin-AviTag™ affinity purification from bacterial lysates. Proteins bound to streptavidin agarose beads were eluted and subject to immunoblotting with antibodies against AviTag™, SopB and SigE. Equivalent amounts of SopB-AviTag and SopB Δ CC-AviTag were affinity purified from bacterial lysates, whereas no untagged SopB was purified (Figure 2). Likewise, similar quantities of SigE co-purified with SopB-AviTag and SopB Δ CC-AviTag, demonstrating that residues 118–142 are not required for the interaction between SopB and SigE.

The CC domain influences SopB secretion, but not translocation

While T3SS1 effectors are primarily translocated into host cells, they can also be secreted into culture medium under certain growth conditions. Because secretion and translocation are distinct transport mechanisms, we next characterized whether either of these processes

was affected by mutation of the SopB CC domain. *Salmonella* were grown under microaerophilic conditions in LB-Miller broth for 8 h. Five strains were directly compared; wild type, $\Delta\text{sopB-sigE}$, $\Delta\text{sopB-sigE}$ pWSKDE, $\Delta\text{sopB-sigE}$ pWSKDE Δ CC and $\Delta\text{sopB-sigE}$ pWSKDE-L³D. Intrabacterial (bacterial lysates) and secreted proteins (culture medium) were collected, separated by SDS-PAGE and subject to immunoblotting with antibodies against SopB (Figure 3A). Confirming the deletion of *sopB*, no SopB signal was detected for the $\Delta\text{sopB-sigE}$ strain. Intrabacterial levels of SopB were increased by 4-fold for $\Delta\text{sopB-sigE}$ pWSKDE bacteria compared to wild type bacteria, in agreement with the multicopy nature of the complementing plasmid. Compared to $\Delta\text{sopB-sigE}$ pWSKDE, less intracellular SopB was detected for $\Delta\text{sopB-sigE}$ pWSKDE Δ CC and $\Delta\text{sopB-sigE}$ pWSKDE-L³D bacteria, 1.7-fold and 1.3-fold respectively. Intrabacterial levels of the cytosolic chaperone, DnaK, were unchanged for all strains. We next evaluated the five *Salmonella* strains for SopB secretion by measuring the fraction of the total SopB pool that is secreted. For wild type and $\Delta\text{sopB-sigE}$ pWSKDE bacteria, approximately one-quarter of the SopB pool was secreted; $26\pm9.7\%$ for wild type and $28\pm10\%$ for $\Delta\text{sopB-sigE}$ pWSKDE. However, SopB secretion efficiency was significantly reduced for both CC mutants, to only $5.5\pm1.9\%$ of the total pool for SopB Δ CC and $9.9\pm2.7\%$ for SopB-L³D. Altogether, these results establish that the CC region of SopB affects both its intrabacterial accumulation and secretion efficiency.

Translocation of SopB can be detected using a 13 amino acid tag derived from human glycogen synthase kinase-3 β (GSK), which is phosphorylated following translocation (Garcia *et al.*, 2006). We used this methodology to compare the translocation of wild type SopB with its CC mutant derivatives. $\Delta\text{sopB-sigE}$ bacteria complemented with pWSKDE-GSK, pWSKDE Δ CC-GSK or pWSKDE-L³D-GSK were grown under conditions that induce invasiveness. The amount of SopB in these bacterial subcultures was initially compared by immunoblotting (Figure 3B). Similar to what was observed in the overnight standing cultures (Figure 3A), the amounts of SopB Δ CC-GSK and SopB-L³D-GSK in the intrabacterial pool were found to be approximately 3-fold less than SopB-GSK. To compensate for this, the MOI was increased by 3-fold for pWSKDE Δ CC-GSK and pWSKDE-L³D-GSK bacteria, so that equivalent amounts of SopB-GSK were present during the initial interaction with host cells. HeLa cell lysates were prepared from a time course of infection (0.5 h – 4 h p.i.) and subject to immunoblotting with phospho-Ser9-GSK3 β and GSK tag antibodies to detect translocated and total (intrabacterial and translocated) SopB-GSK, respectively (Figure 3B). The amounts of total and translocated SopB-GSK accumulated over time, similar to what we have recently reported (Knodler *et al.*, 2009). By comparison, the levels of total and translocated SopB Δ CC-GSK and SopB-L³D-GSK were comparable to those of SopB-GSK at the earliest time points only (0.5 h and 1 h p.i.), but rapidly declined thereafter. The migration pattern of SopB Δ CC and SopB-L³D also differed compared to wild type SopB. Translocated SopB-GSK was primarily detected as three bands, representing unmodified, mono- and di-ubiquitinated species (Knodler *et al.*, 2009, Patel *et al.*, 2009). However, the CC mutants migrated as single bands, at ~60 kDa, suggesting a lack of ubiquitination after delivery to mammalian cells. Collectively, these data show that, although the CC domain is not essential for SopB translocation *per se*, it is required for the post-translational modification and persistence of translocated SopB.

SopB is destabilized upon deletion or mutation of its CC domain

In bacterial cultures, the intrabacterial SopB pool for the CC mutants was reduced compared to their wild type counterpart (Figure 3), suggesting that the stability of SopB was affected for these proteins. To assess this, we measured the intrabacterial stability of SopB, SopB Δ CC and SopB-L³D following chloramphenicol treatment to prevent *de novo* protein synthesis. $\Delta\text{sopB-sigE}$ bacteria complemented with pWSKDE, pWSKDE Δ CC or

pWSKDE-L³D were grown to mid-log phase in LB-Miller broth, then samples were collected at various times post-antibiotic addition. Proteins were separated by SDS-PAGE and subject to immunoblotting with polyclonal SopB antibodies (Figure 4A). For SopB, there was a slow, gradual decline in the intrabacterial pool with time, whereas SopBΔCC and SopB-L³D decayed faster. The calculated half-life of SopB was 80 min, while that of SopBΔCC and SopB-L³D was 20 min and 40 min, respectively. Therefore, the SopB CC mutants have a stability defect inside bacteria.

We also evaluated the stability of SopB, SopBΔCC and SopB-L³D following translocation into mammalian cells. Again, we used a modified pulse-chase experiment in combination with SopB-GSK fusions to estimate their half-lives. HeLa cells were infected with *ΔsopB-sigE* bacteria harboring either pWSKDE-GSK (10 μl subculture), pWSKDEΔCC-GSK (50 μl subculture) or pWSKDE-L³D-GSK (50 μl subculture). Increased MOIs were used to ensure adequate signal intensity in immunoblots for accurate densitometry analysis. At 1.5 h p.i., tetracycline was added to stop bacterial protein synthesis (t₀) and samples were collected at hourly intervals thereafter. Lysates were subject to immunoblot analysis with phospho-Ser9-GSK3β antibodies to detect translocated SopB (Figure 4B). As previously estimated (Knodler *et al.*, 2009), the half-life of translocated SopB was 2 h. By comparison, the half-lives of the CC mutants were much shorter, approximately 55 min. Little to no SopBΔCC-GSK or SopB-L³D-GSK was detected within 2 h of tetracycline addition. Taken together these experiments establish that the CC domain is required for optimal SopB stability, both in *Salmonella* and after translocation into mammalian cells.

The CC domain is not essential for biological activity of SopB

We next assessed whether the CC domain affected the activity of SopB in mammalian cells. SopB is an inositol phosphatase that targets host cell phospholipids. This enzymatic activity is essential for activation of the mammalian pro-survival kinase, Akt/PKB, in epithelial cells (Steele-Mortimer *et al.*, 2000). We therefore used the phosphorylation status of Akt as a reporter for the biological activity of SopB in mammalian cells. Whole cell lysates from HeLa cells expressing Myc-tagged wild type SopB, catalytically inactive SopB (SopB C460S), or the two CC mutants, SopBΔCC and SopB-L³D, were collected and subject to immunoblotting for SopB, phospho-Akt and total Akt. While SopB and SopB C460S appeared as two bands in immunoblots, representing unmodified and mono-ubiquitinated species (Figure 5A; (Knodler *et al.*, 2009)), SopBΔCC and SopB-L³D migrated as a single band only, indicating lack of ubiquitination. Therefore, neither ectopically expressed nor bacterially delivered SopB CC mutants are post-translationally modified in mammalian cells (Figures 3B, 5A). Akt phosphorylation was induced upon ectopic expression of wild type SopB, SopBΔCC or SopB-L³D, but not the catalytically inactive SopB mutant, SopB C460S (Figure 5A). We also examined CC domain-dependence of the biological activity of SopB after translocation by *Salmonella*. HeLa cells were infected with increasing amounts of *ΔsopB-sigE* bacteria expressing SopB-GSK (pWSKDE-GSK) or SopBΔCC-GSK (pWSKDEΔCC-GSK). At 1 h p.i., monolayers were solubilized and subject to immunoblotting for translocated SopB-GSK, total SopB-GSK, phospho-Akt and total Akt. Confirming an absolute requirement for SopB in HeLa cells (Steele-Mortimer *et al.*, 2000), *ΔsopB-sigE* bacteria failed to induce the phosphorylation of Akt, even at a high MOI (Figure 5B). In contrast, increasing the MOI for *ΔsopB-sigE* bacteria expressing SopB-GSK resulted in a proportional increase in the amount of translocated SopB-GSK and phospho-Ser473-Akt, demonstrating the dose-dependence of *Salmonella*-induced Akt phosphorylation. A similar trend was observed for *ΔsopB-sigE* bacteria expressing SopBΔCC-GSK, although equivalent amounts of translocated SopB-GSK induced higher levels of Akt phosphorylation (Figure 5B). Therefore, the CC deletion mutant is able to induce Akt phosphorylation, but less efficiently than wild type SopB.

