The SPI2-encoded SseA chaperone has discrete domains required for SseB stabilization and export, and binds within the C-terminus of SseB and SseD

Daniel V. Zurawski and Murry A. Stein

SseA, a key Salmonella virulence determinant, is a small, basic pl protein encoded within the Salmonella pathogenicity island 2 and serves as a type III secretion system chaperone for SseB and SseD. Both SseA partners are subunits of the surface-localized translocon module that delivers effectors into the host cell; SseB is predicted to compose the translocon sheath and SseD is a putative translocon pore subunit. In this study, SseA molecular interactions with its partners were characterized further. Yeast two-hybrid screens indicate that SseA binding requires a C-terminal domain within both partners. An additional central domain within SseD was found to influence binding. The SseA-binding region within SseB was found to encompass a predicted amphipathic helix of a type participating in coiled-coil interactions that are implicated in the assembly of translocon sheaths. Deletions that impinge upon this putative coiled-coil domain prevent SseA binding, suggesting that SseA occupies a portion of the coiled-coil. SseA occupancy of this motif is envisioned to be sufficient to prevent premature SseB self-association inside bacteria. Domain mapping on the chaperone was also performed. A deletion of the SseA N-terminus, or site-directed mutations within this region, allowed stabilization of SseB, but its export was disrupted. Therefore, the N-terminus of SseA provides a function that is essential for SseB export, but dispensable for partner binding and stabilization.

INTRODUCTION

Type III secretion (TTS) systems are utilized by a range of Gram-negative pathogens to deliver a select set of toxic proteins, termed effectors, into host cells, where they disrupt cellular function (reviewed by Cornelis & Van Gijsen, 2000). They are related to flagellar secretion systems and most likely originated from this system. Virulence-associated TTS systems are composed of distinct functional modules, each assembled from multiple subunits. The modules extending from the bacterial cytosol are: an envelope-spanning secretion apparatus, a surface-exposed needle-like structure, and a translocon that extends from the needle and breaches the host membrane (Cornelis, 2002; Holden, 2002). The translocon is essential for directing effectors to their intracellular targets. All translocons include two proteins that form a multimeric pore within the host membrane (Cornelis & Van Gijsen, 2000). Some specialized translocons contain an additional major subunit that forms a translocon sheath.

Salmonella pathogenicity island 2 (SPI2) encodes a virulence-associated TTS system that translocates an array of effectors required for intracellular survival and systemic disease (reviewed by Holden, 2002; Knodler & Steele-Mortimer, 2003). SPI2 is a member of the Esc phylogenetic group of TTS systems which contain a third, prominent, translocon component that forms a translocon sheath connecting the needle with the translocon pore (Foulquier et al., 2002; Cornelis, 2002). The best-characterized Esc TTS translocon is encoded in the locus for enterocyte effacement (LEE) of enteropathogenic E. coli (EPEC) (Elliot et al., 1998).

The SPI2 translocon consists of SseB, SseC and SseD, based on inferred sequence similarity, comparable subcellular localization, and the same requirement for effector translocation as the LEE translocon subunits (Hensel et al., 1998; Beuzón et al., 1999; Nikolaus et al., 2001; Klein & Jones, 2001). SseC and SseD are envisioned to form a multimeric translocon pore in the vacuolar membrane. SseB shares similarity with the translocon sheath subunit of LEE (EspA),

Abbreviations: EPEC, enteropathogenic E. coli; GST, glutathione S-transferase; LEE, locus for enterocyte effacement; MCS, multiple cloning site; SPI2, Salmonella pathogenicity island 2; TTS, type III secretion.
which self-assembles into a hollow, rod-like structure that connects the translocon pore with the needle (Sekiya et al., 2001; Daniell et al., 2001). Thus, SseB is predicted to also self-assemble into a translocon sheath analogous to that of LEE (Holden, 2002), but efforts to visualize this structure are ongoing. Several findings have been reported that are consistent with SseB functioning as the SPI2 sheath: SseB self-association after export to the cell surface was observed (Nikolaus et al., 2001); SseB is required to anchor SscD to the bacterial surface, indicating that the pore proteins associate with SseB (Nikolaus et al., 2001); and a coiled-coil motif involved in EspA polymerization into a translocon sheath (Delahay et al., 1999) may also be present within SseB (Delahay & Frankel, 2002).

The export of translocon subunits like SseBCD is commonly post-translationally controlled, and synthesized translocon subunits remain within the bacteria until an export signal is received (Beuzon et al., 1998). TTS chaperones are often required for translocon pore protein stability, prior to export, and for export to the bacterial surface to occur (reviewed by Bennett & Hughes, 2000; Parsot et al., 2003). TTS chaperones are energy-independent and transiently associate with one or several export targets. Most TTS chaperones are small, have an acidic pI and contain an amphipathic C-terminal helix. Characterized chaperones are currently discriminated into three groups based on whether their partner is an effector (group I), a pair of translocon pore proteins (group II) or a flagellar component (group III) (Parsot et al., 2003).

