Mutational analysis of *Salmonella* translocated effector members SifA and SopD2 reveals domains implicated in translocation, subcellular localization and function

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Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen causing disease in several hosts. These bacteria use two distinct type III secretion systems that inject effector proteins into the host cell for invasion and to alter maturation of the *Salmonella*-containing vacuole. Members of the *Salmonella* translocated effector (STE) family contain a conserved N-terminal translocation signal of approximately 140 aa. In this study, the STE family member SifA was examined using deletion strategies. Small deletions (approx. 20 residues long) throughout SifA were sufficient to block its secretion and/or translocation into host cells. Transfection of HeLa cells with a GFP-SifA fusion was previously shown to be sufficient to induce formation of Sif-like tubules resembling structures present in *Salmonella*-infected cells. The present study showed that both N- and C-terminal domains of SifA are required for this phenotype. Furthermore, both domains could induce aggregation of Lamp1-positive compartments, provided they were coupled to the minimal C-terminal membrane-anchoring motif of SifA. Mutation or deletion of the conserved STE N-terminal WEK(I/M)xxFF translocation motif of SopD2 disrupted its association with Lamp1-positive compartments, implicating these residues in both effector translocation and subcellular localization. Interestingly, one GFP-SifA deletion mutant lacking residues 42–101, but retaining the WEK(I/M)xxFF motif, targeted the Golgi apparatus. In addition, short peptides containing the signature WEK(I/M)xxFF motif derived from the N-termini of *Salmonella* effectors SopD2, SseJ and SspH2 were sufficient to localize GFP to the Golgi. These studies suggest that *Salmonella* effectors contain multifunctional motifs or domains that regulate several effector traits, including protein secretion/translocation, localization and subversion of host cell systems. Conditions that perturb the tertiary structure of effectors can influence their localization in host cells by liberating cryptic intracellular targeting motifs.

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Abbreviations: BFA, brefeldin A; Lamp1, lysosome-associated membrane protein 1; SCV, *Salmonella*-containing vacuole; Sif, *Salmonella*-induced filament; SPI, *Salmonella* pathogenicity island; STE, *Salmonella* translocated effector; T3SS, type III secretion system; TAPAS-1, tryptophan anchoring phosphatidic acid selective binding domain 1.

INTRODUCTION

The facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium (serovar Typhimurium) is responsible for a range of diseases in different hosts, including gastroenteritis in humans and a typhoid-fever-like disease in mice (Tsolis *et al.*, 1999). From mouse models, infection by serovar Typhimurium arises from bacteria crossing the intestinal epithelial barrier through M-cells or by luminal

sampling of intestinal contents by CD18⁺ phagocytes (Jones *et al.*, 1994; Vasquez-Torres *et al.*, 1999). After breaching the epithelium, serovar Typhimurium goes on to colonize the liver and spleen of infected mice, residing within phagocytic cells at these locations (Richter-Dahlfors *et al.*, 1997; Salcedo *et al.*, 2001).

During the course of disease, serovar Typhimurium relies on two distinct type III secretion systems (T3SSs) to translocate bacterial-encoded virulence factors, termed effectors, directly from the bacterial cytosol into the host cell cytosol (Galan, 2001). The T3SS encoded by *Salmonella* pathogenicity island 1 (SPI-1) is required for invasion of epithelial cells, secreting several effectors that modulate host actin rearrangements and membrane ruffling to facilitate bacterial uptake (Bakshi *et al.*, 2000; Friebe *et al.*, 2001; Galan & Fu, 2000; Galan & Zhou, 2000; Hardt *et al.*, 1998; Stender *et al.*, 2000).

Upon entry into host cells, serovar Typhimurium resides in a membrane-bound compartment termed the *Salmonella*-containing vacuole (SCV) (Dunlap *et al.*, 1991; Knodler & Steele-Mortimer, 2003; Takeuchi, 1967). Expression of a second T3SS, encoded on another pathogenicity island, SPI-2, is induced within the SCV and is essential for intracellular growth and virulence of serovar Typhimurium (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996, 1999; Waterman & Holden, 2003). The SPI-2 T3SS has been shown to be required for many events characteristic of *Salmonella* infection, including the modulation of host endocytic traffic (Brumell *et al.*, 2001b, 2002; Brumell & Grinstein, 2004; Garcia-del Portillo *et al.*, 1993; Knodler & Steele-Mortimer, 2003) and evasion of host cell reactive oxygen species and inducible nitric oxide synthase (Chakravorty *et al.*, 2002; Gallois *et al.*, 2001; Vasquez-Torres *et al.*, 2000, 2001).

SPI2-T3SS effectors are translocated across the SCV membrane and into the host cell (Cirillo *et al.*, 1998; Miao & Miller, 2000; Waterman & Holden, 2003). One such effector, SifA, is essential for inducing the extension of long membranous tubules called *Salmonella*-induced filaments (Sifs) that emanate from the SCV along microtubule networks (Brumell *et al.*, 2002; Garcia-del Portillo *et al.*, 1993; Stein *et al.*, 1996). Transfection of SifA-GFP into host cells is sufficient to mimic the phenotype of the translocated effector, causing aggregation of late endocytic compartments and the formation of Sif-like tubules (Brumell *et al.*, 2001a, 2002). Membrane binding by SifA is mediated by a C-terminal hexapeptide membrane anchor containing an S-acylation site and a CAAX motif serving as a target for host cell prenylation (Boucrot *et al.*, 2003; Reinicke *et al.*, 2005). Sifs are decorated with markers typically found on late endocytic compartments including Rab7 and Lamp1, suggesting that their formation arises in part from the fusion of the SCV with these compartments (Brumell *et al.*, 2003; Brumell & Grinstein, 2004; Garcia-del Portillo *et al.*, 1993). Sifs are associated with rapidly replicating bacteria and their formation peaks at 8–10 h post-infection (Birmingham

et al., 2005). Deletion of *sifA* significantly attenuates serovar Typhimurium virulence, impairs bacterial replication in macrophages, abrogates Sif formation and destabilizes the SCV, suggesting that the control of SCV membrane dynamics is crucial for pathogenicity (Beuzon *et al.*, 2000, 2002; Brumell *et al.*, 2001a; Stein *et al.*, 1996). SifA has been shown to interact with the Rab7 GTPase and may promote the outward growth of Sifs by uncoupling Sif-associated Rab7 from Rab7-interacting lysosomal protein (RILP) (Harrison *et al.*, 2004). Detachment from the centripetally directed dynein motor complex normally associated with RILP may allow Sif extension to proceed throughout the host cell (Harrison *et al.*, 2004). SifA also interacts with a host protein termed 'SifA and kinesin-interacting protein' (SKIP). Recruitment of SKIP to the SCV negatively regulates the recruitment of the plus-end-directed kinesin to this compartment, thus facilitating the inward migration of the SCV towards perinuclear regions of the host cell (Boucrot *et al.*, 2005).

Much less is known about the function of other SPI-2 effectors in comparison to SifA. SopD2 contributes to virulence in mice and acts cooperatively with SifA to promote Sif formation (Brumell *et al.*, 2003; Jiang *et al.*, 2004). Similar to SifA, SopD2 associates with Sifs and late endocytic compartments in infected cells, whether transfected or delivered by serovar Typhimurium itself (Brumell *et al.*, 2003; Jiang *et al.*, 2004). A GFP-fusion to the first 75 aa of SopD2 has been shown to target late endocytic vesicles (Brumell *et al.*, 2003). The SPI-2 effector SseJ also localizes to SCVs and Sifs when ectopically expressed in infected cells. In contrast to SopD2, SseJ appears to inhibit Sif formation through its deacylase activity, which may modify Sif membrane composition (Birmingham *et al.*, 2005; Ohlson *et al.*, 2005). Other SPI-2 effectors, such as SspH2, target the host cytoskeletal system. SspH2 interacts with the actin-binding proteins filamin and profilin and may be involved in regulating the dynamics of actin assembly around the internalized SCV (Miao *et al.*, 2003).

The mechanism by which effector proteins are recognized by T3SSs is not well understood. In general, it appears that effector proteins contain two signals, one that directs protein secretion out of bacteria and into the surrounding medium (secretion signal), and another that is required to further direct protein delivery specifically into host cells (translocation signal) (Ghosh, 2004; Sory *et al.*, 1995). The secretion signal is contained within the 5' region of the gene, in a region encoding approximately the first 20 aa of the secreted substrate, though it is unclear whether the signal acts at the level of mRNA or protein (Anderson & Schneewind, 1997; Ghosh, 2004; Lloyd *et al.*, 2001; Ramamurthi & Schneewind, 2003). Translocation signals appear to reside in regions downstream of secretion signals as well (Miao & Miller, 2000; Sory *et al.*, 1995). In particular, members of the *Salmonella* translocated effector (STE) family, including the SPI-2 effectors SifA, SopD2, SspH2 and SseJ, have a conserved N-terminus of ~140 aa that

mediates translocation into host cells (Brumell *et al.*, 2000, 2003; Miao & Miller, 2000). Significantly, a conserved WEK(I/M)xxFF motif within the N-terminus of STE family members appears to be essential for effector translocation (Miao & Miller, 2000). Interestingly, a fusion of the first 150 aa of SopD2 to GFP retains the ability to localize to late endosomal/lysosomal compartments, indicating that the N-terminus is bifunctional and can mediate translocation and subcellular targeting (Brumell *et al.*, 2003). However, the amino acid residues implicated in directing any of the STE family effectors to their specific subcellular host targets are currently unknown.