We hypothesized that the instability of SopB Δ CC might contribute to its reduced ability to effectively induce Akt phosphorylation at the plasma membrane. We therefore monitored a time course of Akt phosphorylation in the presence of the proteasome inhibitor, MG-132. HeLa cells were infected with Δ *sopB-sigE* bacteria expressing either SopB-GSK (pWSKDE-GSK) or SopB Δ CC-GSK (pWSKDE Δ CC-GSK), in the presence or absence of MG-132. Samples were collected over a 4 h time course and subject to immunoblotting (Figure 5C). For wild type SopB, increasing amounts of translocated SopB were evident with time, in agreement with our earlier study (Knodler *et al.*, 2009). Sustained phosphorylation of Akt was detected up to 4 h p.i., with a peak at 1 h p.i. (Knodler *et al.*, 2005a). Surprisingly, inhibiting proteasomal degradation led to an increased accumulation of translocated SopB and prolonged Akt phosphorylation kinetics, shifting the peak of phosphorylation to 4 h p.i. SopB Δ CC induced low levels of Akt phosphorylation in untreated cells. However, in the presence of MG-132 Akt phosphorylation was restored, with an intensity and longevity comparable to wild type SopB in the absence of MG-132 (Figure 5C). Collectively, these experiments show that translocated SopB and SopB Δ CC are subject to proteasomal degradation and the CC domain of SopB is not essential for its biological activity in mammalian cells.

Membrane-association of SopB requires the CC domain

It has previously been shown that SopB targets host cell membranes after translocation (Marcus *et al.*, 2002, Knodler *et al.*, 2009, Patel *et al.*, 2009). Furthermore, residues 117–167 are required for membrane association of an enhanced green fluorescent protein (EGFP)-SopB fusion as determined by detergent fractionation of transiently transfected cells (Marcus *et al.*, 2002). To further examine the role of the CC domain in membrane association, we constructed an EGFP-full length SopB fusion and various mutants, including the catalytically inactive mutant, EGFP-SopB C460S, and the CC mutants, EGFP-SopB Δ CC (deleted for residues 118–142) and EGFP-SopB L³D (L127D L131D L134D mutations). These plasmid constructs were transfected into HeLa cells and protein localization was then monitored by fluorescence microscopy (Figure 6A). By direct fluorescence microscopy, EGFP-SopB and EGFP-SopB C460S associated with vesicles scattered throughout the cytoplasm, in agreement with previous reports (Dukes *et al.*, 2006). In contrast EGFP-SopB Δ CC and EGFP-SopB L³D were evenly distributed throughout the cytoplasm and also concentrated in membrane ruffles at the periphery of some cells. We next used a sequential detergent fractionation procedure to determine whether SopB and its mutants were membrane associated or cytosolic. HeLa cells were transfected with Myc-SopB, Myc-SopB C460S, Myc-SopB Δ CC or Myc-SopB-L³D for 18 h and then treated with 0.1% (w/v) saponin, which permeabilizes the plasma and internal membranes and releases cytosolic content (Wassler *et al.*, 1987), followed by incubation with 1% (v/v) Triton X-100 (TX-100) to solubilize peripheral and integral membrane proteins. Samples were subject to immunoblotting with antibodies against SopB, the lysosomal membrane protein, LAMP-1, and the cytosolic protein, Hsp27 (Figure 6B). Myc-SopB and Myc-SopB C460S were mostly present in the TX-100 soluble fraction, indicating their membrane association. Notably, the small portion of SopB and SopB C460S present in the saponin-soluble fraction was unmodified, whereas both unmodified and ubiquitinated forms partitioned to the TX-100 soluble fraction. In contrast, Myc-SopB Δ CC and Myc-SopB L³D were primarily detected in the saponin-soluble fraction, hence are cytosolic.

We also tested the intracellular localization of the SopB CC mutants after bacterial translocation. For infected cells, we used a different fractionation technique since TX-100 has been shown to compromise bacterial integrity (Gauthier *et al.*, 2000). HeLa cells were infected with Δ *sopB-sigE* bacteria complemented with pWSKDE-GSK, pWSKDE Δ CC-GSK or pWSKDE-L³D-GSK. At 1 h p.i., cells were subject to mechanical lysis followed by

a low speed centrifugation step to remove intact bacteria and then a high speed centrifugation step to separate cytosolic from membrane proteins (Figure 6C). Equal volumes of the cytosolic and membrane fractions were subject to immunoblotting to detect translocated SopB-GSK, the membrane protein, LAMP-1, and the cytosolic protein, Hsp27. In agreement with previous reports (Marcus *et al.*, 2002, Knodler *et al.*, 2009, Patel *et al.*, 2009), SopB localized exclusively to host cell membranes after translocation, while deletion or mutation of the CC domain caused a significant amount of translocated SopB to partition to the cytosolic fraction (Figure 6C). Together, these data indicate an intact CC domain is required for the membrane localization of SopB in mammalian cells.

CC domains mediate membrane association of other *Salmonella* effectors

We next tested whether CC domains are important for the membrane localization of three other *Salmonella* type III effectors; PipB2, SseJ and SopD2, which are predicted by the COILS program to have a CC domain either at their N-terminus (PipB2) or C-termini (SseJ and SopD2) (Table 1). Specifically, residues 51–66 of PipB2, 308–325 of SseJ and 275–291 of SopD2 are predicted to adopt a CC conformation (data not shown). Using a similar approach to SopB, we constructed EGFP fusions to each of these effectors and also made triple amino acid substitutions to aspartate in positions “a” or “d” of consecutive heptad repeats to disrupt this potential structural motif. The fusion proteins were expressed in HeLa cells and their localization determined by fluorescence microscopy and sequential detergent fractionation (Figure 7).

PipB2 is targeted to host cell membranes and localizes to punctate vesicles that are concentrated at the cell periphery (Figure 7A) (Knodler *et al.*, 2005b). However, we found that the corresponding CC mutant, PipB2 Y54D L57D M61D (PipB2 YLM³D), was spread diffusely throughout the cytoplasm and concentrated in a juxtanuclear position (Figure 7A). SopD2 localizes to vesicular membranes (Brumell *et al.*, 2003) (Figure 7A) and like for PipB2, its association with vesicular structures was abrogated by mutation of the predicted CC domain (SopD2 Y281D Y284D K288D, SopD2 YYK³D) (Figure 7A). SseJ also localizes to cellular membranes (Freeman *et al.*, 2003, Ruiz-Albert *et al.*, 2002) and was detected on the plasma membrane when ectopically expressed (Figure 7A) (Nawabi *et al.*, 2008), but mutation of Asn309 Leu312 Val316 residues (SseJ NLV³D) shifted its localization pattern to the cytoplasm (Figure 7A).

We independently confirmed these observations by differential detergent solubilization. Transfected HeLa cells were subject to sequential incubations with saponin and TX-100 as described above. Samples from both fractions were subject to immunoblotting with antibodies against GFP, the membrane marker, LAMP-1, and the cytosolic protein, Hsp27 (Figure 7B). EGFP-PipB2 was detected only in the TX-100 soluble fraction representing cellular membranes, but the EGFP-PipB2 YLM³D mutant was solely present in the saponin-soluble, or cytosolic, fraction. EGFP-SseJ and EGFP-SopD2 were enriched in the TX-100 soluble fraction whereas their respective CC mutants were present exclusively in the saponin-soluble fraction. Therefore disruption of the potential CC domains of PipB2, SseJ and SopD2 render these proteins cytosolic. Collectively, the fluorescence microscopy and fractionation data support the concept that CC domains in *Salmonella* type III effectors facilitate their targeting to host cell membranes.

