Salmonella pathogenicity island 1 (SPI-1) type III secretion of SopD involves N- and C-terminal signals and direct binding to the InvC ATPase

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Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is an important pathogen and a causative agent of gastroenteritis. During infection, *S. Typhimurium* assembles molecular-needle complexes termed type III secretion (T3S) systems to translocate effector proteins from the bacterial cytoplasm directly into the host cell. The T3S signals that direct the secretion of effectors still remain enigmatic. SopD is a key T3S effector contributing to the systemic virulence of *S. Typhimurium* and the development of gastroenteritis. We have scrutinized the distribution of the SopD T3S signals using *in silico* analysis and a targeted deletion approach. We show that amino acid residues 6–10 act as the N-terminal secretion signal for *Salmonella* pathogenicity island 1 (SPI-1) T3S. Furthermore, we show that two putative C-terminal helical regions of SopD are essential for its secretion and also help prevent erroneous secretion through the flagellar T3S machinery. In addition, using protein–protein interaction assays, we have identified an association between SopD and the SPI-1 T3S system ATPase, InvC. These findings demonstrate that T3S of SopD involves multiple signals and protein interactions, providing important mechanistic insights into effector protein secretion.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is a facultative intracellular pathogen, causing salmonellosis (Hohmann, 2001). During the course of infection, *S. Typhimurium* translocates bacterial virulence ‘effector’ proteins from the bacterial cytoplasm directly into the host-cell cytoplasm via two major type III secretion systems (T3SSs) located in discrete regions of its chromosome or pathogenicity islands (Galán, 2001; Ochman & Groisman, 1996). *Salmonella* pathogenicity island 1 (SPI-1) is located within centisome 63 of the bacterial chromosome (Galán, 1999). SPI-1 is expressed in the initial infectious stage (Wallis & Galyov, 2000), and translocated effectors modulate key host-cell functions, including general signal transduction, cytoskeletal architecture, membrane trafficking and cytokine expression in favour of the pathogen (Hayward & Koronakis, 1999; Lilic *et al.*, 2003; Schlumberger & Hardt, 2005). A second T3SS is encoded within *Salmonella* pathogenicity island 2 (SPI-2) at centisome 31, and is required for systemic infection (Hensel, 2000; Waterman & Holden, 2003).

At least 20 effector proteins are delivered by the SPI-1 T3SS. The mechanism underlying secretion via the T3SS has been an area of controversy for many years. There is still no consensus sequence that describes a universal type III secretion (T3S) signal. An N-terminal secretion signal (NSS) may involve either a signal peptide or, as seen in *Yersinia* spp., an mRNA signal sequence (Anderson *et al.*, 1999; Anderson & Schneewind, 1999; Sorg *et al.*, 2005, 2006). In the signal peptide hypothesis, two essential regions are required for T3S (Cheng *et al.*, 1997; Sory *et al.*, 1995). The first region encodes a secretion signal within the first ~20 amino acids of the effector protein (Cornelis & Van, 2000; Sorg *et al.*, 2005, 2006). The process of T3S was described by Akeda & Galán (2005), who demonstrated that the effector protein SptP in complex with its cognate chaperone, SicP, interacts with the SPI-1 T3SS ATPase, InvC. The InvC ATPase is part of the T3SS injectisome (Muller *et al.*, 2006). Following this interaction, InvC disassociates the substrate–chaperone complex and unfolds SptP, which is then driven through the T3S needle. In *S. Typhimurium*, introduction of a +1 or +2 frameshift at codon 10 of effectors SopE and SptP, to change the protein sequence between residues 11 and 35, significantly reduces secretion through T3S (Lee & Galán, 2004). We have demonstrated that the first 15 amino acids are important for the secretion of SopE independently of the presence of

Abbreviations: CBD, chaperone-binding domain; GST, glutathione S-transferase; NSS, N-terminal secretion signal; SPI-1, *Salmonella* pathogenicity island 1; SPI-2, *Salmonella* pathogenicity island 2; T3S, type III secretion; T3SS, type III secretion system.

the chaperone-binding site (Karavolos *et al.*, 2005). Also, in SipB, residues 3–8 are necessary for its secretion from the bacterial cell (Kim *et al.*, 2007). However, *in silico* comparison of the N-terminal signal regions of known effectors has not led to the identification of any conserved sequences, implying that features such as amphipathicity or secondary structure serve as recognition motifs (Lloyd *et al.*, 2001, 2002; Miao & Miller, 2000; Tampakaki *et al.*, 2004).