Many studies of bacterial effectors have relied on transfection to introduce epitope-tagged versions of effectors into host cells. This may be required due to the unavailability of specific antibodies for immunofluorescence, or low protein copy numbers that preclude their detection. For the most part, the activity and localization of effectors introduced by transfection correlates with that observed for bacterially translocated effectors, as exemplified by SifA (Brumell *et al.*, 2001a, 2002). However, this is not always the case, as the effector SseG targets endosomal membranes, Sifs, SCVs and microtubules when delivered by bacteria (Kuhle & Hensel, 2002; Kuhle *et al.*, 2004), yet localizes to the Golgi when transfected into the same cell type (Salcedo & Holden, 2003). This suggests that the context of effector protein delivery, such as the presence of other effectors, the influence of host responses and the nature of protein fusions, may be important for determining bacterial effector protein localization.

In this study, we performed functional analysis of SifA using deletion strategies to identify regions involved in effector secretion, translocation, localization and modulation of host endocytic trafficking. Although SifA belongs to the STE family, we show that domains throughout the protein, and not only within the N-terminus, are required for secretion and translocation of this effector. We also provide evidence that either N- or C-terminal domains can associate with and aggregate endocytic compartments provided the SifA C-terminal membrane targeting motif is present; however, neither domain is sufficient to generate Sif-like tubules by themselves. In addition, we have determined that residues found within the conserved WEK(I/M)xxFF motif of SopD2 are required for the effector to target late endocytic compartments. Interestingly, a cryptic Golgi-binding peptide containing the above motif is also present in a subset of STE effectors. Our studies demonstrate that STE effectors have domains that possess multiple functions. Furthermore, intracellular targeting motifs within bacterial effectors can be liberated by conditions that may alter protein folding, or by the context in which it is presented.

METHODS

Bacterial strains, plasmids and growth conditions. The *Salmonella enterica* serovar Typhimurium strains used in this work were the wild-type strain SL1344 (Hoiseth & Stocker, 1981), and

AsifA (Stein *et al.*, 1996) and *DssaR* (Brumell *et al.*, 2001a), isogenic mutants derived from SL1344. *Escherichia coli* strains DH5 α or DH10B were used for all molecular cloning experiments. All bacteria were cultured in Luria–Bertani (LB) medium supplemented with chloramphenicol (50 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$) or streptomycin (100 $\mu\text{g ml}^{-1}$) where appropriate.

Plasmids used in this study are described in Table 1 and oligonucleotide sequences are described in Table 2. The plasmid pGFP-SifA was constructed by amplifying *sifA* from SL1344 chromosomal DNA and digesting the PCR product with *KpnI* and *SacI*, followed by ligating it into similarly digested pEGFP-C1. Small, in-frame, internal deletions were constructed in the *sifA* alleles carried on psifA-2HA and pGFP-SifA by an approach analogous to inverse PCR using the oligonucleotides as indicated in Table 1. Following amplification the PCR products were digested with *SpeI* and ligated in an intramolecular fashion to generate plasmids containing the desired deletion. Plasmid pGFP-SifA Δ 1–8 was constructed by ligating a ~ 0.5 kb *KpnI*–*SpeI* fragment derived from pGFP-SifA Δ 8 to a ~ 4.7 kb *KpnI*–*SpeI* fragment derived from pGFP-SifA Δ 1. Plasmid pGFP-SifA Δ 1–5 was constructed by ligating a ~ 0.6 kb *KpnI*–*SpeI* fragment derived from pGFP-SifA Δ 5 to a ~ 4.7 kb *KpnI*–*SpeI* fragment derived from pGFP-SifA Δ 1. Plasmid pGFP-SifA Δ 3–5 was constructed by ligating a ~ 0.6 kb *KpnI*–*SpeI* fragment derived from pGFP-SifA Δ 5 to a ~ 4.8 kb *KpnI*–*SpeI* fragment derived from pGFP-SifA Δ 3. Plasmid pGFP-SifA Δ 9–15 was constructed by ligating a ~ 0.5 kb *SacI*–*SpeI* fragment from pGFP-SifA Δ 9 to a ~ 4.7 kb *SacI*–*SpeI* fragment derived from pGFP-SifA Δ 15. Plasmid pGFP-SifA Δ 9–16 was constructed by ligating a ~ 0.5 kb *SacI*–*SpeI* fragment from pGFP-SifA Δ 9 to a ~ 4.7 kb *SacI*–*SpeI* fragment derived from pGFP-SifA Δ 16.

Deletions and mutations to *sopD2* were generated using overlap extension strategies as outlined previously (Ho *et al.*, 1989). Plasmid pSopD2(W37P,F44R)-GFP, encoding SopD2 bearing W37P and F44R substitutions, was created by amplifying overlapping *sopD2* fragments from psopD2-GFP template in two separate PCRs using primer pairs listed in Tables 1 and 2. The overlapping segments were complementary to each other and contained the specific mutations. These fragments were subsequently pooled together and used as primers for another round of PCR with psopD2-GFP template to generate full-length *sopD2* bearing the desired mutations. After digestion with *Bam*HI and *Xho*I, the mutant gene was ligated into similarly treated pEGFP-N1 to form plasmid pSopD2(W37P,F44R)-GFP. A similar strategy was used to create pSopD2(Δ aa37–44)-GFP, which encodes SopD2 with amino acids 37–44 deleted. However, for this deletion one primer from each primer pair was designed such that its 3' end annealed to the template on one side of the deletion, while the 5' end would anneal to the template DNA on the other side of the deletion (Ho *et al.*, 1989).

Plasmids pSopD2(aa31–64)-GFP, pSopD2(aa31–46)-GFP, pSopD2(aa31–64)-GFP, pSifA(aa25–58)-GFP, pSseJ(aa28–61)-GFP and pSspH2(aa28–61)-GFP encode peptide regions from SopD2, SopD, SifA, SseJ and SspH2, respectively, fused to the N-terminus of GFP. These were constructed using template DNA from psopD2-GFP, psopD-GFP, GFP-SifA (Table 1), or from wild-type serovar Typhimurium SL1344 chromosomal DNA as required. PCR amplicons were digested with *Bam*HI and *Sal*I and ligated into similarly digested pEGFP-N1.

Cell culture, infection of cultured cells and transfections.

HeLa and RAW264.7 cell lines were obtained from the ATCC, and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum at 10% (v/v). Cell cultures were incubated at 37°C and 5% CO₂. Infections of HeLa cells were performed as described previously (Steele-Mortimer *et al.*, 1999). HeLa cells were seeded at 5×10^4 cells per well in a 24-well cell culture

Table 1. Plasmids used in this study

Plasmid	Description	Reference
pEGFP-C1	<i>gfp</i> , for constructing fusions to the C terminus of GFP, Km ^R	Clontech
pEGFP-N1	<i>gfp</i> , for constructing fusions to the N terminus of GFP, Km ^R	Clontech
psifA-2HA	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011), pACYC derivative, Cm ^R	Brumell <i>et al.</i> (2002)
pGFP-SifA	<i>gfp</i> :: <i>sifA</i> , pEGFP-C1 derivative containing <i>sifA</i> amplified using oligonucleotides <i>sifA</i> -f2 and <i>sifA</i> -r2, Km ^R	This study
psifA-2HAΔ1	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ4–63(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 1f2b and <i>sifam</i> 1r, Cm ^R	This study
psifA-2HAΔ2	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ64–123(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 2f and <i>sifam</i> 2r, Cm ^R	This study
psifA-2HAΔ3	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ124–183(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 3f and <i>sifam</i> 3r, Cm ^R	This study
psifA-2HAΔ4	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ184–243(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 4f and <i>sifam</i> 4r, Cm ^R	This study
psifA-2HAΔ5	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ244–303(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 5f and <i>sifam</i> 5r, Cm ^R	This study
psifA-2HAΔ6	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ304–363(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 6f and <i>sifam</i> 6r, Cm ^R	This study
psifA-2HAΔ7	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ433–492(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 7f and <i>sifam</i> 7r, Cm ^R	This study
psifA-2HAΔ8	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ493–552(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 8f and <i>sifam</i> 8r, Cm ^R	This study
psifA-2HAΔ9	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ553–612(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 9f and <i>sifam</i> 9r, Cm ^R	This study
psifA-2HAΔ10	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ613–672(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 10f and <i>sifam</i> 10r, Cm ^R	This study
psifA-2HAΔ11	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ673–732(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 11f and <i>sifam</i> 11r, Cm ^R	This study
psifA-2HAΔ12	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ733–792(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 12f and <i>sifam</i> 12r, Cm ^R	This study
psifA-2HAΔ13	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ793–852(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 13f and <i>sifam</i> 13r, Cm ^R	This study
psifA-2HAΔ14	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ853–912(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 14f and <i>sifam</i> 14r, Cm ^R	This study
psifA-2HAΔ15	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ913–972(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 15f and <i>sifam</i> 15r, Cm ^R	This study
psifA-2HAΔ16	<i>sifA</i> (1–408)::2HA:: <i>sifA</i> (409–1011)Δ973–1008(relative to wt <i>sifA</i>), psifA-2HA derivative using oligonucleotides <i>sifam</i> 16f and <i>sifam</i> 16r2, Cm ^R	This study
pGFP-SifAΔ1	<i>gfp</i> :: <i>sifA</i> Δ4–63(relative to wt <i>sifA</i>), pGFP-SifA derivative using oligonucleotides <i>sifam</i> 1f3 and <i>sifam</i> 1r, Km ^R	This study
pGFP-SifAΔ5	<i>gfp</i> :: <i>sifA</i> Δ244–303(relative to wt <i>sifA</i>), pGFP::SifA derivative using oligonucleotides <i>sifam</i> 5f and <i>sifam</i> 5r, Km ^R	This study
pGFP-SifAΔ8	<i>gfp</i> :: <i>sifA</i> Δ493–552(relative to wt <i>sifA</i>), pGFP::SifA derivative using oligonucleotides <i>sifam</i> 8f and <i>sifam</i> 8r, Km ^R	This study
pGFP-SifAΔ9	<i>gfp</i> :: <i>sifA</i> Δ553–612(relative to wt <i>sifA</i>), pGFP-SifA derivative using oligonucleotides <i>sifam</i> 9f and <i>sifam</i> 9r, Km ^R	This study
pGFP-SifAΔ15	<i>gfp</i> :: <i>sifA</i> Δ913–972(relative to wt <i>sifA</i>), pGFP::SifA derivative using oligonucleotides <i>sifam</i> 15f and <i>sifam</i> 15r, Km ^R	This study
pGFP-SifAΔ16	<i>gfp</i> :: <i>sifA</i> Δ973–1008(relative to wt <i>sifA</i>), pGFP::SifA derivative using oligonucleotides <i>sifam</i> 16f and <i>sifam</i> 16r1, Km ^R	This study
pGFP-SifAΔ1–5	<i>gfp</i> :: <i>sifA</i> Δ4–303(relative to wt <i>sifA</i>), pGFP-SifAΔ1 derivative, Km ^R	This study
pGFP-SifAΔ1–8	<i>gfp</i> :: <i>sifA</i> Δ4–552(relative to wt <i>sifA</i>), pGFP-SifAΔ1 derivative, Km ^R	This study
pGFP-SifAΔ3–5	<i>gfp</i> :: <i>sifA</i> Δ124–303(relative to wt <i>sifA</i>), pGFP-SifAΔ3 derivative, Km ^R	This study
pGFP-SifAΔ9–15	<i>gfp</i> :: <i>sifA</i> Δ913–972(relative to wt <i>sifA</i>), pGFP-SifAΔ15 derivative, Km ^R	This study
pGFP-SifAΔ9–16	<i>gfp</i> :: <i>sifA</i> Δ553–1008(relative to wt <i>sifA</i>), pGFP-SifAΔ16 derivative, Km ^R	This study