CC domains alone are not sufficient for membrane targeting

To determine whether the predicted CC domains were sufficient for membrane targeting, we constructed EGFP fusions to amino acid residues 113–147 of SopB, 41–73 of PipB2, 303–335 of SseJ or 268–300 of SopD2. HeLa cells were transfected with the EGFP-CC domain fusions and their localization determined by direct immunofluorescence and sequential

detergent fractionation. All of the fusion proteins showed localization patterns indistinguishable from EGFP, with fluorescence detected in the nucleus and cytosol (Supplemental Figure S2A). Detergent fractionation confirmed that the EGFP-CC domain fusions, like EGFP, were cytosolic (Supplemental Figure 2B). Therefore the predicted CC domains of *Salmonella* type III effectors are necessary, but not sufficient, to confer membrane targeting.

To test the interchangeability of type III effector CC domains, we constructed a number of chimeric fusion proteins where we replaced the predicted CC domain of one effector with another. One *Salmonella* effector, SseK1, has a region that is predicted by Marcoil to adopt a CC conformation (Table 1), but is not targeted to membranes (Kujat Choy *et al.*, 2004). To see whether the predicted CC domain of SopB could confer membrane targeting of SseK1, we replaced amino acid residues 157–177 of SseK1 with residues 117–137 of SopB and compared the localization of EGFP-SseK1 and EGFP-SseK1-SopBCC after transfection of HeLa cells. By direct immunofluorescence microscopy (Supplemental Figure S2B), EGFP-SseK1 localized to the nucleus and cytosol (Kujat Choy *et al.*, 2004), whereas the SseK1-SopBCC chimera was largely excluded from the nucleus and accumulated primarily in the cytosol. However, both proteins were only detected in the saponin-soluble fraction after detergent fractionation (Supplemental Figure S2B), confirming their cytosolic status.

Having demonstrated that disruption of the predicted CC domains of PipB2, SseJ and SopD2 affected their membrane targeting, we hypothesized that these regions could functionally replace the CC domain of SopB and target it to membranes. To test this, we replaced residues 117–137 of SopB with the regions in these proteins predicted to adopt a CC structure i.e. residues 47–67 of PipB, 309–329 of SseJ or 274–294 of SopD2. Plasmids encoding these chimeric fusions, EGFP-SopB-PipB2CC, EGFP-SopB-SseJCC and EGFP-SopB-SopD2CC, were transfected into HeLa cells and protein localization was monitored by fluorescence microscopy and sequential detergent fractionation. All three chimeras showed the same localization pattern, accumulating in the cytosol and membrane ruffles at leading edges of the plasma membrane (Figure 8A), which is comparable with the SopB CC mutants, SopB Δ CC and SopB L³D, and not wild type SopB. In immunoblots, EGFP-SopB-PipB2CC, EGFP-SopB-SseJCC and EGFP-SopD2CC migrated as single species on immunoblots, indicating a lack of ubiquitination, and were primarily solubilised by saponin (Figure 8B). Collectively, this data shows that none of the chimeric proteins were efficiently targeted to host cell membranes.

Like SopB, the *Shigella* homologue, IpgD, hydrolyzes host cell phospholipids at the plasma membrane, leading to the sustained activation of Akt in infected epithelial cells (Pendaries *et al.*, 2006). This type III effector is predicted by the COILS program to have three CC domains (data not shown), one at the N-terminus in a region analogous to the predicted CC domain of SopB. To investigate whether these residues could function as a membrane targeting determinant in SopB we transfected HeLa cells with a construct that expresses a protein with residues 117–137 of SopB replaced by residues 118–144 of IpgD. We found that EGFP-SopB-IpgDCC exhibited a staining pattern comparable to EGFP-SopB Δ CC and EGFP-SopB L³D, and not wild type SopB, (Figure 8A) and localized primarily in the saponin-soluble fraction (Figure 8B). Therefore the predicted CC domain of IpgD cannot functionally substitute for that of SopB.

Discussion

In this study, we used *in silico* analysis to predict CC domains in the proteome of the pathogenic bacterium, *Salmonella enterica*. We found a high propensity for CC domains in virulence-associated proteins, especially type III effectors and used one of these, SopB, to

experimentally determine the role of CC domains in *Salmonella* type III effectors. We chose SopB since it is a relatively well-characterized effector and its biological activity in mammalian cells can be readily detected. We found that the CC domain is required for the efficient secretion of SopB and enhances SopB stability, both intrabacterial and inside host cells after translocation. In addition, the CC domain promotes SopB membrane association in mammalian cells. There was no requirement for the CC domain in chaperone binding or type III translocation. These results indicate that the CC domain of SopB serves multiple functions, both inside bacteria and host cells.

Previously, Letzelter and others (Letzelter *et al.*, 2006) have shown that the membrane localization domain (MLD) overlaps with the chaperone-binding domain (CBD) for the *Yersinia* effectors YopO, YopE and YopT. Inside bacteria, binding of a class I chaperone to the CBD prevents type III effector aggregation. Upon translocation, the chaperone is no longer associated with the effector, and this freed region acts as a membrane-targeting domain in host cells (Letzelter *et al.*, 2006). As proof of this hypothesis, deletion of the CBD in YopO prevented the interaction with its chaperone, SycO, and its association with the plasma membrane (Letzelter *et al.*, 2006). However, our data suggest that these findings cannot necessarily be extended to *Salmonella* effectors. The SopB Δ CC mutant retains the ability to bind its class I chaperone, SigE (Figure 2), but no longer localizes to host cell membranes (Figure 6), implying that the CBD and MLD do not coincide for SopB. Of note, the CBD/MLD regions of YopO, YopE and YopT are not predicted by COILS (Lupas *et al.*, 1991) to adopt CC conformations. Therefore it appears that the specific role of chaperone binding in masking an aggregation-prone membrane localization signal is divergent for *Salmonella* and *Yersinia* type III effectors.

Translocated SopB targets both plasma and vacuolar membranes (Knodler *et al.*, 2009, Patel *et al.*, 2009) and has inositol phosphatase activity that acts on membrane phospholipids. This enzymatic activity is essential for phosphorylation of the mammalian pro-survival kinase, Akt/PKB, at the plasma membrane (Steele-Mortimer *et al.*, 2000). Molero *et al.* have previously demonstrated that a SopB Δ 118–142 mutant does not activate Akt at 30 min p.i. (Molero *et al.*, 2009). By contrast, here we show that a SopB CC mutant can induce Akt phosphorylation when equivalent amounts of SopB and SopB Δ CC are translocated (Figure 5). Therefore, the CC domain is not essential for the biological activity of SopB, but does contribute to its efficacy. This is most likely related to the defective intracellular localization of the SopB CC mutants. SopB is a peripheral membrane protein (Marcus *et al.*, 2002, Knodler *et al.*, 2009, Patel *et al.*, 2009) and our cellular fractionation data shows that the SopB CC mutants are no longer enriched in membrane fractions. Instead, at steady state, a considerable portion of these mutant proteins partition to the cytosolic fraction (Figure 6), demonstrating that the CC domain enhances the association of SopB with host cell membranes. Overall our data supports the hypothesis that the biological activity of SopB requires a stable association with host cell membranes, and the CC domain increases its membrane affinity, likely via protein-protein or protein-lipid interaction(s). Interestingly, the mammalian inositol phosphatase, myotubularin (Mtmr2), also requires a CC domain to enhance its membrane association (Berger *et al.*, 2003). Homodimerization of the CC domain combines two lipid-binding pleckstrin homology-GRAM (PH-G) domains, increasing the binding affinity of Mtmr2 for membrane phosphoinositides. In an analogous manner, homodimerization of SopB might function to stabilize its interaction with host cell membranes.

Translocated SopB Δ CC and SopB-L³D are not completely deficient in membrane association (Figure 6) implying that additional membrane-targeting domains exist within SopB. In agreement with this, a short hydrophobic domain in the C-terminus of SopB (residues 288–309) was recently identified as being required for its membrane targeting in

host cells (Patel *et al.*, 2009). This is not surprising since CC domains alone are rarely able to impart membrane targeting (Stam *et al.*, 1997, Raiborg *et al.*, 2001) (Supplemental Figure S2) and usually function with other domains to effect the subcellular targeting of mammalian proteins (Buchsbaum *et al.*, 1996, Hume *et al.*, 2006, Stam *et al.*, 1997, Berger *et al.*, 2003). However it was unexpected that predicted CC domains from other *Salmonella* effectors could not substitute for the SopB CC domain in directing membrane association (Figure 8). From these domain-swapping experiments we conclude that it is not simply the secondary structure adopted by a CC domain, but also specific amino acid residues within this region of SopB that provide a protein-protein interface for interaction with other proteins, thus directing its specific subcellular localization. This is analogous to the specificity of pleckstrin homology (PH) domains for distinct membrane phosphoinositides within mammalian cells (Lemmon, 2008). Therefore the targeting of SopB to host cell membranes requires the cooperative function of multiple domains, one of which is the putative CC domain.