Lee & Galán (2004) indicated that the NSS of SptP is not sufficient to mediate secretion through its cognate T3SS, requiring a secondary NSS identified as the chaperone-binding domain (CBD). The CBD is located within the first ~140 amino acids of some secreted proteins and harbours the binding site for the cognate chaperone, which is generally required for efficient transport via the T3S apparatus (Ghosh, 2004; Lee & Galán, 2003). In the case of SopE, Ehrbar and co-workers have noted that an alternative function of the CBD is to prevent erroneous SopE secretion via the flagellar T3SS in the absence of its chaperone, InvB (Ehrbar *et al.*, 2006; Lee & Galán, 2004). It has been shown that effector proteins containing only the N-terminal signal peptide but lacking a CBD are secreted through the flagellar T3SS (Kim *et al.*, 2007; Lee & Galán, 2004). Indeed, the flagellar export machinery has been suggested to be an ancestor of the SPI-1 T3SS (Nguyen *et al.*, 2000), as many components of the SPI-1 and flagellar export apparatuses show high sequence homology and similarity (Foulter *et al.*, 2002; Gophna *et al.*, 2003).

In *S. Typhimurium* T3SS, chaperones are an essential part of the effector secretion mechanism. Most chaperones have additional functions that prevent aggregation (Boyd *et al.*, 2000) and premature interactions in the bacterial cytoplasm (Day *et al.*, 2000). Additional functions include preserving the unfolded state of proteins (Stebbins & Galán, 2001) and protecting them from degradation (Frithz-Lindsten *et al.*, 1995), as well as regulating effector expression (Darwin & Miller, 2001).

The *Salmonella* effector SopD is encoded outside SPI-1 and contributes to inflammation and fluid secretion during gastroenteritis in the bovine model of *Salmonella* infection (Jones *et al.*, 1998). Genome sequence analysis has also revealed the presence of a SopD homologue termed SopD2, which possesses a different intracellular function to that of SopD (Brumell *et al.*, 2003). Expression of *sopD* is maintained at later stages of infection when other SPI-1 effectors are not expressed, indicating its involvement in systemic disease in mice (Brumell *et al.*, 2003). Additionally, the bacteria require SopD for optimal replication in mouse macrophages (Jiang *et al.*, 2004b). SopD appears to be acting as a 'dual effector' of both SPI-1 and SPI-2 T3SSs. Thus, SopD is found in the host-cell cytosol not only during the early stages of infection but also later in the *Salmonella*-containing vacuole (SCV) (Brumell *et al.*, 2003). Moreover, SopD and SopB act cooperatively to enhance membrane fission and to promote macro-

pinocytosis during *S. Typhimurium* invasion (Bakowski *et al.*, 2007). Wood *et al.* (2004) have suggested that SopD consists of a single compact domain and does not have a cognate bacterial chaperone, implying that an alternative, chaperone-independent mechanism may be used for its secretion via the T3S apparatus.

In this study, we have dissected the contribution of various SopD domains in T3S. We have identified an NSS essential for SPI-1 T3S. Interestingly, a C-terminal region is essential to prevent misdirected secretion through the flagellar apparatus. We show that for successful secretion, SopD interacts with the SPI-1 T3SS ATPase, InvC. Finally, we propose a model for the secretion of SopD through the SPI-1 T3SS which further adds to our current mechanistic understanding of effector secretion signals.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1. *S. Typhimurium* and isogenic mutant strains were grown in Luria–Bertani (LB) medium for 12 h at 37 °C, and diluted 1:100 into fresh medium with 0.3 M NaCl and 5 mM arabinose. Bacterial cells were grown for 4 h at 37 °C and 200 r.p.m. under conditions that stimulated expression of the SPI-1 T3SS (Bajaj *et al.*, 1996). Strains carrying mutations in *fliGHI*, *invC* and *invA* were constructed using the λ -red system (Datsenko & Wanner, 2000). *Escherichia coli* strain DH5 α was used as a cloning host, while *E. coli* strain BL21 was used for protein overexpression. *E. coli* was routinely grown at 37 °C in LB medium. The following antibiotics were used at the indicated concentrations: ampicillin, 100 μ g ml⁻¹; kanamycin, 50 μ g ml⁻¹.