Table 1. cont.

Plasmid	Description	Reference
psopD2-2HA	<i>sopD2::2HA</i> , pACYC derivative, Cm ^R	Brumell <i>et al.</i> (2003)
psopD2-GFP	<i>sopD2::gfp</i> , pEGFP-N1 derivative, Km ^R	Brumell <i>et al.</i> (2003)
pSopD2(aa31–64)-GFP	<i>sopD2(91–192)::gfp</i> (relative to wt <i>sopD2</i>), pEGFP-N1 derivative using oligo-nucleotides sopD2-JS1 and sopD2-JS2, Km ^R	This study
pSopD2(aa31–46)-GFP	<i>sopD2(91–138)::gfp</i> (relative to wt <i>sopD2</i>), pEGFP-N1 derivative using oligo-nucleotides sopD2-JS1 and sopD2-JS3, Km ^R	This study
pSopD2(Δ aa37–44)-GFP	<i>sopD2(Δ108–132)::gfp</i> (relative to wt <i>sopD2</i>), pEGFP-N1 derivative using oligo-nucleotide pairs JB0111, sopD2 Δ 37–44upstream and JB0112, sopD2 Δ 37–44downstream, Km ^R	This study
pSopD2(W37P,F44R)-GFP	<i>sopD2::gfp</i> encoding W37P and F44R substitutions (relative to wt SopD2), pEGFP-N1 derivative using oligonucleotide pairs JB0111, sopD2-W37PF44R and JB0112, sopD2-W37PF44Rrev, Km ^R	This study
psopD-GFP	<i>sopD::gfp</i> , pEGFP-N1 derivative, Km ^R	Brumell <i>et al.</i> (2003)
pSopD(aa31–64)-GFP	<i>sopD(91–192)::gfp</i> (relative to wt <i>sopD</i>), pEGFP-N1 derivative using oligo-nucleotides JS-SopD-FW and JS-SopD-RV, Km ^R	This study
pSspH2(aa28–61)-GFP	<i>sspH2(82–183)::gfp</i> (relative to wt <i>sspH2</i>), pEGFP-N1 derivative using oligo-nucleotides JS-SspH2-FW and JS-SspH2-RV, Km ^R	This study
pSseJ(aa28–61)-GFP	<i>sseJ(82–183)::gfp</i> (relative to wt <i>sseJ</i>), pEGFP-N1 derivative using oligonucleotides JS-SseJ-FW and JS-SseJ-RV, Km ^R	This study
pSifA(aa25–58)-GFP	<i>sifA(73–174)::gfp</i> (relative to wt <i>sifA</i>), pEGFP-N1 derivative using oligonucleotides JS-SifA-FW and JS-SifA-RV, Km ^R	This study

plate containing coverslips 16–24 h before infection. The bacteria used for HeLa cell infections were exponential-phase *Salmonella* grown for 3 h in LB medium. Bacteria were pelleted at 10 000 g for 2 min and resuspended in PBS. The inoculum was diluted and added to HeLa cells at a m.o.i. of 100:1 at 37 °C for 10 min. The cells were then washed extensively with PBS, and growth medium containing 100 µg gentamicin ml⁻¹ was added until 2 h post-infection, at which point the cells were washed and growth medium containing 10 µg gentamicin ml⁻¹ was added for the duration of the experiment.

RAW264.7 cells were infected in triplicate with opsonized, stationary-phase bacteria. Bacterial cultures were grown for 16 h at 37 °C and then opsonized in 20% normal human serum in DMEM for 25 min at 37 °C. RAW264.7 cells were inoculated with approximately 50 bacteria per cell followed by centrifugation for 5 min at 600 g. Infected cells were incubated for 20 min at 37 °C, 5% CO₂, washed three times with PBS and then cultured for 90 min in DMEM containing 100 µg gentamicin ml⁻¹. Cells were then washed as above and incubated in DMEM containing 10 µg gentamicin ml⁻¹ for an additional 20 h. At 2 and 20 h post-infection, infected cells were washed and then lysed in 0.25 ml 1% Triton X-100, 0.1% SDS in PBS. Lysates were diluted and plated in replicate onto LB agar for enumeration of c.f.u.

For transfection, HeLa cells were seeded at 5 × 10⁴ cells per well into 24-well cell culture plates containing coverslips. After 16–24 h, between 0.5 and 1 µg DNA was used to transfect HeLa cells with Eugene 6 transfection reagent (Roche) according to the manufacturer's instructions. Following transfection, cells were cultured for a further 16–20 h. To enumerate the aggregation of Lamp1⁺ compartments or formation of Sif-like tubules, HeLa cells transfected with plasmids encoding various GFP-SifA deletion derivatives were fixed and immunostained for Lamp1 (see below). The numbers of transfected cells that contained Lamp1⁺ aggregates or Sif-like tubules were determined for at least 100 cells, and each experiment

was performed at least three times. The assessment of swollen/aggregated Lamp1 compartments is based on a subjective increase in Lamp1⁺ compartment size. The mean ± SD for these experiments is presented.

Immunofluorescence staining and microscopy. Immunofluorescence staining was carried out as described previously (Coombes *et al.*, 2003). Samples were fixed in 2.5% paraformaldehyde/PBS solution (pH 7.4) for 10 min at 37 °C. Samples were then washed twice with PBS prior to being blocked and permeabilized in 10% normal goat serum, 0.2% saponin in PBS (SS-PBS) for 1–16 h. Primary and secondary antibodies were overlaid on coverslips in SS-PBS for 1 h, followed by three washes with PBS. Coverslips were mounted onto glass slides using DakoCytomation fluorescent mounting medium. Confocal microscopy was performed using a Zeiss Axiovert microscope (63× objective) and Zeiss LSM software. Images were imported into Adobe Photoshop 7 and assembled in Adobe Illustrator CS for labelling. For live cell imaging, HeLa cells were seeded onto 2.5 cm coverslips at 2.0 × 10⁵ cells per well in a 6-well culture plate and transfected with 1.5 µg pSopD2(aa31–64)-GFP (Table 1) as described above. After approximately 16 h, seeded coverslips were transferred to RPMI medium (supplemented with L-glutamine, HEPES, no bicarbonate; Wisent). Cells were incubated with 5 µg brefeldin A (BFA) ml⁻¹ for 70 min, followed by washout of the drug and replacement with fresh RPMI. Cells were imaged at 3 min intervals during the course of BFA treatment and during the recovery period following drug removal using a Leica DMIRE2 fluorescent microscope equipped with Openlab 3.1.7 software (Improvision).

Antibodies and reagents. The anti-Lamp1 (H4A3) and anti-tubulin (E7) antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa (Department of Biological Sciences, Ames, IA, USA). H4A3 was used at 1:50–1:100 for immunofluorescence.