Previously, two targeting mechanisms had been identified for the membrane association of bacterial proteins in host cells: (i) the covalent attachment of fatty acids or lipids promoting membrane binding affinity, (ii) motifs that mediate membrane binding via protein-protein interactions. We propose that CC domains represent a third membrane-targeting determinant for type III effectors. Examples of the first category include bacterial proteins with sequence motifs that are recognized by host cell enzymes responsible for post-translational modifications; prenylation of CAAX-like motifs at the C-terminus of the *Salmonella* type III effector SifA (Reinicke *et al.*, 2005) and numerous *L. pneumophila* type IV effectors (Ivanov *et al.*, 2010) is required for their membrane anchoring. Similarly, modification of eukaryotic-like acylation sites in three type III effectors from the plant pathogen *Pseudomonas syringae* drives their efficient membrane association (Nimchuk *et al.*, 2000). In the second category, small protein modules facilitate the membrane recruitment of bacterial proteins. For example, short hydrophobic stretches in the N-termini of YopE from *Y. pseudotuberculosis* and ExoS from *P. aeruginosa* serve as membrane localization signals in host cells (Krall *et al.*, 2004). A region in the N-terminus of SopD that is conserved in the *Salmonella* translocated effector (STE) family, the WEKI(I/M)xxFF motif, is required for membrane targeting of this effector (Bakowski *et al.*, 2007), but whether it is involved in the membrane association of other STE family members has not been investigated. Finally, mutations in a predicted α -helix in the C-terminus of the *P. aeruginosa* type III cytotoxin ExoS disrupt its plasma membrane localization (Veesenmeyer *et al.*, 2010). Interestingly, of these three categories, only targeting determinants that lead to a permanent post-translational modification such as acylation or lipidation are sufficient to mediate membrane attachment. For example, the C-terminal hexapeptide of SifA containing the CAAX motif (Boucrot *et al.*, 2003) and a CAAX motif in conjunction with a nearby stretch of positively charged amino acids for *Legionella* effectors (Ivanov *et al.*, 2010) is sufficient for their membrane targeting. However, while the N-terminal hydrophobic regions of YopE and ExoS are sufficient for directing membrane association (Krall *et al.*, 2004), SopD requires a CC-domain in addition to its WEKI(I/M)xxFF motif for membrane targeting (Bakowski *et al.*, 2007). Furthermore, a CC-rich region of LidA encompassing residues 191–541 is sufficient for membrane association (Derre *et al.*, 2005), but not the predicted CC domains of *Salmonella* effectors (this manuscript, (Bakowski *et al.*, 2007)).

Targeting to specific subcellular compartments is essential for type III effector function. Here we have shown that CC domains of four *Salmonella* type III effectors, SopB, PipB2, SopD2 and SseJ, are required for their membrane association. SopD is another *Salmonella* effector that binds to host cell membranes (Bakowski *et al.*, 2007) and is predicted to contain a CC domain at its C-terminus (Table 1). When heterologously expressed in mammalian cells, a SopD truncation mutant lacking the predicted CC domain localized to the cytosol

(Bakowski *et al.*, 2007). Additionally, the T3SS1 translocon component and effector, SipC/SspC, is predicted to have multiple CC domains (Table 1). Individual point mutations in one CC domain at the C-terminus of SipC/SspC disrupted its plasma membrane association after bacterial translocation (Scherer *et al.*, 2000). Therefore, CC domains facilitate the membrane localization of at least six *Salmonella* type III effectors. Recently, a high prevalence of CC domains was also predicted for type IV effectors (Gazi *et al.*, 2009). Although, little data exists validating a connection between CC domains and the membrane association of type IV effectors, the *Legionella pneumophila* effector LidA provides one example (Derre *et al.*, 2005). Collectively, the *in silico* analyses and experimental data support a correlation between the presence of α -helical CC domains in type III and type IV effectors and their ability to associate with host cell membranes. Notably, a few *Salmonella* effectors are not predicted to have CC domains, but do target host cell membranes (SopE2, PipB, SseF, SseG; Table 1), so must utilize CC-independent targeting mechanisms for their intracellular localization.

Several studies have demonstrated that monoubiquitination regulates the function of SopB in mammalian cells (Marcus *et al.*, 2002, Patel *et al.*, 2009) and up to nine lysine residues in SopB serve as ubiquitin conjugation sites (Patel *et al.*, 2009, Knodler *et al.*, 2009). Here we demonstrate that the SopB CC mutants, SopB Δ CC and SopB L³D, are defective for monoubiquitination. Another SopB mutant, SopB Δ 288–309 (Patel *et al.*, 2009), and the chimeric proteins SopB-PipB2CC, SopB-SseJCC, SopB-SopD2CC and SopD-IpgDCC (Figure 8) are also not ubiquitinated and fail to associate with host cell membranes after translocation. Within the predicted CC domain are four lysine residues (Lys118, Lys125, Lys128, Lys129) and one lysine residue is within the hydrophobic domain (Lys297) but none of these residues are targets of ubiquitin (Patel *et al.*, 2009, Knodler *et al.*, 2009). This argues that lack of ubiquitination of the SopB mutants and chimeras is an indirect effect of their inability to associate with membranes. Interestingly, this bears a striking similarity to the mammalian endosomal scaffold protein, CIN85. CIN85 directly interacts with the membrane phospholipid, phosphatidic acid (Zhang *et al.*, 2009), and is monoubiquitinated by the ubiquitin ligase, Cbl, on endosomal membranes (Haglund *et al.*, 2002). Deletion of a predicted CC domain in the C-terminus of CIN85 abolishes phosphatidic acid binding and reduces interaction with Cbl. As a result, the CC deletion mutant of CIN85 is cytosolic and not monoubiquitinated (Haglund *et al.*, 2002, Zhang *et al.*, 2009). Further studies are required to clarify which ubiquitin ligase is responsible for the modification of SopB when it is bound to membranes.

Based upon previous reports that translocated SopB is not subject to polyubiquitination and degradation by the proteasome (Marcus *et al.*, 2002, Patel *et al.*, 2009), we did not expect MG-132 treatment to affect SopB stability and activity (Figure 5). However, proteasome inhibition blocked the degradation of translocated SopB and SopB Δ CC and significantly enhanced the longevity of SopB- and SopB Δ CC-induced signaling in epithelial cells, suggesting that translocated SopB is subject to polyubiquitination. Indeed, we have previously reported that immunoprecipitation of translocated SopB indicates mono-, di- and poly-ubiquitinated species (Knodler *et al.*, 2009). Notably, a similar function for proteasomal regulation of bacterial protein activity in host cells has been reported for two other T3SS1 effectors, SopE and SptP (Kubori *et al.*, 2003). Therefore, in addition to the documented monoubiquitination-dependent effects on SopB localization (Patel *et al.*, 2009), our data supports a role for polyubiquitination in regulating the kinetics of SopB activity inside mammalian cells.

In conclusion, we report that a high percentage of *Salmonella* T3SS effectors are predicted to have one or more CC domains. Using SopB as a model type III effector, we identified that this domain has a multi-faceted role, and is central in defining the cellular location of

translocated SopB. We extended this functional characterization to other *Salmonella* effectors and demonstrated that CC motifs facilitate membrane association of these proteins too. Given the high prevalence of putative CC domains in type III and type IV effectors, we believe that this is a commonly used mechanism for targeting bacterial proteins to host cell membranes.

Experimental Procedures

Plasmids and bacterial strains

Salmonella enterica serovar Typhimurium SL1344 (Hoiseth *et al.*, 1981) was the wild-type strain used in this study. The Δ *sopB-sigE* mutant has been described previously (Knodler *et al.*, 2006).

The complementing plasmid, pWSKDE, (Knodler *et al.*, 2009) is a low copy number plasmid that encodes SopB and its cognate chaperone, SigE, under the control of the *sopB* promoter. To create the CC deletion mutant, pWSKDE Δ CC, residues 118–142 of SopB were removed by overlap extension PCR (Horton *et al.*, 1989), using pWSKDE as a template. The initial round of PCR used SigD-Hind-F (see Supplementary Table 3 for oligonucleotide sequences) with SigD d118-142-R and SigD d118-142-F with SigD-Nhe-R. These amplicons were purified and used as a template for a second round of PCR with SigD-Hind-F and SigD-Nhe-R. The resulting fragment was digested with HindIII/NheI and cloned into HindIII/XbaI digested pWSK29 (Wang *et al.*, 1991). The pWSKDE-L³D mutant (pWSKDE L127D L131D L134D) was generated by site-directed mutagenesis with the Quikchange kit (Stratagene).