Recently, it has been demonstrated that SseA is a chaperone for SseB and SseD, but not SseC. SseA was shown to be required for SseBD stability and for SseBD export to the bacterial surface when low pH was encountered (Zurawski & Stein, 2003; Ruiz-Albert et al., 2003). SseA was also demonstrated to bind SseB by co-precipitation (Zurawski & Stein, 2003; Ruiz-Albert et al., 2003) and SseD by yeast two-hybrid analysis (Ruiz-Albert et al., 2003). Currently, the relationship of SseA to other characterized virulence chaperones is unclear. SseA is a chaperone for a putative translocon sheath protein and a single, putative translocon pore protein. This constitutes a unique set of binding partners when compared to the other classified chaperones (Parsot et al., 2003). Furthermore, a range of SseA physical features, such as its basic pI and partial membrane association, are also atypical of virulence chaperone proteins (Zurawski & Stein, 2003). To gain insight into the relationship of SseA to other chaperones, we addressed how SseA interacts with its partners.

In the present study, the regions required for SseA binding to SseB and SseD were identified. In the case of SseB, the location of this binding site allows a relationship with flagellar chaperones and a mechanism for an antiopolymerization role to be postulated. We also determined that the N-terminal portion of SseA is dispensable for binding and stabilizing SseB, but is essential for SseB export to the surface of the bacterium.

**METHODS**

**Genetic constructions.** Bacterial strains used in this study that were reported previously are listed in Table 1. The majority of strains constructed for the present study were derived from PCR products obtained with the primer pairs listed in Table 2. The PCR products were first cloned to the initial plasmid listed in Table 2. Other constructs were made by subcloning the insert from the initial plasmid to generate those constructions listed as subclones in Table 2. In all cases, the final plasmid constructs were sequenced. The molecular reagents, host strains, bacterial media and growth conditions were described previously (Zurawski & Stein, 2003). The parent plasmids are listed in Table 2. Briefly, pEG202 and pG4S vectors were used to generate the LexA and B42 fusion proteins, pGEX6P-1 for the glutathione S-transferase (GST) fusion proteins and pBAD30 for the arabinose-inducible constructs. Antibiotics for selection were 25 mg chloramphenicol (Cam) ml⁻¹, 100 mg ampicillin (Amp) ml⁻¹ and 25 μg kanamycin (Kan) ml⁻¹. A brief outline of the specific constructions generated follows.

The SseB constructions were made as follows. The sseB1–196 allele was subcloned from pCR2.1::sseB1–196 (Zurawski & Stein, 2003) to pEG202 using the EcoRI site within the pCR2.1 multiple cloning site (MCS) and the incorporated Xhol site. The SseB N-terminal or C-terminal truncation constructs were generated with the sseBα primer pairs listed in Table 2. The PCR product was subcloned from pCR2.1 to pEG202 using the EcoRI site within the pCR2.1 MCS and the incorporated Xhol site. SseB–GST-fusion constructs were generated by subcloning the PCR product from pCR2.1 using the primer-incorporated BamHI and Xhol into the same sites in pGEX6P-1.

The SseA constructions were made as follows. The sseA1–108 PCR product was cloned to pCR2.1 and subcloned to pGEX6P-1 using primer-incorporated BamHI and Xhol sites or to pG4S using the EcoRI site within the pCR2.1 MCS and the incorporated Xhol site. The SseA truncation constructs were made by subcloning the PCR product generated with the sseAα primer pairs from pCR2.1 into pG4S or pGEX6P-1 using the primer-incorporated BamHI and Xhol sites. For construction of the inducible complementation allele, sseAα1–31, the upstream primer contained an ATG start and a 5’ sequence that added an EcoRI site, the ribosome-binding site (rbs), an ATG start and a BgII site. The downstream primer included a SalI site. This product (rbs::sseAα1–31) was subcloned into the pBAD30 EcoRI and SalI sites to generate pBAD30rbs::sseAα1–31, also referred to as pSAα1–31. Site-directed point mutations within the N-terminus of SseA were constructed via inverse PCR with pSA (Zurawski & Stein, 2003). The sseAQ17D,Q31D primer pairs (Table 2) were utilized and the inverse PCR was performed as described for the SseD internal deletions that follow.