Table 2. Oligonucleotides used in this study

Name	Sequence (5'–3')
sifA-f2	ATGGAGCTCTGCCGATTACTATAGGGAATGG
sifA-r2	ATGGGTACCTTATAAAAAACAACATAAACAGCCG
sifam1f2b	ATGACTAGTCATATTAATCTCACTTATACTGGAGT
sifam1f3	ATGACTAGTCAGAGCTCGAGATCTGAG
sifam1r	ATGACTAGTACGAAAGAAGCATGGTG
sifam2f	ATGACTAGTATTCCTTGGGGAGTTGGTAAGG
sifam2r	ATGACTAGTGGCAAAGCAAAAGCGGAC
sifam3f	ATGACTAGTAGTAGAAAAAAGAAAGTCTTTAAT
sifam3r	ATGACTAGTCGAGAGCGGCTTACAGA
sifam4f	ATGACTAGTTGTGGGGGCGCGTTCGG
sifam4r	ATGACTAGTGATAGATTTCAGGTTTCATAATCCTC
sifam5f	ATGACTAGTTCTTTGCGATGCGCAGGCT
sifam5r	ATGACTAGTGATCAAAACGAAGAGAACGAATTG
sifam6f	ATGACTAGTCATGATGCCAAGAATAATGGTG
sifam6r	ATGACTAGTGAAGTCATGGGGAATCTTTATTT
sifam7f	ATGACTAGTCATTTGTGGATGCGATTTT
sifam7r	ATGACTAGTTGTCTCAATCCTGCTGGC
sifam8f	ATGACTAGTTAATGTGGAAGGGAGGGG
sifam8r	ATGACTAGTTTATATACTGAATTGAGAAAAGGAC
sifam9f	ATGACTAGTATACCCCTCTATGTTGTCTAATGG
sifam9r	ATGACTAGTTACCTGGCAGCGAAAATTCAG
sifam10f	ATCACTAGTGTTGCTTTTCTTGCG
sifam10r	ATGACTAGTAATATATCCGAAAGTACTCAGCAA
sifam11f	ATCACTAGTCGCATGGTGTAAAAATGC
sifam11r	ATGACTAGTAAACAGCTTGAAATACCACCAC
sifam12f	ATCACTAGTTAATCCACACATCGCCATT
sifam12r	ATGACTAGTGTTTTACTAGCGGATAAGACG
sifam13f	ATGACTAGTCTCTTTTACCATTTTTCATAGG
sifam13r	ATGACTAGTAGTATGTTAGCTGAGATAGTCGAAG
sifam14f	ATGACTAGTTTGGCTGGTGCTGGGAT
sifam14r	ATGACTAGTATAGACCCCCAGGCTATACAA
sifam15f	ATGACTAGTTCTAAAAATGGCGTGAAAAACC
sifam15r	ATGACTAGTTCAGAACAACAAAGCGGCTG
sifam16f	ATGACTAGTGCGAACGTGTAGCGTGTT
sifam16r1	ATGACTAGTTAAGGATCCACCGGATCTAGATAA
sifam16r2	ATGACTAGTTAAGTCGACCGATGCCCTT
sopD2-JS1	CGCGTCGACAGCATGGCGCTATACATGGGGGTATG
sopD2-JS2	CGCGGATCCGCTTCACATCCATGGATGAG
sopD2-JS3	CGCGGATCCGCTGTTCTGAAGCAGTCCTTAAATC
JB0111	CCGCTCGAGAGCATGCCAGTTACGTTAAGTTTGGTAAT
JB0112	CGTGGATCCAGTATAAGCATATTGCGACAACCTCGACTT
sopD2Δaa37-44upstream	GTTTTTGTGTGTTCTTACCCCATGTATAGCGC
sopD2Δaa37-44downstream	CTATACATGGGGGTAAGAACACAAAAACAAG
sopD2-W37PF44R	GGGTACCGGATAGATTTAAGGACTGCCGCAGAACAC
sopD2-W37PF44Rrev	GTGTTCTGCGGCAGTCCTTAAATCTATCCGGTACCC
JS-SopD-FW	CGCGTCGACAGCATGGCAATCCATATGGGGGGTTGGG
JS-SopD-RV	CGCGGATCCGCGCCCTGCCCGTGAATGATGGAGTG
JS-SseJ-FW	CGCGTCGACAGCATGCTTCCGGAATTAAGTTTATGGG
JS-SseJ-RV	CGCGGATCCGCTTCTGATGGTGTTAAAGATTAAAGATACATTCG
JS-SifA-FW	CGCGTCGACAGCATGGCAATGGAAGTTTATGGG
JS-SifA-RV	CGCGGATCCGCGCGTTCGGCAAACAACATCTCATG
JS-SspH2-FW	CGCGTCGACAGCATGCCCCCTGAAATGAGTTCCTGGG
JS-SspH2-RV	CGCGGATCCGCGGCCGCGGGTGACAAATCGTCC

Rabbit anti-Lamp1 antibody (Affinity BioReagents) was used at 1:200 for immunofluorescence. Anti-tubulin antibody was used at a dilution of 1:5000 for Western blots. The anti-giantin antibody was used at 1:1000 for immunofluorescence and was obtained from Dr H. P. Hauri, University of Basel, Switzerland. The anti-HA epitope antibody (Covance) was used at 1:400 for immunofluorescence and 1:2000 for Western blots. Antibodies against DnaK and calnexin were obtained from Stressgen and used at 1:3500 and 1:2000, respectively, for Western blots. Alexa-568-conjugated goat anti-mouse and anti-rabbit antibodies were used at 1:200 and were purchased from Molecular Probes. Horseradish-peroxidase-labelled anti-mouse antibodies (Jackson ImmunoResearch Laboratories) were used at a concentration of 1:5000.

In vitro secretion assays. *In vitro* secretion assays were carried out as described previously (Coombes *et al.*, 2004). Bacteria were cultured until stationary phase in a modified M9 minimal medium optimized for SPI2 gene expression and SPI2 type III secretion (Beuzon *et al.*, 1999; Nikolaus *et al.*, 2001). Overnight cultures of the required *Salmonella* strains grown in LB broth were washed twice in low-phosphate, low-magnesium medium (LPM) and then inoculated 1:50 in 3 ml LPM at pH 5.8. LPM medium consisted of 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 38 mM glycerol (0.3% v/v), 0.1% Casamino acids, 8 µM MgCl₂, 337 µM PO₄³⁻, and 80 mM MES (for titration to pH 5.8). Cultures were grown for 4–6 h at 37 °C with shaking, after which the OD₆₀₀ was measured. Bacteria were pelleted by centrifugation for 2 min at 12 000 r.p.m. (4 °C) and the supernatant was passed through a 0.22 µm filter and precipitated with trichloroacetic acid (10%, v/v, final concentration) at 4 °C for 4–16 h.

The trichloroacetic-acid-insoluble fraction was collected by centrifugation, washed with ice-cold acetone, and solubilized with a volume of 2 × SDS-sample buffer (100 mM Tris/HCl, pH 6.8, 20% glycerol, 4% SDS, 0.002% bromophenol blue and 200 mM dithiothreitol) adjusted according to the OD₆₀₀ of the original culture. When necessary, solubilized secreted proteins were neutralized with an appropriate volume of non-titrated Tris. The bacterial pellet fraction from above was also dissolved in a volume of 2 × SDS-sample buffer adjusted according to the OD₆₀₀ of the original culture. Proteins from equivalent numbers of bacterial cells, as determined by OD₆₀₀ readings, were separated on 10% or 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and then blocked in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) powdered non-fat milk for 1 h at room temperature. Blots were incubated with mouse anti-HA monoclonal or mouse anti-DnaK monoclonal antibody in TBST plus 5% non-fat milk. Secondary antibodies conjugated to horseradish peroxidase were used at a 1:5000 dilution in TBST for 1 h at room temperature. Antibody complexes were detected using enhanced chemiluminescence (Amersham Biosciences).

Host cell fractionation. HeLa cells for fractionation were seeded at 2 × 10⁶ per dish in 100 mm dishes, and were infected as described above. Cells were fractionated as previously described (Gauthier *et al.*, 2000). In brief, cells were scraped and disrupted mechanically by passage through a 22-gauge needle in a homogenization buffer containing 250 mM sucrose, 3 mM imidazole and 0.5 mM EDTA (pH 7.4). The cell suspension was centrifuged at low speed (3000 g for 15 min at 4 °C) to pellet bacteria and unbroken cells (fraction designated 'P' in these studies), followed by ultracentrifugation (41 000 g for 20 min at 4 °C) to separate cellular membranes (designated 'M') from the cytosolic fraction (designated 'C'). Each fraction was dissolved in 1 × SDS sample buffer and loaded onto 10% polyacrylamide gels for electrophoresis. Gels were transferred to PVDF membranes for Western blotting experiments.

RESULTS

Deletions within SifA disrupt its secretion and translocation

In general, the N-termini of various bacterial T3SS effectors have been implicated in effector secretion and translocation (Cornelis & Van Gijsegem, 2000; Miao & Miller, 2000), while the actual functional effector domain appears to reside in the C-terminal half of the protein (Hueck, 1998). SifA has been classified as a member of the STE family, as it contains a conserved N-terminal domain of approximately 140 aa that has been shown to be involved in translocation of another STE protein, SspH1 (Fig. 1a) (Miao & Miller, 2000). The C-terminus of SifA bears similarity to several Gram-negative bacterial proteins, and contains a prenylation motif required for membrane insertion (Fig. 1a) (Boucrot *et al.*, 2003; Brumell *et al.*, 2002; Reinicke *et al.*, 2005). Addition of an internal, double haemagglutinin tag (2 × HA; herein referred to as 2HA) between the conserved N-terminal type III secretion signal and the C-terminal portion of SifA resulted in a protein (SifA-2HA) that retained all known functions, including secretion, translocation, localization to endocytic compartments and induction of Sif formation (Brumell *et al.*, 2002; Jiang *et al.*, 2004). This suggested that SifA might follow the generalized model of T3SS effectors, and comprise at least two distinct domains, one implicated in its delivery into host cells, and the other in modulating host cell targets.

To gain more insight into functional domains of SifA, a panel of deletion mutants was constructed. A series of 16 genes encoding scanning internal deletions of SifA were constructed in psifA-2HA (Fig. 1b). Plasmids encoding these deletion mutants were transformed into a Δ sifA strain of serovar Typhimurium to determine their effects on the defective replication of this strain previously described in the RAW264.7 macrophage cell line (Brumell *et al.*, 2001a). It was found that all deletions constructed in psifA-2HA resulted in reduced replication compared to the control strain containing the unmodified parental plasmid, which displayed expected replication levels within host cells (Fig. 2a). The psifA-2HA deletion plasmids were also unable to complement Sif formation and SCV maintenance in a chromosomal Δ sifA strain when used to infect HeLa cells for 10 h (results not shown). Similarly, none of the sifA-2HA deletion alleles acted as dominant negatives on Sif formation and SCV maintenance when expressed from wild-type serovar Typhimurium used to infect HeLa cells (results not shown).