Overlap extension PCR was also used to construct pSopB-AviTag, a plasmid that expresses a SopB-AviTagTM fusion (Avidity Inc.) under the control of the *sopB* promoter. Two PCR fragments were generated using pWSKDE as a template with the oligonucleotides SigD-Hind-F and SigD-Rv-AP to amplify *sopB* and its promoter region, and AP-SigD-Fw and SigD-Nhe-R to amplify *sigE*. The resulting amplicons were gel purified and used in a second round of PCR with the oligonucleotides SigD-Hind-F and SigD-Nhe-R. This amplicon was gel purified, digested with HindIII/NheI and ligated into HindIII/XbaI-digested pWSK29 to generate pSopB-AviTag. pSopB Δ CC-AviTag was created in a similar manner, but using pWSKDE Δ CC as a template.

To detect translocated SopB by immunoblotting, a 13-residue peptide tag derived from human GSK-3 β was fused to the C-terminus of SopB. This plasmid, pWSKDE-GSK, has been described previously (Knodler *et al.*, 2009). The corresponding CC deletion mutant, pWSKDE Δ CC-GSK, was constructed as described for pWSKDE-GSK (Knodler *et al.*, 2009), except that pWSKDE Δ CC was used as the template for amplification. The pWSKDE-L³D-GSK mutant was generated by Quikchange site directed mutagenesis (Stratagene).

pCMV-Myc-SopB encodes for N-terminal Myc-tagged SopB (Knodler *et al.*, 2009) and was used for ectopic expression studies. Overlap extension PCR was used to create Myc-SopB deleted for the CC domain, pCMV-Myc-SopB Δ CC. The initial PCR products were amplified from *S. Typhimurium* genomic DNA with the oligonucleotides Myc-SigD-F and SigD Δ 118-142R, SigD Δ 118-142-F and pGAD-SigD-R. These two amplicons were mixed for the second round of PCR with the oligonucleotides Myc-SigD-F and pGAD-SigD-R. The amplicon was digested with EcoRI and BglII and ligated into the corresponding sites of pCMV-Myc (Clontech). pCMV-Myc-SopB L³D and pCMV-Myc-SopB-C460S were generated by Quikchange site-directed mutagenesis (Stratagene).

Alternatively, the codon usage of *sopB* was optimized for expression in human cells and this plasmid, EX-SopB-M29 (GeneCopoeia), was used as a template for amplification. To create EGFP-HuSopB, the humanized *sopB* coding sequence was amplified from EX-SopB-M29 with the oligonucleotides Kpn-HuSigD-F and BglII-HuSigD-R. The amplicon was purified, digested with KpnI/BglII and ligated into KpnI/BamHI digested pEGFP-C1 (Clontech). To generate EGFP-HuSopB Δ CC, EX-SopB-M29 was used as a template for amplification with the oligonucleotides Kpn-HuSigD-F and HuSigD d118–142-R, and HuSigD d118-142-F and BglII-HuSigD-R. These two amplicons were mixed together for a second round of PCR with Kpn-HuSigD-F and BglII-HuSigD-R. The PCR product was purified, digested with KpnI/BglII and ligated into KpnI/BamHI digested pEGFP-C1. EGFP-HuSopB C460S and EGFP-HuSopB L³D were generated by Quikchange site-directed mutagenesis (Stratagene).

For ectopic expression of other *Salmonella* effectors, EGFP fusions were constructed. EGFP-PipB2 has been described previously (Knodler *et al.*, 2005b). *sseJ* was amplified from *S. Typhimurium* genomic DNA with the oligonucleotides GFP-SseJ-F and GFP-SseJ-R. The resulting amplicon was digested with BglII/KpnI and ligated into the corresponding sites of pEGFP-C1 (Clontech) to create EGFP-SseJ. *sopD2* was amplified with the oligonucleotides GFP-SopD2-F and GFP-SopD2-R, the amplicon digested with BglII/KpnI and ligated into BglII/KpnI-digested pEGFP-C1 to create EGFP-SopD2. Likewise, *sseK1* was amplified with EGFP-SseK1-Bgl and EGFP-SseK1-Kpn, digested and ligated into BglII/KpnI-digested pEGFP-C1 to create EGFP-SseK1. EGFP-PipB2 Y54D L57D M61D (EGFP-PipB2 YLM³D), EGFP-SseJ N309D L312D V316D (EGFP-SseJ NLV³D) and EGFP-SopD2 Y281D Y284D K288D (EGFP-SopD2 YYK³D) were generated by site-directed mutagenesis.

To assess the sufficiency of the CC domains for membrane localization, the predicted CC domains of SopB, PipB2, SseJ and SopD2 were fused to the C-terminus of EGFP. The following oligonucleotide pairs were used in PCR amplification with *S. Typhimurium* SL1344 genomic DNA; HuSopB-113F and HuSopB-147R, EGFP-PipB2-41F and EGFP-PipB2-73R, EGFP-SopD2-268F and EGFP-SopD2-300R, EGFP-SseJ-303F and EGFP-SseJ-335R. The amplicons were digested with BglII/KpnI and ligated into BglII/KpnI-digested pEGFP-C1 to create EGFP-SopBCC, EGFP-PipB2CC, EGFP-SopD2CC and EGFP-SseJCC, respectively.

Overlap extension PCR was used to replace amino acids 117–137 of SopB with the predicted CC domains of IpgD, PipB2, SseJ and SopD2. EGFP-HuSopB was used as a template for amplification with the oligonucleotides Kpn-HuSigD-F and HuSopB-IpgDCC-R, HuSopB-PipB2CC-R, HuSopB-SseJCC-R or HuSopB-SopD2CC-R, and BglII-HuSigD-R with HuSopB-IpgDCC-F, HuSopB-PipB2CC-F, HuSopB-SseJCC-F or HuSopB-SopD2CC-F. The resulting amplicons were gel purified and mixed for a second round of PCR with Kpn-HuSigD-F and BglII-HuSigD-R. The PCR products were gel purified, digested with KpnI/BglII and ligated into KpnI/BamHI digested pEGFP-C1 to create EGFP-HuSopB-IpgDCC, EGFP-HuSopB-PipB2CC, EGFP-HuSopB-SseJCC and EGFP-HuSopB-SopD2CC, respectively. To replace amino acid residues 157–177 of SseK1 with the SopB CC domain, EGFP-SseK1 was used as a template with EGFP-SseK1-Bgl and SseK1-SopBCC-R, and EGFP-SseK1-Kpn and SseK1-SopBCC-F. The resulting amplicons were purified and mixed for a second round of PCR with EGFP-SseK1-Bgl and EGFP-SseK1-Kpn, followed by BglII/KpnI digestion and ligation into pEGFP-C1 to create EGFP-SseK1-SopBCC. All plasmid sequences were verified by DNA sequencing.

In silico prediction of CC-containing proteins in *S. Typhimurium*

The predicted ORFs of *S. Typhimurium* SL1344 were obtained from the Sanger Institute (http://www.sanger.ac.uk/Projects/Salmonella/SL1344_web.tab) and putative functions

assigned by BLAST based upon the *S. Typhimurium* LT2 proteome. 4741 annotated ORFs (Ibarra *et al.*, 2010) were analyzed for CC propensities using two prediction programs, Pepcoil and Marcoil. Pepcoil calculates the probability of a CC domain for windows of 14, 21 or 28 residues in a protein sequence using the method of Lupas *et al.* (Lupas *et al.*, 1991). Here we have used Pepcoil with a window size of 14 and a probability of 1.0. Marcoil is a hidden Markov model-based program that predicts the presence and location of CC domains in protein sequences (Delorenzi *et al.*, 2002). Marcoil was used with parameters by default and a threshold of 2.0. In addition, all known *S. Typhimurium* type III effectors (McGhie *et al.*, 2009, Niemann *et al.*, 2011) were analyzed for CC domains using Marcoil and Pepcoil algorithms as described above.

To evaluate the performance of the CC predictions, two additional data sets comprising 420 proteins with well-characterized CC domains (true-positive data set) were compared to 420 randomly generated protein sequences with no known CC domains (true-negative data set). This comparison was useful to calculate the following values: (1) true-positives (TP), proteins with at least one CC structure identified by any method; (2) false-positives (FP), proteins whose well-known CC was not identified; (3) false-negatives (FN), proteins with a CC structure not identified by any method; (4) sensitivity of the fraction of proteins recovered in the inferred CC search and was calculated as $S_n = TP / (TP + FN)$; (5) positive predictive value (PPV) is the fraction of the proteins and CC motifs that belong to the well-known data set, calculated as $PPV = TP / (TP + FP)$; and (6) accuracy (Ac) is the PPV and S_n average, calculated as $Ac = (S_n + PPV) / 2$. Results for performance evaluation are as follows:

	PEPCOIL	MARCOIL
TP (420)	340	420
TN (420)	0	21
S_n	0.81	1
PPV	0.81	0.95
Ac	0.9	0.97

Overall both methods detected true-positive proteins with a high accuracy. In order to have a better prediction performance, we defined proteins with at least one CC motif as “positive” if they were identified by both prediction algorithms.