Plasmid constructions. All recombinant DNA techniques used were based on standard protocols (Sambrook *et al.*, 1989). The PCR primers (Invitrogen) used are listed in Table 2. The PCRs were carried out using Phusion High-Fidelity DNA polymerase (Finnzymes), and ligations were performed using a T4 DNA ligase (Fermentas).

To identify the NSS of SopD, we constructed a set of vectors expressing variable lengths of the SopD effector using oligonucleotide primers listed in Table 2. A set of DF primers was used to amplify the full length of the SopD-coding region. The resulting product was digested with *EcoRI*/*HindIII* and cloned into *EcoRI*/*HindIII*-digested pJBT, giving pJWDF, which expressed the SopD protein fused with a C-terminal strep-tag under the control of the inducible arabinose promoter of pBAD24. A first 20 aa N-terminal deletion (SopD _{Δ 1-20}) was created using primer set D20317, following ligation of the *EcoRI*/*HindIII* PCR fragments into *EcoRI*/*HindIII*-digested pJBT, resulting in pJWD21317. To create a series of N-terminal truncations of SopD deleting amino acids 6–20, 11–20, 16–20 and 6–10, the pJWDF plasmid was also used as a template in the inverse PCRs performed with a set of D620, D1120, D1620 or D610 primers to generate pJWD620, pJWD1120, pJWD1620 or pJWD610, respectively.

To investigate the role of the C terminus of SopD on its secretion through the T3SS, PCR fragments containing 200 and 305 N-terminal amino acids of SopD were cloned using *EcoRI*/*HindIII* digestion into similarly digested pJBT, generating pJWD200 and pJWD305, respectively. To construct pJWD201220 and pJWD268302, pJWDF containing the full-length SopD was used as a template for the inverse PCRs using D201220 and D268302 primer sets, respectively. All plasmids encoding recombinant versions of SopD were verified by DNA sequencing (GATC Biotech AG).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Reference or source
S. Typhimurium strains		
SL1344	Wild-type $\Delta prgH$ <i>invG::Tnp_{phoA}</i>	Hoiseth & Stocker (1981) This study Lodge <i>et al.</i> (1995)
RM69	<i>SPI-1::Kan^R</i>	Murray & Lee (2000)
SW001	<i>fliGHI::Kan^R</i>	This study
SW002	<i>invC::Kan^R</i>	This study
SW003	<i>invA::Kan^R</i>	This study
E. coli strains		
DH5 α	<i>fhuA2</i> Δ (<i>argF-lacZ</i>)U169 <i>phoA glnV44</i> ϕ 80 Δ (<i>lacZ</i>)M15 <i>gyrA96 recA1</i> <i>relA1 endA1 thi-1 hsdR17</i>	Gibco-BRL
BL21	F ⁻ , <i>ompT</i> , <i>hsdS_B</i> (r _B ^m β), <i>dcm</i> , <i>gal</i> , <i>lon</i>	Novagen
Plasmids		
pKD46	Red recombinase expression plasmid	Datsenko & Wanner (2000)
pKD13	Template plasmids for kanamycin cassette	Datsenko & Wanner (2000)
pGEX-2T	Expression vector with GST fusion, Amp ^R	GE Healthcare
pGEX-2K	pGEX-2T derivative containing <i>XbaI</i> and <i>HindIII</i> sites	This study
pGWC	pGEX-2T derivative encoding full-length InvC	This study
pBAD24	Expression vector with arabinose-inducible promoter, Amp ^R	Guzman <i>et al.</i> (1995)
pJBT	pBAD24 derivative with C-terminal strep-tag	Karavolos <i>et al.</i> (2005)
pJWDF	pJBT derivative with full-length SopD (317 aa)	This study
pJWD21317	pJBT derivative encoding SopD _{Δ1-20}	This study
pJWD620	pJBT derivative encoding SopD _{Δ6-20}	This study
pJWD1120	pJBT derivative encoding SopD _{Δ11-20}	This study
pJWD1620	pJBT derivative encoding SopD _{Δ16-20}	This study
pJWD610	pJBT derivative encoding SopD _{Δ6-10}	This study
pJWD200	pJBT derivative encoding SopD _{Δ201-317}	This study
pJWD201220	pJBT derivative encoding SopD _{Δ201-220}	This study
pJWD268302	pJBT derivative encoding SopD _{Δ268-302}	This study
pJWD305	pJBT derivative encoding SopD _{Δ306-317}	This study

Plasmid pGWC, encoding glutathione S-transferase (GST)–InvC, was constructed by inserting and ligating a 1300 bp, GC primers-amplified PCR fragment encoding full-length InvC digested with *XbaI* and *HindIII* into similarly cut vector pGEX-2K. The sequence of *invC* in pGWC was verified by DNA sequencing (GATC-Biotech AG).