The inability to restore the intracellular growth of the Δ sifA strain to levels comparable to the Δ sifA strain containing unmodified psifA-2HA may be due to defects in secretion or translocation of each SifA-2HA mutant. Using an *in vitro* secretion assay optimized for SPI-2 gene expression and SPI-2 T3SS secretion, we observed that the majority of deletions to SifA abrogated its secretion [Fig. 2b; compare bacterial pellet fractions (P) with corresponding supernatants (S)]

Fig. 1. Schematic of serovar Typhimurium effectors SifA and SopD2. (a) SifA and SopD2 share a conserved N-terminal region involved in translocation into host cells (Brumell *et al.*, 2000; Miao & Miller, 2000). SifA also contains a prenylation motif involved in host membrane targeting at its C-terminus (Boucrot *et al.*, 2003; Reinicke *et al.*, 2005). The position of an in-frame insertion of tandem HA-tags (2×HA-tag) encoded in plasmid psifA-2HA is indicated in SifA (Brumell *et al.*, 2002). The late-endosome/lysosome (LE/Lys)-binding region of SopD2, and a predicted C-terminal coiled-coil (CC) region are also shown. Asterisks indicate the relative position of a conserved WEK(I/M)xxFF motif found in members of the STE family. (b) Scale diagram indicating deletions constructed in each mutant SifA used in this study. The top line indicates scale in amino acids and represents wild-type SifA. Lines below represent SifA mutants, with the deleted region (20 aa in each case) indicated as a gap in the thick line. Mutant designations are labelled to the left and were constructed in both psifA-2HA and pGFP-SifA. Note that no deletion was constructed in the region of amino acids 122–144 due to the presence of the 2×HA epitope tag in SifA-2HA.

containing secreted proteins]. Western blotting using the anti-HA antibody showed that each SifA-2HA mutant was synthesized in the bacteria (Fig. 2b), confirming that our secretion results were not due to a lack of mutant protein being expressed. We note that limited amounts of mutant SifA-2HA were secreted from bacteria transformed with psifA-2HAΔ11, psifA-2HAΔ15 and psifA-2HAΔ16

compared to control bacteria carrying psifA-2HA (Fig. 2b). Interestingly, the mutant protein SifA-2HAΔ2, bearing a deletion of its conserved WEK(I/M)xxFF translocation domain common to STE effectors (Miao & Miller, 2000), was also secreted by bacteria into the culture supernatant.

To detect the delivery of effector protein into host cells, we performed sensitive immunoblot-based translocation assays in HeLa cells using both wild-type serovar Typhimurium SL 1344 and a *ΔssaR* strain deficient in SPI-2 type III secretion. Fig. 2(c) shows a representative assay testing the translocation of a SifA-2HA deletion derivative, namely the SifA-2HAΔ2 mutant. Whereas wild-type SifA-2HA was delivered by the wild-type strain and could be detected in the host cell membrane fraction (M) by immunoblotting, SifA-2HAΔ2 was not. As expected, neither SifA-2HA nor SifA-2HAΔ2 was translocated from the *ΔssaR* strain (SPI-2 T3SS defective) (Fig. 2c). The two signals that appear upon detection of wild-type SifA-2HA in lane 1 (Fig. 2c) likely represent post-translationally modified and unmodified forms of SifA-2HA. It is established that SifA undergoes prenylation and S-acylation within the host (Reinicke *et al.*, 2005). Hence, the untranslocated SifA-2HA remaining within bacteria due to the SifA-2HAΔ2 mutation or the lack of a functional SPI-2 T3SS (*ΔssaR*) was detected as the sole lower molecular mass signals common to lanes 4, 7 and 10 (Fig. 2c). The remaining SifA-2HA mutants were also tested using this immunoblot-based translocation assay, and were not translocated. This included mutants with deletions at the C-terminus outside of the conserved STE family protein translocation region (data not shown). Furthermore, immunofluorescence microscopy revealed that none of the SifA-2HA deletion proteins were translocated into host HeLa cells at 10 or 18 h postinfection when expressed from either a *ΔsifA* or a wild-type strain (data not shown). Collectively, these results show that regions throughout SifA, and not only within its N-terminus, are required for its secretion and/or translocation.

N- and C-terminal domains of SifA each have roles in targeting and/or aggregation of Lamp1⁺ compartments, but cannot induce Sif-like tubule formation by themselves

Transfection of SifA-GFP results in its association with Lamp1⁺ vesicles that induces their aggregation and filamentation into Sif-like tubules, similar to bacterial delivery of the effector (Boucrot *et al.*, 2003; Brumell *et al.*, 2001a, 2002). A previous study has identified an 11 aa membrane-anchoring motif at the C-terminus of SifA (Boucrot *et al.*, 2003). Fusion of this motif to a cytosolic protein, GFP-SifB, was sufficient to target the protein to membrane compartments when transfected into HeLa cells (Boucrot *et al.*, 2003). However, it is not clear whether these compartments were Lamp1⁺ and thus indicative of the actual subcellular targets of SifA.

To determine the domains in SifA involved in targeting and aggregating Lamp1⁺ vesicles, as well as in inducing Sif-like

Fig. 2. Short deletions in SifA block its secretion and translocation into host cells. (a) Complementation of a chromosomal $\Delta sifA$ genotype with psifA-2HA deletion constructs. Replication of bacteria in RAW264.7 macrophages is expressed as the number of intracellular bacteria at 20 h divided by the number at 2 h. The infecting strain is indicated below the graph, with the transformed plasmid indicated in parentheses. Results shown are means $\pm$ SEM ($n = 3$). (b) Secretion of mutant SifA-2HA proteins into culture supernatants. The indicated strains were cultured until stationary phase in a minimal medium optimized for SPI-2 gene expression and SPI2 type III secretion. Bacteria were then centrifuged out of suspension and lysed in SDS sample buffer, and the culture supernatant proteins were precipitated using trichloroacetic acid and dissolved in SDS sample buffer. Samples were then resolved on 12% polyacrylamide gels, Western blotted and probed with anti-HA and anti-DnaK antibodies. Lanes are labelled 'P' for whole cell bacterial samples and 'S' for precipitated culture supernatant sample. DnaK was used as a control for cytosolic contamination of the culture supernatants. (c) Assessment of SifA-2HA Δ 2 translocation into host cells. HeLa cells were infected for 18 h with SL1344 wild-type or $\Delta ssaR$ (SPI-2 T3SS-deficient mutant) transformed with psifA-2HA or psifA-2HA Δ 2, as indicated. These were then fractionated into a low-speed pellet fraction designated 'P', a high-speed pellet designated 'M' and a supernatant fraction designated 'C' as described in Methods. Fraction P primarily consists of high-order polymers, nuclei, unbroken cells and bacteria, fraction M consists mainly of host cell membranes and fraction C consists of soluble host cell cytoplasmic molecules. DnaK was used as a marker for potential bacterial contamination of fractions M and C. Calnexin is an endoplasmic reticulum integral membrane protein and β -tubulin is a cytoplasmic protein.

tubule formation, we constructed a GFP-fusion to the N-terminus of SifA (GFP-SifA). Several deletions to SifA were also constructed for localization studies in HeLa cells. Specifically, we tested the fusions GFP-SifA Δ 9–15 (lacking aa 185–324 of wild-type SifA), GFP-SifA Δ 9–16 (lacking the C-terminus of SifA from aa 185), GFP-SifA Δ 1–8 (lacking aa 2–184), GFP-SifA Δ 1–5 (lacking aa 2–101), and GFP-SifA Δ 3–5 (lacking aa 42–101) (Fig. 3a). GFP-SifA Δ 9–15 was designed to retain the C-terminal amino acids involved in membrane anchoring (Boucrot *et al.*, 2003). HeLa transfectants were immunostained for Lamp1 to visualize colocalization and/or aggregation effects of each GFP-SifA fusion on late endocytic compartments.

As expected, wild-type GFP-SifA was associated with Lamp1⁺ vesicles, many of which appeared swollen and aggregated, as revealed by confocal microscopy and quantification (Fig. 3b, c). In addition, the fusion protein induced the formation of Sif-like tubules (Fig. 3b, d). These phenotypes elicited by GFP-SifA were consistent with those observed previously using a similar construct (Boucrot *et al.*, 2003), and with SifA-GFP (Brumell *et al.*, 2001a), where GFP was fused to the C-terminus of the effector. Hence, the functionality of both SifA fusions was similar (Fig. 3c). GFP-SifA Δ 9–15 also produced a similar phenotype to the wild-type control (Fig. 3b, c), indicating that much of the C-terminus of SifA (from aa 185 to aa 324) is not required for binding and aggregating Lamp1⁺ compartments. However, GFP-SifA Δ 9–15 could not induce the formation of Sif-like tubules. Removal of the SifA membrane-targeting motif from GFP-SifA Δ 9–15, resulting in GFP-SifA Δ 9–16 (Fig. 3a), rendered the protein distributed throughout the cytosol (Fig. 3b). Furthermore, GFP-SifA Δ 9–16 did not induce aggregation of Lamp1⁺ vesicle activities, similar to GFP transfectant controls (Fig. 3b, c). These results show that the N-terminal half of SifA is able to aggregate Lamp1⁺ compartments provided the minimal SifA membrane-anchoring motif is present.