Structural modeling of SopB

A computational model of the three-dimensional structure of SopB was built by the CPHmodels 2.0 Server (<http://www.cbs.dtu.dk/services/CPHmodels>) using a probable sugar ABC transporter from *Streptococcus pneumoniae* (PDB entry 2w7y:A) as a template. SopB and 2w7y:A are 31% identical at the amino acid level. In order to refine the model, it was minimized using Gromacs server (<http://lorenz.immstr.pasteur.fr/gromacs>). Finally, the RAMPAGE program (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) was used to validate the stereochemical quality of the resulting three-dimensional model. After analyzing the Ramachandran plot, 92.4% of the residues are in favored and allowed regions and only 7.6% are in disallowed regions. All the bond distances, angles and dihedrals fulfill the normal limits for polypeptide chains. The model includes 463 of the 561 residues of SopB. The lack of a signal peptide in the template protein was likely responsible for the inability to model the first 18 amino acid residues of SopB.

SopB and SigE affinity co-precipitation

SopB and SopB Δ CC binding to SigE were analyzed by a pulldown assay. Wild-type *Salmonella*, Δ sopB-sigE, Δ sopB-sigE complemented with plasmid borne SopB-AviTag and SigE (pSopB-AviTag), or SopB Δ CC-AviTag and SigE (pSopB Δ CC-AviTag) were grown shaking (225 rpm) overnight at 37°C in LB-Miller broth, then subcultured 1:33 into 10 ml of fresh LB-Miller broth. After 3.5 h shaking at 37°C (OD₆₀₀~3.5), bacteria were pelleted by centrifugation and resuspended in 1 ml phosphate buffered saline (PBS) supplemented with protease inhibitor cocktail set II (Calbiochem), followed by sonication in an ice bath for 2 min (cycles of 20 sec pulse/10 sec rest) using a S4000 sonicator (Misonix). Bacterial extracts were cleared by centrifugation at 16,000 $\times g$ for 20 min at 4°C. Equivalent amounts of SopB-AviTag and SopB Δ CC-AviTag were used as the reaction mixture input (3.3-fold more SopB Δ CC-AviTag culture), determined from immunoblotting of bacterial extracts, and incubated with 60 μ l of streptavidin-agarose beads (Invitrogen) for 2 h at 4°C. Beads were washed three times with PBS containing 0.5% (v/v) Triton X-100. Bound proteins were eluted by boiling in 1.5 $\times$ SDS-PAGE sample buffer, and separated on 12% or 15% SDS-PAGE gels and transferred to 0.2 μ m or 0.45 μ m nitrocellulose (Bio-Rad). Blots were blocked in Tris-buffered saline supplemented with 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) skim milk powder (TBST-milk) for 2 h at room temperature. Immunoblotting was with mouse monoclonal anti-AviTagTM antibodies (Avidity Inc.; 1:10,000) or rabbit polyclonal anti-SigE antibodies ((Darwin *et al.*, 2001); 1:2,000). Blots were washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG or horse anti-mouse IgG (Cell Signaling Technology; 1:10,000) in TBST-milk for 1 h at room temperature. Immunoblotting with affinity purified rabbit polyclonal anti-SopB antibodies ((Marcus *et al.*, 2002); 1:40,000) was as described previously (Knodler *et al.*, 2006). Blots were developed using enhanced chemiluminescence according to manufacturer's protocol (SuperSignal West Femto Maximum Sensitivity detection system, Thermo Scientific) and imaged using either a Kodak Image Station 440 system or Kodak BioMax Light film.

Secretion assays

Wild-type or Δ sopB-sigE *Salmonella* harboring pWSKDE, pWSKDE Δ CC or pWSKDE-L³D were grown under microaerophilic conditions for 8 h in 10 ml LB-Miller broth for the analysis of intracellular and secreted protein levels. Preparation of samples has been described previously (Knodler *et al.*, 2006). Aliquots of intracellular (15 μ l, equivalent to 0.6% original sample) and secreted (6 μ l, equivalent to 2.3% original sample) proteins were separated by SDS-PAGE, transferred to 0.45 μ m nitrocellulose (Bio-Rad) and subject to immunoblotting with rabbit polyclonal anti-SopB antibodies ((Marcus *et al.*, 2002); 1:40,000) and mouse monoclonal anti-*Escherichia coli* DnaK antibodies (Stressgen; 1:20,000). Blots were incubated with affinity purified HRP-conjugated goat anti-rabbit or horse anti-mouse IgG (Cell Signaling Technology; 1:20,000) and developed using enhanced chemiluminescence as above. To calculate SopB secretion efficiency, identical exposure times were used for chemiluminescence detection of intracellular and secreted protein samples and band intensity was quantified using a Kodak Image Station 440 system with 1D Image Analysis software. Secretion efficiency was calculated as follows: secreted/[(3.8 $\times$ intracellular)+secreted] $\times$ 100%.

Bacterial infection of mammalian cells

HeLa adenocarcinoma epithelial cells (ATCC CCL-2) were grown in Eagle's modified medium (Mediatech, Herndon, VA) containing 10% (v/v) heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA). Cells were seeded in 24-well tissue-culture treated plates (Costar) 12–18 h prior to infection. Cells were serum-starved for 3 h prior to infection and incubated in serum-free media post-infection. Preparation of invasive bacteria and infection conditions has been described in detail previously (Knodler *et al.*, 2006). For all infections,

three-fold more SopB Δ CC and SopB-L³D bacteria (MOI~150) than SopB bacteria (MOI~50) were added to monolayers to compensate for the deficit in mutant SopB protein levels in bacterial subcultures. For some experiments, cells were pretreated for 45 min with 10 μ M MG-132 (Calbiochem), and the inhibitor was maintained for the duration of the experiment.

Akt phosphorylation in epithelial cells

HeLa cells were seeded in 6-well tissue-culture treated plates (Costar) at 2.4×10^5 cells/well the day prior to the experiment. Cells were serum-starved for 3 h prior to infection, and maintained in serum-free media for the duration of the experiment. Cells were infected with invasive bacteria as described above (MOI~50 for SopB, MOI~150 for SopB Δ CC and SopB-L³D). Infected monolayers were solubilized in boiling 1.5 $\times$ SDS-PAGE sample buffer and proteins separated by SDS-PAGE. Immunoblotting was with rabbit monoclonal antibodies against phospho-Akt (Ser473) (D9E, Cell Signaling Technology; 1:20,000) and Akt (pan) (11E7, Cell Signaling Technology; 1:2,000) as described previously (Knodler *et al.*, 2009).

Stability assays

The intra-bacterial stability of SopB, SopB Δ CC and SopB-L³D was measured by a modified-pulse chase experiment (Knodler *et al.*, 2006). Δ *sopB-sigE* bacteria harboring pWSKDE, pWSKDE Δ CC or pWSKDE-L³D were inoculated from freshly streaked LB agar plates into 10 ml LB-Miller broth containing 50 μ g/ml carbenicillin. Bacteria were grown, standing, at 37°C for 4–5 h to mid-log phase (OD₆₀₀~0.4). Chloramphenicol (60 μ g/ml) was then added to stop *de novo* protein synthesis and at various times post-antibiotic addition 1 ml aliquots were collected, centrifuged at $16,000 \times g$ for 2 min and the pellets resuspended in 100 μ l (pWSKDE Δ CC or pWSKDE-L³D) or 200 μ l (pWSKDE) boiling 1.5 $\times$ SDS-PAGE sample buffer. Proteins (15–20 μ l from each sample) were separated by SDS-PAGE and subject to immunoblotting with rabbit polyclonal antibodies against SopB (1:20,000) as described previously (Knodler *et al.*, 2006).

The stability of SopB was also measured after its translocation into host cells (Knodler *et al.*, 2009). Serum-starved HeLa cells were infected with Δ *sopB-sigE* bacteria harboring pWSKDE-GSK (10 μ L inoculum, MOI~50), pWSKDE Δ CC-GSK (30 μ L inoculum, MOI~150) or pWSKDE-L³D-GSK (30 μ L inoculum, MOI~150). At 1.5 h p.i., 10 μ g/ml tetracycline was added to stop bacterial protein synthesis. At hourly intervals thereafter, infected cells were lysed in 150 μ l boiling 1.5 $\times$ SDS-PAGE sample buffer. Samples were subject to immunoblotting with rabbit polyclonal anti-phospho-GSK-3 β (Ser9) (Cell Signaling Technology; 1:2,000) or rabbit polyclonal anti-GSK-3 β tag (Cell Signaling Technology; 1:5,000) antibodies as described previously (Knodler *et al.*, 2009).