The SseD constructions were made as follows. The sseD1–195 PCR products were directly cloned to pGEX6P-1 and pEG202 using the primer-incorporated EcoRI and Xhol sites. The SseD internal deletions were generated via inverse PCR of pCR2.1::sseD1–195 using a different sseDα primer listed in Table 2. Inverse PCR was performed essentially as reported before (Suvarnampunya et al., 2003) with the following modifications. The thermocycled PCR mix was cleaned with QiaPrep spin column (Qiagen) and the eluate treated with T7 polynucleotide kinase to add terminal phosphates, followed by ligation with T4 DNA ligase. A portion of the ligated inverse PCR mix was used for PCR with the sseD1–195 primer set. The PCR products containing the internal deletions were smaller and were agarose-gel purified. The purified truncation products were cloned to pCR2.1 and subcloned to pEG202 using the primer-incorporated EcoRI and Xhol sites.

The SPI2 effector constructs were generated as follows. The spiC1–133 PCR product was directly cloned to pGEX6P-1 using the incorporated BamHI and Xhol sites. The sifA1–33r PCR product was cloned to pGEX6P-1 using the incorporated EcoRI and Xhol sites.
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. typhimurium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL1344</td>
<td>hisG46</td>
<td>Parental S. typhimurium strain</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>ΔsseA (ST282)</td>
<td>ΔsseA</td>
<td>SseB/SseD stability and export abrogated</td>
<td>Zurawski &amp; Stein (2003)</td>
</tr>
<tr>
<td>ΔsseA/pSA (ST286)</td>
<td>ΔsseA/pBAD30 :: sseA1–108</td>
<td>Ara-inducible, full-length SseA; SseB export restored</td>
<td>Zurawski &amp; Stein (2003)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Top10</td>
<td>F− mcrA Δ(mrr–hsdRMS–mcrBC) Δ80lacZAM15 ΔlacX74 recA1 deoR araD139 Δ(ara–leu)7697 galU galK rpsL(Strβ) endA1 mupG</td>
<td>Host for PCR cloned products in pCR2.1</td>
<td></td>
</tr>
<tr>
<td>DH5x</td>
<td>endA1 hsdR17 (rK mK) supE44 thi-1 recA1 gyrA1 (NalR) relA1 ΔlacIYA–argF U169 deoR [Δ80 lacΔ(lacZ)M15]</td>
<td>Host for routine genetic constructions</td>
<td>Lab. strain collection</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>F− ompT hsdS8 (rK mK) gal dcm (DE3) pLysS</td>
<td>Cam'; host for protein production</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGY48 p1840</td>
<td>MATα ura3 his3 trp1 integrated lexAop–LEU2 integrated lexAop–lacZ</td>
<td>Host for pJG4-5 constructs</td>
<td>Richman et al. (1999)</td>
</tr>
<tr>
<td>W303-1A</td>
<td>MATα his3-11,5 leu2-3,112 trp1-1 ade2-101 ura3-1 can1-100</td>
<td>Host for pEG202 constructs</td>
<td>Richman et al. (1999)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>Selection/purpose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR2.1</td>
<td>Kan' Amp'/Parental Topo–TA vector</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pGEX6P-1</td>
<td>Amp'/Parental GST fusion vector</td>
<td></td>
<td>Amersham</td>
</tr>
<tr>
<td>pBAD30</td>
<td>Amp'/Parental arabinose inducible vector</td>
<td></td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pJG4-5</td>
<td>Amp'/Parental B42 ‘acid blob’ fusion vector</td>
<td></td>
<td>Gyuris et al. (1993)</td>
</tr>
<tr>
<td>pEG202</td>
<td>Amp'/Parental LexA fusion vector</td>
<td></td>
<td>Richman et al. (1999)</td>
</tr>
<tr>
<td>pEG202 (Cla4)</td>
<td>Amp'/B42–Cla4 fusion vector; positive control</td>
<td></td>
<td>Richman et al. (1999)</td>
</tr>
<tr>
<td>pEG202 (cde42G122G122,G188S)</td>
<td>Amp'/LexA–cde42G122G122,G188S fusion vector; positive control</td>
<td></td>
<td>Richman et al. (1999)</td>
</tr>
</tbody>
</table>

Yeast methodologies and two-hybrid screening. Yeast strains listed in Table 1 were routinely grown at 30 °C in Yeast Extract-Peptone-Glucose medium. Standard methodologies were used for yeast transformations (Adams et al., 1997) and yeast matings (Kolonin et al., 2000). pJG4-5 constructs were transformed into the EGY48 p1840 strain while pEG202 constructs were transformed into the W303-1A strain. Transformants were selected on synthetic complete (SC) dropout medium supplemented with 2 % glucose and either a −Trp or −His amino acid supplement (Qbiogene). The matings to produce daughters with both bait and prey vectors were selected on SC with 2 % glucose and −Trp −His amino acid supplement (SC Trp−, His−). Positive control constructs, pEG202 (Cdc42G122G122,G188S) and pJG4-5 (Cla4) were detailed in a previous study (Richman et al., 1999). The negative controls were generated by mating LexA–SseB2–196 or LexA–SseD2–195 containing constructs with the pJG4-5 vector.