We next examined the effects of N-terminal deletions to SifA using GFP-SifA constructs. Both microscopy and quantification showed that GFP-SifA Δ 1–8 efficiently produced Lamp1⁺ vacuolation and aggregation (Fig. 3b, c). However, despite this ability, GFP-SifA Δ 1–8 was not localized exclusively to Lamp1⁺ vesicles, and appeared in the cytosol as well (Fig. 3b). GFP-SifA Δ 1–5, which contained additional SifA N-terminal residues (aa 102–184) that were excluded from GFP-SifA Δ 1–8 (Fig. 3a), also presented a cytosolic distribution with little colocalization with Lamp1⁺ vesicles (Fig. 3b). Curiously, GFP-SifA Δ 1–5 did not induce significant aggregation of Lamp1⁺ compartments as GFP-SifA Δ 1–8 did, in comparison to the negative control GFP (Fig. 3c). Significantly, these results indicate that the C-terminal half of SifA, which includes the membrane-targeting motif, is not sufficient to properly target and/or aggregate late endocytic compartments, and that determinants within the N-terminus are required as well.

Interestingly, GFP-SifA Δ 3–5 appeared concentrated at a region adjacent to the nucleus and did not colocalize with Lamp1⁺ compartments (Fig. 4). HeLa cells expressing GFP-SifA Δ 3–5 had significantly fewer Lamp1⁺ aggregates, even compared to cells transfected with the unmodified GFP vector (Fig. 3c). In contrast to both wild-type SifA-GFP and GFP-SifA, none of the GFP-SifA deletions could induce the formation of Sif-like tubules upon transfection of HeLa cells (Fig. 3d). Overall, our results suggest that both the N- and C-terminal domains of SifA contribute to its localization to, and aggregation of, Lamp1⁺ compartments. Furthermore, tubulation of Lamp1⁺ compartments into Sif-like tubules appears to require a complete SifA protein.

GFP-SifA Δ 3–5 targets the Golgi complex

The localization of GFP-SifA Δ 3–5 was strikingly different from that of the other GFP-SifA constructs. The fusion protein did not colocalize with Lamp1⁺ compartments, and was found predominantly at a juxtanuclear position and on vesicles surrounding the nucleus (Fig. 4, top panels). The concentration of GFP-SifA Δ 3–5 adjacent to the nucleus suggested the fusion might be preferentially targeted to the Golgi apparatus. Indeed, the majority of GFP-SifA Δ 3–5 colocalized with giantin (Fig. 4, bottom panels), an established marker of the Golgi (Linstedt & Hauri, 1993; Seelig *et al.*, 1994). In contrast to GFP-SifA Δ 1–5, which was predominantly in the cytosol (Fig. 3b), the Golgi-targeting GFP-SifA Δ 3–5 contained the first 41 aa of SifA (Fig. 3a). This suggested that determinants within the first 41 residues of SifA might redirect its subcellular localization in certain conditions, such as in the context of deletion mutations.

A conserved N-terminal motif in SopD2 is involved in membrane association and is sufficient to target the Golgi

Similar to SifA, the SPI-2 effector SopD2 localizes to Lamp1⁺ endocytic compartments when transfected as a GFP-fusion, or when delivered by the serovar Typhimurium T3SS (Brumell *et al.*, 2003). We verified that such localization could occur simultaneously in HeLa cells by first transfecting SopD2-GFP and then infecting the cells with a Δ sopD2 serovar Typhimurium mutant delivering plasmid-encoded SopD2-2HA. Both SopD2-GFP and SopD2-2HA were colocalized with Lamp1⁺ vesicles (Fig. 5b, middle row panels). Thus, SopD2 fusions to either epitope tag did not disrupt its targeting of late endocytic compartments.

Since the N-terminus of SifA contained residues that could influence its subcellular localization, we examined whether this region in other STE family members might do the same. The first 200 aa of SopD2 have been shown to be required for its translocation into host cells, while the membrane-association domain of this effector has been mapped to within the first 75 aa of the protein (Fig. 5a) (Brumell *et al.*, 2003). Hence, both translocation and subcellular targeting domains are found within the same terminus of the protein. Partial protein sequence alignment of the N-termini of STE

Fig. 3. Modulation of host cell endosome trafficking by SifA involves multiple domain activities. (a) Schematic of larger deletions constructed in GFP-SifA. Thin lines and numbers indicate positions of amino acids deleted in SifA. Full-length SifA is 336 aa. (b) Localization of GFP-SifA mutant proteins. HeLa cells were transfected with plasmids expressing the indicated GFP-SifA deletion mutant (green) for 16–20 h, fixed, immunostained with antibody to Lamp1 (red), and analysed by confocal microscopy. Bottom panels show the merge between corresponding GFP and Lamp1 signals, with colocalization indicated in yellow. Arrows indicate positions of swollen and/or aggregated Lamp1⁺ compartments. The arrowhead indicates Sif-like tubules. Scale bars, 10 μ m. (c). SifA domain functions on aggregation of Lamp1⁺ compartments. HeLa cells were transfected, fixed and stained as in (b) and the incidence of Lamp1-positive aggregates was determined. The results shown are means $\pm$ SD of three separate experiments. Asterisks indicate $P < 0.05$. (d). SifA domain functions on Sif-like tubule formation. HeLa cells were transfected, fixed and stained as in (b) and the percentage of cells exhibiting Sif-like tubules was determined. The results shown are means $\pm$ SD of three separate experiments. Asterisks indicate $P < 0.05$.

Fig. 4. A SifA truncation mutant can target the Golgi. HeLa cells were transfected with a plasmid expressing GFP-SifA Δ 3–5 (See Fig. 2a for details) for 16–20 h, fixed, immunostained with antibody to Lamp1 (red, top panels) or to the Golgi protein giantin (red, bottom panels) and analysed by confocal microscopy. Arrows indicate colocalization of GFP-SifA Δ 3–5 with giantin. Size bars, 10 μ m.

family members SifA, SopD2, SseJ, SspH2 and SopD shows the conserved WEK(I/M)xxFF motif previously identified as an important signal for effector translocation (Miao & Miller, 2000). From the effectors used in our protein alignment, the consensus sequence of this conserved motif is W(E/D)(K/R)xxxF; however, we will continue to use the previously established consensus of WEK(I/M)xxFF for discussion purposes (Miao & Miller, 2000). Full-length protein sequence alignments (data not shown) indicate that the region encompassing this motif is one of the most conserved amongst STE members; hence we chose to examine this region in SopD2 (WDRFKDCF) (Fig. 5a) to determine its role, if any, in protein localization. GFP fusions to SopD2 bearing a complete deletion of its WEK(I/M)xxFF motif [SopD2(Δ aa37–44)-GFP] or simultaneous substitutions of W37P and F44R at highly conserved positions [SopD2(W37P,F44R)-GFP] were constructed and used in HeLa transfection studies. W37P and F44R substitutions were selected to disrupt a predicted helical conformation that spans the WEK(I/M)xxFF motif (Brumell *et al.*, 2001a).

Confocal microscopy showed that wild-type SopD2-GFP colocalized with Lamp1⁺ vesicles (Fig. 6a, top panels), as

previously observed (Brumell *et al.*, 2003). In contrast, both SopD2(Δ aa37–44)-GFP and SopD2(W37P,F44R)-GFP appeared distributed throughout the cytosol and did not localize to Lamp1⁺ compartments (Fig. 6a, middle and bottom panels). Hence, residues within the conserved WEK(I/M)xxFF motif of SopD2 are required for membrane association to Lamp1⁺ compartments.

We next sought to determine whether the conserved STE region encompassing the WEK(I/M)xxFF motif had any inherent subcellular targeting properties. A fusion of GFP to SopD2 residues 31–64 (containing the peptide WDRFKDCF) (Fig. 5a) was constructed and transfected into HeLa cells. Transfected SopD2(aa31–64)-GFP did not colocalize with Lamp1⁺ vesicles, nor with SopD2-2HA translocated into the same cell by bacteria (Fig. 5b, bottom panels). Interestingly, SopD2(aa31–64)-GFP was concentrated to a juxtanuclear position (Fig. 5b, bottom left panel; Fig. 6b, top left panel), similar to GFP-SifA Δ 3–5 (Fig. 4). However, the localization of SopD2(aa31–64)-GFP was much more distinct, and did not appear on other vesicles surrounding the nucleus as observed with GFP-SifA Δ 3–5. The position of SopD2(aa31–64)-GFP suggested it targeted

Fig. 5. SopD2 contains a conserved WEK(I/M)xxFF motif implicated in intracellular targeting. (a) The conserved WEK(I/M)xxFF motif of SopD2 is underlined. Shown are residues 31–64 of SopD2, selected for fusion with GFP. Asterisks indicate conserved residues selected for further mutagenesis. (b) Transfected SopD2-GFP colocalizes with translocated SopD2-2HA and Lamp1-positive compartments, but SopD2(aa31–64)-GFP does not. HeLa cells were transfected with plasmids expressing GFP alone (top panels), or GFP-fusions to wild-type SopD2 (middle panels) or SopD2(aa31–64) (bottom panels) for 16–20 h. Cells were then infected with a Δ sopD2 strain of serovar Typhimurium SL 1344 containing a plasmid encoding SopD2-2HA. After 8 h of infection, cells were fixed, permeabilized, and co-immunostained with antibodies to HA-tag (red) and Lamp1 (blue). Images show flat projections of assembled confocal sections. Pink signals indicate colocalization of translocated SopD2-HA and Lamp1 signals. White signals indicate colocalization of translocated SopD2-HA, transfected SopD2-GFP and Lamp1 (arrow). Size bars, 10 μ m.

the Golgi, which was confirmed by its extensive colocalization with the Golgi markers giantin (Fig. 6b, top panels) and GM130 (data not shown). Treatment of HeLa cells

expressing SopD2(aa31–64)-GFP with the Golgi-disrupting drug BFA resulted in the dispersal of fluorescent signal throughout the cytosol (Fig. 6c). The effects of BFA were

reversible, with SopD2(aa31–64)-GFP signal returning to its original position following washout of the drug (Fig. 6c). A GFP fusion to a smaller SopD2 peptide composed of only 16 aa (aa 31–46) and retaining the residues WDRFKDCF was also sufficient to specifically target the Golgi, as shown by its colocalization with giantin (Fig. 6b, bottom panels). SopD2(aa31–46)-GFP also did not colocalize with Lamp1⁺ compartments (data not shown), similar to SopD2(aa31–64)-GFP. Overall, these results show that residues within the conserved WEK(I/M)xxFF motif of SopD2 are involved in directing this STE family member to endocytic vesicles. Furthermore, SopD2 peptides containing this conserved motif can target the Golgi.