Mechanical fractionation of infected cells

The partitioning of SopB and the SopB CC mutants to host cell membranes was assayed by the mechanical fractionation of infected HeLa cells as described previously (Knodler *et al.*, 2003), with minor modifications. HeLa cells were seeded in 10 cm tissue culture treated dishes (Corning), serum-starved for 3 h prior to infection and maintained in serum-free media throughout. HeLa cells were infected with 50 μ l invasive Δ *sopB-sigE* bacteria complemented with pWSKDE-GSK or 400 μ l Δ *sopB-sigE* bacteria complemented with pWSKDE Δ CC-GSK or pWSKDE-L³D-GSK per 10 cm dish (four dishes per condition). At 1 h p.i., cells were mechanically disrupted in homogenization buffer containing protease inhibitor cocktail set III and phosphatase inhibitor cocktail set II (EMD Biosciences). Intact nuclei and bacteria and unbroken cells were removed by a low speed centrifugation at $6,000 \times g$ for 10 min at 4°C, and the supernatant was subject to high-speed ultracentrifugation at

100,000 $\times g$ for 30 min at 4°C to separate host cell membranes from cytosol. Equal volumes of these samples were subject to immunoblotting with rabbit polyclonal anti-phospho-GSK-3 β antibodies (Ser9) (Cell Signaling Technology; 1:2,000), mouse monoclonal anti-LAMP1 antibodies (H4A3 developed by J.T. August and J.E.K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa; 1:5,000) and mouse monoclonal anti-Hsp27 antibodies (G31, Cell Signaling Technology, 1:20,000) as described previously (Knodler *et al.*, 2009).

Transient transfection of mammalian cells

Plasmid DNA was prepared using the Perfectprep® Plasmid Midi kit according to the manufacturer's instructions (Eppendorf). HeLa cells were seeded in 6-well plates or on acid-washed glass coverslips in 24-well tissue culture plates 8 h prior to transfection with FuGENE® 6 reagent (Roche). Cells were lysed in boiling 1.5 $\times$ SDS-PAGE sample buffer or fixed for microscopy 18–24 h post-transfection.

Detergent fractionation of transfected cells

For analysis of the membrane association of *Salmonella* effector proteins and their CC mutants when ectopically expressed, transfected HeLa cells were subject to sequential detergent extraction with 0.1% (w/v) saponin and 1% (v/v) TX-100 as described previously (Marcus *et al.*, 2002). Saponin- and TX-100-soluble fractions were analyzed by immunoblotting with rabbit polyclonal anti-GFP antibodies (Invitrogen; 1:50,000) and mouse monoclonal anti-LAMP1 and anti-Hsp27 antibodies (see above).

Fluorescence microscopy of transfected cells

Transfected cells were fixed in 2.5% (w/v) paraformaldehyde for 10 min at 37°C. DNA was labeled with 5 μ M DRAQ5™ (Biostatus Limited) for 5 min at room temperature and coverslips mounted in ProLong Gold Antifade reagent (Invitrogen). Images (1024 $\times$ 1024 pixels, 0.35 μ m optical sections) were acquired on a Zeiss LSM510 or LSM710 confocal microscope equipped with a Plan APOCHROMAT 63 $\times$ /1.4 NA objective. Flat maximum intensity projections of 3–6 sections were assembled using LSM510 AIM v4.2 or Zen 2008 software (Carl Zeiss MicroImaging) and final figures were prepared using Adobe Photoshop CS and Canvas X.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SopB is predicted to contain a CC domain. (A) A ribbon model of the three dimensional structure of SopB (463 of 561 residues). This computational model was generated by the CPHmodels 2.0 Server and displayed using Pymol software. A CC motif was identified in α -helix 10, corresponding to amino acid residues 118–138 of SopB (residues 100–120 in the model), and is denoted in red. (B) A helix wheel projection of residues Thr117 to Leu145 of SopB. Heptad-repeat positions are labeled *a* to *g*. Two SopB CC mutants were constructed based upon this prediction; residues 118–142 were deleted (SopB Δ CC mutant) and three leucine residues in the predicted CC domain (Leu127, Leu131, Leu134; indicated in red) were mutated to aspartate (SopB-L³D mutant).

Figure 2.

SopB binds to SigE independently of its CC domain. $\Delta sopB$ -*sigE* bacteria (ΔBE) and $\Delta sopB$ -*sigE* complemented with plasmid borne SopB and SigE (SopB), SopB-AviTag and SigE (SopB-AviTag) or SopB Δ CC-AviTag and SigE (SopB Δ CC-AviTag) were grown in LB-Miller broth to an OD_{600} ~3.5. Bacteria were disrupted by sonication and extracts clarified by centrifugation. Equivalent amounts of SopB-AviTag and SopB Δ CC-AviTag were used as the reaction mixture input. Soluble proteins were incubated with streptavidin-agarose beads. Bound proteins were eluted by boiling in 1.5 $\times$ SDS-PAGE sample buffer, and separated by SDS-PAGE. Immunoblotting was with anti-SopB, anti-AviTagTM and anti-SigE antibodies.

Figure 3.

Secretion, but not translocation, is disrupted for SopB CC mutants. (A) Intrabacterial and secretion profiles for various *Salmonella* strains; wild type (wt), Δ *sopB-sigE*, (Δ BE), Δ *sopB-sigE* complemented with pWSKDE (SopB), pWSKDE Δ CC (Δ CC) or pWSKDE-L³D (L³D). Bacteria were grown under microaerophilic conditions in LB-Miller broth for 8 h at 37°C. Intracellular and secreted proteins (equivalent to 0.6% and 2.3% of the original sample, respectively) were separated by SDS-PAGE and analyzed by immunoblotting with anti-SopB and anti-DnaK antibodies. Equal exposure times were used for development of SopB intrabacterial and secreted immunoblots and band intensity quantified by densitometry. Quantification of data is shown in the graph underneath. Grey bars represent

intrabacterial SopB levels compared to those for Δ *sopB-sigE* pWSKDE (SopB) bacteria (set to 100%). White bars represent SopB secretion efficiency. Results are means $\pm$ SD from three separate experiments. (B) Upper panel; Δ *sopB-sigE* bacteria complemented with pWSKDE-GSK (SopB), pWSKDE Δ CC-GSK (Δ CC) or pWSKDE-L³D-GSK (L³D) were grown under invasive conditions. Intrabacterial levels of SopB and DnaK in these subcultures were analyzed by SDS-PAGE and immunoblot analysis (3-fold less SopB Δ CC and SopB-L³D compared to wild type SopB). Lower panel; HeLa cells were infected with bacterial subcultures (MOI~50 for SopB and MOI~150 for SopB Δ CC and SopB-L³D) and at the indicated times, monolayers were solubilized, proteins separated by SDS-PAGE and subject to immunoblotting with antibodies directed against phospho-GSK-3 β (translocated SopB-GSK) and GSK-3 β tag (total SopB-GSK). Molecular mass markers are indicated on the left.

A Intrabacterial

B Translocated

Figure 4.

Disruption of the CC domain destabilizes SopB. (A) Δ *sopB-sigE* bacteria were complemented *in trans* with pWSKDE (SopB), pWSKDE Δ CC (SopB Δ CC) or pWSKDE-L³D (SopB-L³D). Bacteria were grown statically in LB-Miller broth at 37°C to mid-log phase (OD₆₀₀~0.4) and then 60 μ g/ml chloramphenicol was added to stop bacterial protein synthesis (t₀). Aliquots were removed at the indicated times (in min), centrifuged and pellets resuspended in boiling 1.5 $\times$ SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and subject to immunoblotting with anti-SopB antibodies. Graph shows densitometric analysis from three separate experiments (mean $\pm$ SD). Closed circles, SopB; open circles, SopB Δ CC; open squares, SopB-L³D. (B) HeLa cells were infected with Δ *sopB-sigE* bacteria harboring pWSKDE-GSK (SopB), pWSKDE Δ CC-GSK (SopB Δ CC) or pWSKDE-L³D-GSK (SopB-L³D). At 1.5 h p.i., 10 μ g/ml tetracycline was added to infected monolayers to stop bacterial protein synthesis (t₀). Whole cell lysates were collected at hourly intervals by adding boiling 1.5 $\times$ SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and immunoblotting was antibodies against phospho-GSK-3 β to detect translocated SopB-GSK. Graph shows densitometric analysis from three separate experiments (mean $\pm$ SD). Closed circles, SopB; open circles, SopB Δ CC; open squares, SopB-L³D.

Figure 5.