Protein secretion analysis. To assess protein secretion, *Salmonella* strains harbouring various expression constructs were grown as described above. Whole cells and culture supernatants were separated by centrifugation at 13 000 *g* for 10 min. The culture supernatants were filter-sterilized (0.22 μ m pore-size), and proteins were pre-

Table 2. Primer sets used in this study

Primer set	Forward primer sequence (5'–3')*	Reverse primer sequence (5'–3')*
DF	GgaattcACCATGCCAGTCACCTTTAAGTTTCG	aagcttTGTCAGTAATATATTACGACTG
D20317	GgaattcACCATGGCTCATCTGTTAAGCGCAG	aagcttTGTCAGTAATATATTACGACTG
D620	cccgggGCTCATCTGTTAAGCGCAG	cccgggTAAAGTGACTGGCATGGTGAA
D1120	cccgggGCTCATCTGTTAAGCGCAG	cccgggATGATTACCGAACTTAAAGT
D1620	cccgggGCTCATCTGTTAAGCGCAG	cccgggAAGCGTATAATTTTGATGATTA
D610	cccgggCAAAATTATACGCTTAATGAAAGT	cccgggTAAAGTGACTGGCATGGTGAA
D200	GgaattcACCATGCCAGTCACCTTTAAGCTTCG	GaagcttAGTGAGTCCTGCCATTCGAC
D201220	tctagaAGTGAGTCCTGCCATTCGAC	tctagaAACTTTGAGCTACGACTCAG
D268302	cccggg TTGCACATTGGTAAAGATGGGTG	cccggg ATTTGTTGGTTTAAACTCTGAAAA
D305	GgaattcACCATGCCAGTCACCTTTAAGTTTCG	aagcttAATGTGCAAAGAACGTTTCATGGG
GC	tctagaAAAACACCTCGTTTACTGCAATA	aagcctTTAATTCTGGTCAGCGAATGCATT

*Lower-case type indicates an engineered restriction enzyme site.

precipitated with 10%, v/v, TCA and acetone (Jiang *et al.*, 2004a). Whole-cell and precipitated culture supernatant samples were electrophoresed on 12% SDS-PAGE gels and analysed by Western blotting.

Protein–protein interaction analysis. Bacterial cultures containing pGWC (GST–InvC) were grown in LB medium at 37 °C to OD₆₀₀ ~0.4, supplemented with 0.1 mM IPTG and incubated at 37 °C for an additional 4 h. Bacteria were harvested by centrifugation (3600 r.p.m., 10 min, 4 °C). The bacterial pellet was resuspended in lysis buffer [PBS, 1% Triton X-100, 10 mM DTT, 2 mM EDTA, one tablet of Protease Inhibitor Cocktail (Roche)], and the cells were lysed by sonication. Cell debris was removed by centrifugation. The supernatant was passed through a 0.45 µm pore-size filter.

Glutathione–Agarose gel lyophilized powder (Sigma) was swollen in PBS for 30 min at room temperature. After swelling, the agarose beads were placed into a SigmaPrep spin column (Sigma). The bacterial cleared lysate from strains overexpressing GST–InvC was bound with swelling resin. Glutathione agarose beads coated with GST–InvC were mixed with cleared extracts of *E. coli* BL21 strains harbouring pJWDF (SopD–strep-tag), pJWD21317 (SopD_{Δ1-20}–strep-tag), pJWD200 (SopD_{Δ201-317}–strep-tag), pJWD201220 (SopD_{Δ201-220}–strep-tag) or pJWD268302 (SopD_{Δ268-302}–strep-tag) and incubated at 4 °C for 12 h. The columns were washed three times with PBS, 0.1% Tween 20 (PBS-T). Elution was performed with 10 mM glutathione. Eluted proteins were analysed by SDS-PAGE and immunoblotting.