A conserved N-terminal peptide present in a subset of STE effectors can target the Golgi

To determine the subcellular localization characteristics of WEK(I/M)xxFF motif-containing peptides from other STE family members, GFP-fusions to such peptides from SseJ, SspH2, SopD2 and SifA were constructed (Fig. 7). Like SifA, the localization of each of these STE effectors upon transfection has already been established. SseJ localizes to globular membranous Lamp1⁺ compartments that have a composition similar to that of SCVs (Ruiz-Albert *et al.*, 2002), SspH2 associates with regions of active actin polymerization such as membrane ruffles (Miao *et al.*, 2003), and SopD, a SPI-1 effector, appears to be cytosolically distributed (Brumell *et al.*, 2003).

Transfection of HeLa cells with SspH2(aa28–61)-GFP and SseJ(aa28–61)-GFP resulted in a noticeable fraction of each fusion localizing to an area adjacent to the nucleus, with the remaining protein diffusely distributed throughout the cytosol (Fig. 7a, b). Colocalization with giantin confirmed that SspH2(aa28–61)-GFP and SseJ(aa28–61)-GFP could be targeted to the Golgi (Fig. 7a, b), although not to the same extent as SopD2(aa31–64)-GFP (Fig. 6b). Interestingly, SopD(aa31–64)-GFP was found nearly exclusively within the nucleus (Fig. 7c). Although some of the fusion was also observed in a region next to the nucleus, it did not exhibit significant colocalization with the Golgi marker giantin (Fig. 7c). SifA(aa25–58)-GFP was distributed in a network surrounding the nucleus, with only limited colocalization with giantin (Fig. 7d). Much of the SifA(aa25–58)-GFP signal was extended beyond that of giantin; however, its perinuclear distribution did not significantly colocalize

with an endoplasmic reticulum marker, protein disulphide isomerase (data not shown).

Although both SifA(aa25–58)-GFP and GFP-SifAΔ3–5 contained the conserved WEK(I/M)xxFF motif, we noted that they each had a distinct localization upon transfection (compare Figs 4 and 7d). This suggested that, while the WEK(I/M)xxFF can function to direct subcellular targeting, the overall context of the protein in which this motif appears may play an important role in influencing its ultimate localization. In support of this, GFP-SifAΔ1, with amino acids 2–21 of SifA deleted but retaining the conserved WEK(I/M)xxFF motif, localized exclusively to the nucleus upon transfection and was not colocalized with any Lamp1⁺ compartments (Fig. 7e). Overall, our results show that a subset of STE effectors contain N-terminal motifs that can target the Golgi. Furthermore, mutations such as deletions may liberate other cryptic subcellular localization motifs in bacterial effectors.

DISCUSSION

This is believed to be the first study to examine the distribution of functional domains in the STE family member SifA. To exert its effects, SifA must be secreted through the SPI-2 T3SS, engage and tubulate late endocytic compartments, and interact with specific regulators of microtubule motors (Beuzon *et al.*, 2000; Boucrot *et al.*, 2005; Brumell *et al.*, 2001a, b, 2002; Harrison *et al.*, 2004; Stein *et al.*, 1996). As such, it is expected that several functional domains would exist within this effector to regulate its activities. Evidence for this can be seen with the SifA-2HA construct used in our studies. This fusion consists of a 20 aa insertion (encoding tandem HA epitopes) following amino acid 136 of wild-type SifA (Brumell *et al.*, 2002). That this protein is translocated efficiently and permits a Δ sifA mutant to induce Sif formation suggested, in previous studies, that SifA consists of at least two domains approximated by N- and C-terminal halves that could be separated by the 2HA insertion (Brumell *et al.*, 2002).

To date, the best-characterized SifA region is its membrane-anchoring C-terminus, containing a 6 aa sequence with homology to CAAX and Rab geranylgeranyl transferase prenylation motifs (Boucrot *et al.*, 2003; Reinicke *et al.*, 2005). Biochemical studies have shown that this motif is a site for isoprenoid attachment, with an adjacent cysteine residue modified by S-acylation (Reinicke *et al.*, 2005).

Fig. 6. A conserved N-terminal motif in SopD2 is involved in membrane association and is sufficient to target the Golgi. (a) HeLa cells were transfected with plasmids expressing the indicated SopD2-GFP mutants (green) for 16–20 h, fixed, and immunostained with antibody to Lamp1 (red). Right panels show the merge between corresponding GFP and Lamp1 signals, with colocalization indicated in yellow. Arrows indicate colocalization of SopD2-GFP with Lamp1⁺ compartments. Size bars, 10 μ m. (b) HeLa cells transfected with plasmids expressing fusions of SopD2 peptides (aa31–64 or aa31–46) to GFP for 16–20 h, fixed, and immunostained with antibody to giantin (red). Arrows indicate colocalization of SopD2-GFP with giantin. Size bars, 10 μ m. (c). HeLa cells expressing GFP-SopD2(aa31–64) were treated with the Golgi-disrupting drug BFA and observed using live cell imaging. BFA was removed after 70 min and cells were allowed to recover in drug-free medium. Size bar, 10 μ m.

Fusion of an 11 aa C-terminal SifA sequence containing this CAAX motif is sufficient to target a GFP fusion to uncharacterized membranes upon transfection of HeLa cells (Boucrot *et al.*, 2003). Our results extend the above studies and demonstrate that the N-terminal half of SifA is sufficient to mediate targeting and aggregation of Lamp1⁺ compartments, provided it is fused to the SifA prenylation motif. Moreover, our results also show that the C-terminal half of SifA containing the membrane-anchoring motif is not sufficient for localizing the effector to Lamp1⁺ compartments. This was evident with GFP-SifAΔ1–5 and GFP-SifAΔ3–5, each having reduced association with Lamp1⁺ vesicles despite retaining the C-terminal membrane anchor. This indicates that SifA targeting to appropriate host cell compartments is a combined result of membrane anchoring via prenylation and by other regions distributed throughout SifA, including determinants found within the N-terminus. Collectively, this would provide an effective concentration of SifA at the site of action and induce the aggregation of Lamp1⁺ compartments.

GFP-SifA truncation derivatives that retained the ability to aggregate Lamp1⁺ vesicles could not tubulate these compartments, in contrast to unmodified GFP-SifA. Thus both N- and C-terminal domains of SifA are required for the formation of Sif-like tubules. To date, several studies have linked SifA effector function to mediating interactions with microtubule motors and/or motor adaptors to control the membrane dynamics of SCVs and/or Sifs (Boucrot *et al.*, 2005; Guignot *et al.*, 2004; Harrison *et al.*, 2004). Therefore the effector domain(s) of SifA that regulate these activities may span regions encompassing the entire protein, as compared to the more discrete C-terminal effector domains found in other translocated bacterial proteins (Hueck, 1998).

Our studies also demonstrate that a completely intact SifA is required for its proper secretion and translocation. SifA translocation appears to involve regions in addition to those described for other effectors. Deletions in the N-terminal 140 aa disrupted translocation, consistent with SifA possessing a proteinaceous N-terminal or 5' mRNA-encoded secretion signal and a chaperone-dependent signal (Ramamurthi & Schneewind, 2003). However, our results are also consistent with regions downstream of amino acid 140 being required for SifA translocation, lying beyond the N-terminal translocation domain common to the STE family (Miao & Miller, 2000). Other bacterial effectors have been characterized to have translocation signals localized in their C-termini, including the serovar Typhimurium SPI-1

effector SipC (Chang *et al.*, 2005) and translocated intimin receptor (Tir) from enterohaemorrhagic *E. coli* (EHEC O157:H7) (Allen-Vercoe *et al.*, 2005).

Previous results have shown that various truncated SifA fusions to CyaA were not translocated into host cells and tended to be unstable (Miao & Miller, 2000). For the most part, the deletions used in our study did not appear to negatively affect SifA-2HA levels in serovar Typhimurium; hence, the differences in secretion compared to wild-type protein were not likely due to poor protein synthesis or stability. Although translocation into host cells was disrupted, we note that there was still some detectable secretion of several mutant proteins into the growth medium. The continued secretion of SifA-2HAA2 indicates that the WEK(I/M)xxFF is not required for SifA secretion. The secretion of an effector is not indicative of its ability to be translocated into a host cell, suggesting that other factors are needed. This is evidenced by YopE and YopH effector proteins from *Yersinia* species. While YopE and YopH secretion signals were mapped within the first 15 and 17 residues of each respective protein, additional residues encompassing the first 50 and 71 residues of YopE and YopH, respectively, were needed for their translocation into host cells (Sory *et al.*, 1995). Hence, residues in addition to those directing secretion may be required to properly interact with specific chaperones and/or the translocon pore to efficiently direct effector translocation into a host cell. It is also possible that certain SifA-2HA mutants were secreted through the flagellar apparatus, as described previously with deletion mutants in serovar Typhimurium effectors SopE and SptP (Lee & Galan, 2004). As translocation is dependent on a T3SS apparatus, the secretion of an effector through a flagellar apparatus would therefore not result in its delivery into a host cell.