The CC domain is not essential for biological activity of SopB. (A) HeLa cells were transfected with plasmids expressing Myc-SopB, Myc-SopB C460S, Myc-SopBΔCC or Myc-SopB-L³D for 18 h, or none (-). Monolayers were solubilized in boiling 1.5× SDS-PAGE sample buffer, proteins separated by SDS-PAGE and subject to immunoblotting with antibodies against SopB, phospho-Akt and total Akt. (B) HeLa cells were infected with increasing amounts of $\Delta sopB$ -sigE bacteria (ΔBE) or $\Delta sopB$ -sigE bacteria complemented with pWSKDE-GSK (SopB) or pWSKDEΔCC-GSK (SopBΔCC). At 1 h p.i., monolayers were solubilized and proteins subject to immunoblotting with antibodies against phospho-Akt (activated Akt), Akt pan (total Akt), phospho-GSK-3β (translocated SopB-GSK) and GSK-3β tag (total SopB-GSK). (C) HeLa cells were infected with $\Delta sopB$ -sigE bacteria complemented with pWSKDE-GSK (SopB) or pWSKDEΔCC-GSK (SopBΔCC). Where indicated, monolayers were pretreated and infected in the presence of the proteasome inhibitor, MG-132 (10 μM). At various times p.i., monolayers were solubilized and proteins subject to immunoblotting as for (B). Molecular mass markers are indicated on the left.

Figure 6.

The CC domain directs efficient membrane-association of SopB. (A) HeLa cells were transfected with plasmids expressing EGFP-HuSopB, EGFP-HuSopB C460S, EGFP-HuSopB Δ CC or EGFP-HuSopB-L³D for 20 h. Monolayers were fixed and DNA stained with DRAQ5TM. Confocal microscope images show EGFP-SopB fusions in grey scale and DNA in blue. Scale bar, 10 μ m. (B) HeLa cells were transfected with plasmids expressing Myc-SopB, Myc-SopB C460S, Myc-SopB Δ CC or Myc-SopB-L³D, harvested and subject to sequential detergent fractionation. Equal volumes of the saponin-soluble (S, cytosolic proteins) and TX-100 soluble (T, membrane proteins) fractions were separated by SDS-PAGE and subject to immunoblotting with antibodies against SopB, LAMP-1 (membranes)

and Hsp27 (cytosol). (C) HeLa cells were infected with $\Delta sopB$ -*sigE* bacteria harboring pWSKDE-GSK (SopB), pWSKDE Δ CC-GSK (Δ CC) or pWSKDE-L³D-GSK (L³D). At 1 h p.i., infected cells were mechanically fractionated and subject to low-speed centrifugation to remove intact bacteria, and then high-speed ultracentrifugation to separate host cell membranes (M) from cytosol (C). Equal volumes of each fraction were subject to immunoblotting with antibodies against phospho-Ser9-GSK3 β (translocated SopB-GSK), LAMP-1 (membranes) and Hsp27 (cytosol).

Figure 7.

CC domains mediate the membrane association of other *Salmonella* effectors. (A) HeLa cells were transfected with plasmids expressing EGFP-PipB2, EGFP-PipB2 YLM³D, EGFP-SseJ, EGFP-SseJ NLV³D, EGFP-SopD2 or EGFP-SopD2 YYK³D for 24 h. Monolayers were fixed and DNA stained with DRAQ5TM. Confocal microscope images show EGFP-effector fusions in grey scale and DNA in blue. Scale bar, 10 μ m. (B) HeLa cells were transfected as described in (A). Cells were harvested and subject to sequential detergent fractionation. Equal volumes of the saponin-soluble (S, cytosolic proteins) and TX-100 soluble (T, membrane proteins) fractions were separated by SDS-PAGE and subject to immunoblotting with antibodies against GFP, LAMP-1 (membranes) and Hsp27

(cytosol). The asterisk indicates a non-specific band in saponin-soluble fractions that cross-reacts with the anti-GFP antibody.

Figure 8.

CC domains are not functionally interchangeable. (A) HeLa cells were transfected with plasmids expressing EGFP-HuSopB or the following chimeras, EGFP-HuSopB-IpgDCC, EGFP-HuSopB-PipB2CC, EGFP-HuSopB-SseJCC or EGFP-HuSopB-SopD2CC for 24 h. Monolayers were fixed and DNA stained with DRAQ5TM. Confocal microscope images show EGFP-SopB fusions in grey scale and DNA in blue. Scale bar, 10 μ m. (B) HeLa cells were transfected as described in (A), harvested and subject to sequential detergent fractionation. Equal volumes of the saponin-soluble (S, cytosolic proteins) and TX-100 soluble (T, membrane proteins) fractions were separated by SDS-PAGE and subject to immunoblotting with antibodies against GFP, LAMP-1 (membranes) and Hsp27 (cytosol).

Table 1

Salmonella T3SS1 and T3SS2 effectors with predicted CC domains.

Translocated by	Effector	CC domain?	Predicted CC domain (amino acid residues)		Chaperone	Membrane-associated?
			Marcoil	Pepcoil		
T3SS1	AvrA	Yes	42–52 259–300			
	SipA/SspA	Yes	22–36 142–173 594–630	144–168 595–613	InvB ^a	Yes ^j
	SipB/SspB	Yes	97–326 529–592	151–175 214–232	SicA ^b	Yes ^j
	SipC/SspC	Yes	74–115 192–228 288–326 343–408		SicA ^b	Yes ^j
	SopA	Yes	37–44 368–384 617–633	368–384	InvB ^c	
	SopB/SigD	Yes	100–169	117–138	SigE ^d	Yes ^k
	SopD	Yes	163–177			Yes ^l
	SopE	Yes	12–55		InvB ^c	Yes ^j
	SopE2	No			InvB ^c	Yes ^j
	SptP	Yes	259–295	275–294	SicP ^e	Yes ⁱ
T3SS2	CigR (STM3762)	No				
	GogB	No				No ^m
	GtgA (STM11026)	No				
	StcD (STM2139)	No				
	PipB	No				Yes ⁿ
	PipB2	Yes	49–66		StcA ^f	Yes ⁿ
	SifA	No				Yes ^o

Translocated by	Effector	CC domain?	Predicted CC domain (amino acid residues)		Chaperone	Membrane- associated?
			Marcoil	Pepcoil		
	SlrB	No				Yes ^p
	SptC	No				
	SopD2	Yes	139–219 275–299	274–298		Yes ⁱ
	SpvB	No				
	SpvC	No				
	SrrH/SseI	No				
	SseF	No			SscB ^g	Yes ^q
	SseG	No				Yes ^q
	SseJ	Yes	257–272 292–327	307–325		Yes ^p
	SseK1	Yes	132–178			No ^f
	SseK2	Yes	152–182			
	SseK3 (sb26)	Yes	101–127 132–155			Yes ^s
	SseL	No			SscA ^f	Yes ^t
	SspH2	Yes	551–567 622–652 676–691 713–735	673–693 720–736		Yes ^u
	SteC	Yes	365–429			
T3SS1 & T3SS2	GigE	No				
	SspH1	Yes	534–572 589–602 628–677	40–55 586–604 631–651		No ^{i,v}
	SlrP	Yes	154–286 547–602 640–657 680–758	159–191 638–658		
	SpvD	No				
	SteA	No				Yes ^w
	SteB	No				

Translocated by	Effector	CC domain?	Predicted CC domain (amino acid residues)		Chaperone	Membrane- associated?
			Marcoil	Pepcoil		
	SteE (STM2585)	No				

All known *Salmonella* effectors (McGhie *et al.*, 2009, Niemann *et al.*, 2011) were analyzed for the presence of CC domains using Marcoil and Pepcoil algorithms. CC motif(s) were considered to be present when predicted by either of the two programs. Known cognate chaperones and membrane association in host cells is indicated.

- ^a(Bronstein *et al.*, 2000);
- ^b(Tucker *et al.*, 2000);
- ^c(Ehrbar *et al.*, 2004)
- ^d(Darwin *et al.*, 2001);
- ^e(Ehrbar *et al.*, 2003);
- ^f(Fu *et al.*, 1998);
- ^g(Cooper *et al.*, 2010);
- ^h(Dai *et al.*, 2004);
- ⁱ(Cain *et al.*, 2004);
- ^j(Scherer *et al.*, 2000);
- ^k(Marcus *et al.*, 2002);
- ^l(Bakowski *et al.*, 2007);
- ^m(Coombes *et al.*, 2005);
- ⁿ(Knodler *et al.*, 2003);
- ^o(Brumell *et al.*, 2002);
- ^p(Freeman *et al.*, 2003);
- ^q(Kuhle *et al.*, 2002);
- ^r(Kujat Choy *et al.*, 2004);
- ^s(Brown, 2011);

t (Rytönen *et al.*, 2007);

u (Quezada *et al.*, 2009);

v (Haraga *et al.*, 2003);

w (Van Engelenburg *et al.*, 2010)