Western blotting analysis. After SDS-PAGE, proteins were transferred onto nitrocellulose transfer membranes using a Mini Trans-Blot cell (Bio-Rad). The transferred membrane was incubated with a mouse strep-tag antibody (IBA) overnight at 4 °C. After washing three times with PBS-T, the membrane was incubated with a goat anti-mouse horseradish peroxidase-labelled secondary antibody (Sigma) at room temperature for 1 h. The membrane was washed three times with PBS-T, and detection was carried out using the EZ-ECL Chemiluminescence Detection kit (Geneflow) according to the manufacturer's instructions. The chemiluminescent signal was detected using Kodak BioMax light film (Sigma-Aldrich). When needed, the blotting membranes were also probed with a custom-made rabbit polyclonal anti-LuxS antibody followed by a goat anti-rabbit horseradish peroxidase-labelled secondary antibody (Sigma) to account for bacterial lysis or non-specific leakage.

RESULTS

The N-terminal region is essential for T3S of SopD

To investigate the role of the N-terminal region of SopD in T3S, we constructed full-length or truncated versions of SopD fused with a C-terminal strep-tag under the control of the inducible arabinose promoter (P_{BAD}) (Karavolos *et al.*, 2005). Following induction with arabinose, secreted proteins were analysed by SDS-PAGE and Western blotting using a specific strep-tag antibody. Secretion of full-length and truncated SopD was investigated in the parent (SL1344), a T3S-defective (*ΔprgH*) strain and a flagellar secretion-defective (*fliGHI*) strain to confirm secretion specificity through the respective T3S machineries. The *fliGHI* deletion was used to minimize possible regulatory effects on SPI-1 T3SS expression reported to occur upon disruption of *fliHDC* (Eichelberg & Galán, 2000).

Full-length SopD was secreted into the supernatant of the SL1344 parent and *fliGHI* mutant strains, but was not detected in the secreted protein fraction of the isogenic *ΔprgH*, indicating SPI-1 T3SS-dependent secretion of the SopD strep-tag hybrid protein (Fig. 1a). Several studies have concluded that the first ~20 amino acid residues of the T3S effector proteins contribute signals for their routing into the secretory pathway. A truncated version of SopD lacking the N-terminal 20 aa (SopD_{Δ1-20}, pJWD21317) was not detected in the supernatant of the parent strain (Fig. 1b). To further delineate the N-terminal signal of SopD, a series of SopD amino acid truncations in residues 6–10, 11–20, 16–20 and 6–20 were constructed to create SopD_{Δ6-10}, SopD_{Δ11-20}, SopD_{Δ16-20} and SopD_{Δ6-20}, respectively (Table 1). SopD_{Δ11-20} and SopD_{Δ16-20} were successfully secreted and detected in parent strain culture supernatant in an SPI-1 T3S-dependent manner (Fig. 1c, d). Strikingly, the truncated version of SopD lacking amino acids 6–10 (SopD_{Δ6-10}) failed to be secreted into the culture supernatant (Fig. 1e).

Secretion of a C-terminal truncation of SopD is SPI-1 T3SS-independent

Although several effectors contain a CBD within the first ~140 amino acids, a CBD for SopD has not yet been identified. We investigated the ability of the N-terminal 200 amino acids of SopD to direct T3S in the parent (SL1344), a T3S-defective (*ΔprgH*) strain and a flagellar secretion-defective (*fliGHI::Kan^R*) strain. SopD_{Δ201-317} (pJWD200) was detected in culture supernatants from the parent and surprisingly the *ΔprgH* strain but was absent from supernatants of the flagellar secretion-defective strain (*fliGHI::Kan^R*) (Fig. 2a). This observation suggests that secretion of SopD_{Δ201-317} is an SPI-1 T3SS-independent event mediated via erroneous secretion through the flagellar apparatus (Fig. 2a). Furthermore, SopD_{Δ201-317} secretion in a panel of *Salmonella* strains lacking SPI-1 T3SS function (*invC*, *invG*, *invA* and *spi-1*) was similar to that of the parent SL1344 (Fig. 2b). To rule out cell leakage, we also probed for the intracellular enzyme LuxS, which was undetectable in the same supernatant fractions (Fig. 2b).

The C-terminal α-helical regions are important for SPI-1 T3S of SopD

Earlier studies have demonstrated that α-helical secondary structures are associated with protein–protein interactions, including those involved in T3S (Delahay & Frankel, 2002). A prediction of the secondary structure of the SopD C terminus (amino acids 200–317) using ANTHEPROT (Deleage *et al.*, 2001; Gibrat *et al.*, 1987) revealed that it contains two putative long α-helical regions situated between residues 200–218 (helix I) and 268–302 (helix II) (Fig. 3a). Indeed, the C terminus of the effector protein SipB includes an amphipathic α-helix, which is required for secretion through the SPI-1 T3SS (Kim *et al.*, 2007).