Residues within the conserved WEK(I/M)xxFF motif have been shown to be essential for translocation of various STE family members (Miao & Miller, 2000). We provide evidence that this motif has at least two roles, in both translocation and protein localization in host cells. Using SopD2 as a model STE effector, we demonstrated that either deletion or mutation of residues in the conserved N-terminal WEK(I/M)xxFF motif abrogates its localization to late endocytic compartments. Significantly, this appears to be the first report to identify specific residues within a short, defined translocation domain that can direct subcellular localization. Other studies have shown a relationship between translocation domains and subcellular targeting

Fig. 7. A conserved N-terminal peptide present in SspH2 and SseJ can target the Golgi. HeLa cells were transfected with plasmids expressing GFP-fusions to a conserved N-terminal region found in various serovar Typhimurium effectors, including (a) SspH2(aa28–61), (b) SseJ(aa28–61), (c) SopD(aa31–64), and (d) SifA(aa25–58). At 16–20 h after transfection, cells were fixed and immunostained with antibodies to giantin (red). Right panels in (a–d) show the merge between corresponding GFP and giantin signals, with colocalization indicated in yellow. Arrows indicate colocalization of SspH2(aa28–61)-GFP and SseJ(aa28–61)-GFP with giantin. Size bars, 10 μm. (e) Nuclear localization of GFP-SifAΔ1. HeLa cells were transfected with a GFP-SifAΔ1 expressing plasmid for 16–20 h, fixed, and immunostained with antibody to Lamp1 (red). The right panel shows the merge between corresponding GFP and Lamp1 signals.

(Knodler *et al.*, 2003; Miao *et al.*, 2003); however, those studies focused on relatively large protein regions (>150 residues) and did not identify specific residues involved in protein localization. Our results reinforce the notion that small regions in bacterial effectors can mediate multiple activities. Such multifunctional domains have been observed in other bacterial effector proteins, including the 80 residue N-terminus of the *Shigella flexneri* T3SS substrate IpaC, where the secretion signal, chaperone-binding region, IpaB-binding domain and IpaC invasion function are all encoded (Harrington *et al.*, 2003). More recently, a small C-terminal peptide region (LFNEF) within the non-STE effector PipB2 was identified to be important for both targeting and function of the effector on late endocytic and lysosomal compartments (Knodler & Steele-Mortimer, 2005). We note that the SopD2(Δ a37–44)-GFP fusion did appear to induce some aggregation of Lamp1⁺ compartments in a small fraction of transfected cells. Hence, some function may be retained despite the inability of the mutant fusion protein to overtly co-localize with these compartments. It is possible that the overexpression of SopD2(Δ a37–44)-GFP can partially overcome its inability to specifically co-localize with Lamp1⁺ compartments, and induce their aggregation in some transfected cells.

Curiously, peptides containing the WEK(I/M)xxFF motif from a subset of STE effectors, particularly SopD2, are targeted to the Golgi apparatus upon transfection. Ectopic expression of the non-STE effector SseG results in its association with the Golgi as well (Salcedo & Holden, 2003). A 55 aa region predicted to be a transmembrane domain has been identified as the SseG Golgi-targeting region (Salcedo & Holden, 2003); however, protein sequence alignments show there is no similarity between this region and the WEK(I/M)xxFF-containing peptides used in our study (Fig. 8). The biological significance, if any, of Golgi targeting by WEK(I/M)xxFF-containing peptides is not known. It is possible that certain STE effectors are first directed to the

secretory pathway before redistribution to other cell regions, but such an intermediate localization step has not been observed. Numerous proteins residing in the endosomal system transit through the endoplasmic reticulum and Golgi; however, identifying these intermediates by methods such as immunofluorescence can be difficult (Cook *et al.*, 2004). Since SifA is modified by prenylation and S-acylation, it is possible that this effector may be processed through the endoplasmic reticulum (Reinicke *et al.*, 2005). Hence, it is conceivable that certain effectors may need to pass through the host cell secretory pathway before interacting with their final intracellular targets.

We note that the conserved WEK(I/M)xxFF motifs have similarity to a previously characterized phosphatidic acid (PA)-binding domain found in PDE4A1 cAMP-specific phosphodiesterase implicated in Golgi targeting (Baillie *et al.*, 2002). Golgi-specific localization of PDE4A1 is likely a result of binding to enriched PA formed from phospholipase D localized in the Golgi (Baillie *et al.*, 2002; Freyberg *et al.*, 2001, 2003). The PDE4A1 peptide region, referred to as TAPAS-1 (tryptophan anchoring phosphatidic acid selective binding domain 1), is an 11 aa sequence proposed to form an amphipathic α -helix that interacts with PA-containing surfaces by insertion of the peptide in a parallel orientation to membrane surfaces (Baillie *et al.*, 2002). Fusion of a 25 aa TAPAS-1-containing fragment to GFP was shown to target the protein almost exclusively to the Golgi (Baillie *et al.*, 2002), similar to our observations with SopD2 peptides containing their cognate WDRFKDCF motif. Strikingly, sequence alignment reveals that the SopD2 WEK(I/M)xxFF motif (sequence **WDRFKDCF**) shares four identical residues (bold) and two polar/charged residues (underlined) with the TAPAS-1 microdomain (PWLVGWWD**QFKR**) (Fig. 8). However, preliminary studies probing phosphoinositide- and PA-spotted nitrocellulose strips with a GST fusion to the SopD2 Golgi-targeting peptide have not shown specific binding to PA under the conditions tested.

Fig. 8. Partial CLUSTAL W multiple alignment of STE proteins SopD2, SopD, SseJ, SspH2 and SifA showing the conserved WEK(I/M)xxFF motif (residues under black bar). Asterisks indicate conserved W37 and F44 residues found in the WEK(I/M)xxFF motif. The STE family member residues shown were all fused to GFP for localization studies. Residues underneath the dotted line correspond to SopD2 residues 31–46. Also aligned are the Golgi-targeting sequences determined for serovar Typhimurium effector SseG (Salcedo & Holden, 2003) and Uukuniemi virus (Bunyaviridae) glycoprotein G1 (Andersson & Pettersson, 1998), as well as the phosphatidic acid (PA)-binding domain of rat PDE4A1 cAMP-specific phosphodiesterase (Baillie *et al.*, 2002). Arrows indicate identical residues between the Golgi-targeting motif of SopD2 and the PA-binding domain of PDE4A1.

Interestingly, a region within the Golgi-targeting C-terminus of Uukuniemi virus G1 membrane glycoprotein (Andersson & Pettersson, 1998) shares some similarity with both TAPAS-1 and WEK(I/M)xxFF motifs (Fig. 8; residues underneath black bar). GFP fusions to viral G1 peptides (as small as 30 residues) containing the region resembling the WEK(I/M)xxFF consensus have also been shown to target the Golgi (Andersson & Pettersson, 1998). That bacterial and viral proteins may have evolved to contain similar TAPAS-1-like sequences to host proteins is intriguing, and may present evidence of convergent evolution to exploit various host cell systems. The specificity of TAPAS-1-like peptides for the Golgi, including the SopD2 peptides used in the present study, might serve as a useful cell biology tool in future studies requiring Golgi localization.

It remains possible that the distinct localization patterns we observed with our constructs may have resulted from the liberation of cryptic subcellular targeting signals by deletion of surrounding residues that would normally mask or override them. For example, our GFP-SifAΔ1 mutant was localized to the nucleus, a compartment where SifA is not normally observed. Nuclear localization has also been observed with a GFP-SifA mutant lacking its membrane-anchoring motif (Boucrot *et al.*, 2003). Thus, in the context of the present study, the Golgi and nuclear-specific targeting of SopD2 and SifA peptides, respectively, may represent artefacts due to drastic deletions. In addition, the method of introducing effectors into host cells can influence protein localization. This is evidenced by the Golgi-specific localization of transfected SseG compared to its association with SCV and Sifs when translocated by bacteria (Salcedo & Holden, 2003). Furthermore, it is possible that some deletions may cause protein misfolding that can affect effector protein function and localization. Hence, the overall context of the effector and how it is presented to the host cell, including the nature of deletions or mutations, the presence of other effectors, the quantity of effector protein present in the cell and its delivery by bacterial translocation or by transfection, may influence the targeting of the protein to possibly produce artefacts, and should be carefully considered when engaging in bacterial effector protein studies.

Overall, we have identified regions of SifA required for various aspects of its biological function. It is apparent that regions distributed throughout SifA are required for secretion and translocation. We provide evidence that both N- and C-terminal halves of SifA play roles in mediating the binding and aggregation of Lamp1⁺ vesicles. However, neither domain was sufficient to induce the formation of Sif-like tubules. Thus, SifA is composed of N- and C-terminal domains that must act cooperatively to effectively tubulate late endocytic compartments. As such, this may make precise structure–function assignments to SifA more challenging than for other effectors. SopD2 also appears to contain a multifunctional N-terminus that mediates both translocation and subcellular localization. Significantly, we show that residues in the conserved STE

family WEK(I/M)xxFF motif are involved in subcellular targeting by SopD2. Hence, discrete translocation domains can also function in protein localization. Finally, we show that peptides present in a subset of STE family members can target discrete subcellular locations, suggesting that cryptic binding motifs within bacterial effectors can be liberated depending on the context of their presentation within the host cell.

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