Fig. 1. Identification of the N-terminal signal for T3S of SopD. (a) Expression and secretion of full-length SopD (SopD₁₋₃₁₇) was assessed in SL1344 wild-type, SL1344Δ*prgH* and SL1344*fltGH::Kan^R*. Full-length SopD secretion is SPI-1-mediated, since there was no secretion in SL1344Δ*prgH*. (b) Assessment of the role of regional N-terminal truncations of SopD (SopD_{Δ1-20}, SopD_{Δ6-20}, SopD_{Δ11-20} and SopD_{Δ16-20}) on its secretion in SL1344 wild-type. (c) Secretion of SopD_{Δ11-20} is SPI-1-mediated, since there was no secretion in SL1344Δ*prgH*. (d) Secretion of SopD_{Δ16-20} is SPI-1-mediated, since there was no secretion in SL1344Δ*prgH*. (e) Assessment of the expression and secretion of SopD_{Δ6-10} in SL1344 wild-type indicates that amino acids 6–10 are essential for SPI-1 T3S of SopD. Experiments were repeated at least three times and a representative is shown.

Fig. 2. Role of the C-terminal domain of SopD in T3S. (a) Evaluation of expression and secretion of a C-terminally truncated version of SopD (SopD_{Δ201-317}) in SL1344 wild-type, SL1344Δ*prgH* and SL1344*fltGH::Kan^R* shows diminished secretion in SL1344*fltGH::Kan^R* lacking a functional flagellar secretion apparatus. (b) Expression and secretion of a truncated version of SopD lacking its C-terminal domain (SopD_{Δ201-317}) was unaffected in various SPI-1 secretion-deficient strains (*invC::Kan^R*, *invG::TnpA*, *invA::Kan^R* and *spi-1::Kan^R*). The control protein LuxS was not secreted in culture supernatants. Experiments were repeated at least three times and a representative is shown.

Initially we constructed a C-terminal 12 amino acid deletion of SopD, leaving intact the two putative helical domains (Fig. 3a, SopD $_{\Delta 306-317}$). Secretion of SopD $_{\Delta 306-317}$ into the culture supernatant was similar to that of full-length SopD, demonstrating that the last 12 residues on the C-terminal end of SopD do not affect its secretion through SPI-1 T3S (Fig. 3b).

We hypothesized that the putative helical structural motifs in the C-terminal regions of SopD physically associate with the secretion machinery during T3S. To investigate the importance of the putative helical regions we designed SopD versions lacking helix I (residues 200–220), helix II

(residues 268–302) or both helices (Fig. 3a). Secretion was assessed in the parent (SL1344), a T3S-defective ($\Delta prgH$) strain and a flagellar secretion-defective (*fliGHI::Kan^R*) strain. Deletion of helix I (SopD $_{\Delta 201-220}$) led to no secretion (Fig. 3c). However, deletion of helix II (SopD $_{\Delta 268-302}$) led to marginal secretion in the flagellar secretion-defective strain (*fliGHI::Kan^R*) (Fig. 3d).

SopD interacts with InvC during SPI-1 T3S

InvC is a class AAA ATPase, forming a hexameric ring on the inner membrane, and is the SPI-1 T3SS energizer. Class

Fig. 3. Identification of C-terminal signals directing T3S of SopD. (a) Schematic diagram of SopD constructs used in this study. Numbers indicate positions of amino acids deleted. Broken lines indicate deleted regions representing helices I and II. Black boxes represent strep-tag-coding regions. Full-length SopD is 317 aa. Secretion via SPI-1 or flagellar T3S is indicated by a plus (secretion) or a minus (no secretion). (b) Deletion of the last C-terminal 12 aa of SopD (SopD $_{\Delta 306-317}$) has no effect on its expression and secretion in SL1344 wild-type and SL1344*fliGHI::Kan^R* but leads to diminished secretion in SL1344 $\Delta prgH$, indicating SPI-1-dependent T3S of SopD $_{\Delta 306-317}$. (c) Expression of SopD lacking helix I (SopD $_{\Delta 201-220}$) in SL1344 wild-type, SL1344 $\Delta prgH$ and SL1344*fliGHI::Kan^R* shows no secretion. (d) Expression of SopD lacking helix II (SopD $_{\Delta 268-302}$) in SL1344 wild-type, SL1344 $\Delta prgH$ and SL1344*fliGHI::Kan^R* shows slight secretion in the strain lacking flagellar apparatus. Experiments were repeated at least three times and a representative is shown.

AAA ATPases utilize the energy released from ATP to unfold proteins and pass them through a channel at the centre of their ring (Sauer *et al.*, 2004). Akeda & Galán (2005) demonstrated that InvC binds the chaperone–substrate complex. Although InvC binds to SPI-1 T3S substrates indirectly, a recent study has shown that the N terminus of MxiC, a *Shigella flexneri* T3SS substrate, interacts with the ATPase Spa47 (Botteaux *et al.*, 2009). Direct binding between a T3S ATPase and its substrate can therefore be an alternative mechanism for T3S of effectors.

We reasoned that SopD T3S may also involve direct contact with the SPI-1 T3SS ATPase, InvC. To verify our hypothesis, we generated a GST–InvC fusion protein (pGWC, Table 1). Cytosolic extracts from an *E. coli* BL21 strain overexpressing full-length SopD with a C-terminal strep-tag (pJWDF) were tested for the ability to bind GST-immobilized InvC. Elutions from GST–InvC-containing columns contained full-length SopD, while elutions from GST-only columns contained no detectable SopD, indicating a possible interaction between SopD and the SPI-1 T3SS ATPase, InvC (Fig. 4a).

To further scrutinize the interaction with InvC, cytosolic extracts from an *E. coli* BL21 strain overexpressing strep-tagged SopD $_{\Delta 1-20}$ or SopD $_{\Delta 201-317}$ were tested for the ability to bind GST-immobilized InvC. Only SopD $_{\Delta 1-20}$ was present in elutions from GST–InvC-containing columns. The inability of SopD $_{\Delta 201-317}$ to bind the column suggests that the C-terminal amino acids 201–317 of SopD are essential for the interaction with InvC (Fig. 4b).

In view of the role of the C-terminal region of SopD in interacting with InvC, we proceeded to determine the role of the two C-terminal helices of SopD. Cytosolic extracts from an *E. coli* BL21 strain overexpressing helix I-deleted strep-tagged SopD (SopD $_{\Delta 200-220}$) or helix II-deleted strep-tagged SopD (SopD $_{\Delta 268-302}$) were tested for the ability to bind GST-immobilized InvC. Elutions from GST–InvC-containing columns indicated the presence of SopD $_{\Delta \text{helix I}}$ but not SopD $_{\Delta \text{helix II}}$, suggesting that the helix II region (residues 268–302) of SopD is essential for the interaction with InvC (Fig. 4c).

DISCUSSION

The signals that target bacterial effector proteins into host cells through T3SS injectisomes remain poorly understood. Earlier investigations have identified T3S-related domains in several effectors (Mota *et al.*, 2005; Sory *et al.*, 1995; Tree *et al.*, 2009; Wang *et al.*, 2008). These include the first ~20 amino acids and also an additional region located within the first ~140 amino acid which binds the specific cognate chaperone (Ghosh, 2004). For example, Russmann *et al.* (2002) found that amino acid residues 4–7 of the SPI-1 T3SS substrate InvJ mediate its secretion in a T3SS-dependent manner. In addition, amino acid residues 3–8 of SipB also function as an NSS, routing this effector for secretion through the T3S pathway (Kim *et al.*, 2007).

Fig. 4. Helix II is important for SopD interaction with InvC. (a) Eluted fractions from GST- and GST–InvC-coated beads after incubation with the cleared lysate of a strain expressing full-length SopD–strep-tag were analysed by SDS-PAGE and immunoblotting as described in Methods. (b) The C-terminal part of SopD is important for its interaction with InvC. Eluted fractions from GST- and GST–InvC-coated beads after incubation with the cleared lysate of strains expressing strep-tagged SopD, SopD $_{\Delta 201-317}$ or SopD $_{\Delta 1-20}$ were analysed by SDS-PAGE and immunoblotting as described in Methods. Respective protein bands are highlighted by arrows. Experiments were repeated at least three times and a representative is shown. (c) Helix II of SopD is essential for its interaction with InvC. Eluted fractions from GST–InvC-coated beads after incubation with the cleared lysate of strains expressing tagged versions of SopD lacking helix I (SopD $_{\Delta 201-220}$; SopD $_{\Delta \text{helix I}}$) or helix II (SopD $_{\Delta 268-302}$; SopD $_{\Delta \text{helix II}}$) were analysed by SDS-PAGE and immunoblotting as described in Methods. Experiments were repeated at least three times and a representative is shown.

We examined the importance of the N-terminal region of the *Salmonella* effector protein SopD in its secretion through the T3S machinery using a set of deletions. We show that SopD requires an NSS that encompasses N-terminal residues 6–10 for successful secretion through the SPI-1 T3SS (Fig. 1e). The reduced cytoplasmic levels of SopD constructs missing the N-terminal amino acids 6–10 and 6–20 may reflect their reduced stability in the cytoplasm after arabinose induction. In addition, we observed reduced secretion of SopD_{Δ11–20}, which may be attributable to reduced secretion efficiency. Our data also point out an additional control level in the targeting of SopD towards the correct secretion apparatus. Indeed, the SopD NSS in combination with a C-terminal domain (residues 200–317) is needed to prevent secretion of SopD through the flagellar T3SS (Fig. 2a). Erroneous secretion through the flagellar T3S has also been observed in SptP and SopE upon removal of the CBD (Lee & Galán, 2004).

To facilitate SPI-1 T3SS secretion, the effector protein SptP in complex with its chaperone SicP is recognized by the membrane-associated protein InvC to facilitate secretion of the effector and simultaneous dissociation of the chaperone (Akeda & Galán, 2005). To date, the cognate chaperone and CBD of SopD have not been identified. Previous data from size-exclusion chromatography indicate that SopD forms a monomer and hence is unlikely to have a specific chaperone (Wood *et al.*, 2004). Using GST-immobilized SopD, we have been unable to detect a specific interaction with proteins from *Salmonella* cytoplasmic extracts under the conditions used (data not shown).

We have also highlighted the importance of the C-terminal region of SopD and particularly the role of the two putative helices (helix I and helix II). In contrast, an earlier study has shown that the first 202 amino acids of SopD fused to the N-terminal domain of adenylate cyclase, *cya*, are sufficient for secretion in *Salmonella* Dublin (Jones *et al.*, 1998). We speculate that the differences observed may be due to the size or nature of the reporter fusion partner (8 amino acid strep-tag versus the bulky, 43 kDa Cya N-terminal region). Also, the resulting hybrid SopD–Cya protein may encode or mimic signals that result in secretion through the SPI-1 or other systems.

Here we show that both helices are required for SPI-1 T3SS secretion of SopD (Fig. 3a). This is independent of the presence of the final C-terminal 12 aa of SopD (Fig. 3b). Deletion of either helix leads to loss of secretion via the SPI-1 T3SS (Fig. 3a, c, d). However, the observation that the deletion of helix II leads to marginal secretion in the flagellar mutant suggests the existence of additional cryptic signals directing secretion of SopD via an alternative system. Remarkably, the absence of only one helix is enough to prevent flagellar (and SPI-1) secretion if the other helix is present (Fig. 3a).

Finally, we demonstrate that SopD associates with the T3S ATPase InvC (Fig. 4a). Remarkably, the interaction involves the C-terminal region of SopD, which comprises

two α -helical domains (amino acids 201–220 and 268–302; Figs 3a and 4b). In particular, helix II (amino acids 268–302) is essential for binding to the InvC ATPase (Fig. 4c). In contrast to the C-terminal region interaction in *Salmonella*, in *Yersinia*, the InvC ATPase homologue YscN interacts with the N terminus of the effector YopR (Sorg *et al.*, 2006). This may reflect differences in size, domain organization or timing of secretion of the two effectors.

It has been suggested that due to the maximum diameter of the central channel of the T3SS needle apparatus (estimated to be ~28 Å) it would be necessary to unfold translocating proteins prior to release through the needle (Marlovits *et al.*, 2004). Interaction of InvC with SopD could mediate the unfolding of SopD, leading to ejection through the needle using proton motive force at the expense of ATP (Galán, 2008).

In summary, we have identified important N- and C-terminal regions of the effector protein SopD required for its secretion through SPI-1 T3S. We show that an N-terminal signal consisting of amino acids 6–10 is needed to target SopD to the SPI-1 T3S apparatus. We also reveal the role of two putative helical domains in the C-terminal region of SopD (amino acids 201–302) in preventing erroneous secretion through flagellar T3S. In particular, helix II (amino acids 268–302) is necessary for the interaction with the InvC ATPase and contributes to secretion through the T3S injectisome. The elucidation of signalling motifs leading to the SPI-1 T3SS-dependent secretion of substrates reveals new insights in our understanding of effector protein secretion.

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