Evaluation of the stability of SseA, B or D fusion proteins within induced daughter cells was performed via an alkali lysis protocol (Yaffe & Schatz, 1984). Briefly, daughters were subcultured into SC −Trp, −His medium supplemented with 2 % raffinose. They were then subcultured into SC −Trp, −His medium supplemented with 2 % raffinose and 2 % galactose, and grown to ~0.8 OD600. Cells were lysed and total protein was precipitated with trichloroacetic acid (TCA) followed by an acetone wash. Pellets were resuspended in sample buffer volumes to render them comparable based on starting OD600 values and immunoblotted (see below).

Qualitative assessment of the two-hybrid interaction was attained by filter lift assays developed with X-Gal, as previously described (Davis et al., 1998). Filter lifts were qualitatively analysed after development for 6 h at 30 °C. Filter lifts that remained white were scored as negative while the darkest blue, represented by daughters with the strongly interacting pair of Cla4/Cdc42G122G122,G188S (Richman et al., 1999), were scored as four plus. Quantification of reporter activity was measured in liquid assays using the Y-PER β-galactosidase kit (Pierce). The strength of reporter activation was calculated as previously described (Miller, 1997).
<table>
<thead>
<tr>
<th>Primer set</th>
<th>Forward (5′–3′)*</th>
<th>Reverse (5′–3′)*</th>
<th>Initial plasmid†</th>
<th>Subclones</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SseD</strong></td>
<td>AAGGGgaattcATTGAGCTTAGTACGTACG</td>
<td>AGGAAAActcagTTACCTCGTTAATGCGTACG</td>
<td>pGEX6p-1 : : sseD</td>
<td>pEG202 : : sseD</td>
</tr>
<tr>
<td><strong>spiC</strong></td>
<td>AAGGGgaattcATTGAGCTTAGTACGTACG</td>
<td>AGGAAAActcagTTACCTCGTTAATGCGTACG</td>
<td>pGEX6p-1 : : sspC</td>
<td>None</td>
</tr>
<tr>
<td><strong>sifA</strong></td>
<td>GGGAAGgaattcATTGAGCTTAGTACGTACG</td>
<td>AGGAAAActcagTTACCTCGTTAATGCGTACG</td>
<td>pGEX6p-1 : : sifA</td>
<td>None</td>
</tr>
<tr>
<td><strong>sseA</strong></td>
<td>ggattcATTGAGCTTAGTACGTACG</td>
<td>CCCTTggatccTCTTCAGGAAACATCTTG</td>
<td>pGEX6p-1 : : sseA</td>
<td>None</td>
</tr>
<tr>
<td><strong>sseB</strong></td>
<td>ggattcATTGAGCTTAGTACGTACG</td>
<td>CCCTTggatccTCTTCAGGAAACATCTTG</td>
<td>pGEX6p-1 : : sseB</td>
<td>None</td>
</tr>
<tr>
<td><strong>sseC</strong></td>
<td>ggattcATTGAGCTTAGTACGTACG</td>
<td>CCCTTggatccTCTTCAGGAAACATCTTG</td>
<td>pGEX6p-1 : : sseC</td>
<td>None</td>
</tr>
<tr>
<td><strong>sseD</strong></td>
<td>ggattcATTGAGCTTAGTACGTACG</td>
<td>CCCTTggatccTCTTCAGGAAACATCTTG</td>
<td>pGEX6p-1 : : sseD</td>
<td>None</td>
</tr>
<tr>
<td><strong>sseE</strong></td>
<td>ggattcATTGAGCTTAGTACGTACG</td>
<td>CCCTTggatccTCTTCAGGAAACATCTTG</td>
<td>pGEX6p-1 : : sseE</td>
<td>None</td>
</tr>
<tr>
<td><strong>sseF</strong></td>
<td>ggattcATTGAGCTTAGTACGTACG</td>
<td>CCCTTggatccTCTTCAGGAAACATCTTG</td>
<td>pGEX6p-1 : : sseF</td>
<td>None</td>
</tr>
</tbody>
</table>

*Lower case indicates an engineered restriction enzyme site and bold an engineered stop codon.
†Designates the plasmid initially constructed with the PCR product generated. Additional constructs made by subcloning this insert are listed in the adjacent Subclones column.
precipiation assays. The expression of GST–rSseD 1–195, GST–
Immunodetection. Sample preparation, electrophoresis, transfer to nitrocellulose and detection were performed as detailed before (Zurawski & Stein, 2003). Proteins were separated by SDS-PAGE using 10 % or 16.5 % Tris-Tricine or 12 % Tris-glycine gels. Total protein in polyacrylamide gels was stained with BioSafe Coomassie blue (Bio-Rad). Immunodetection of SseA or SseA-fusion proteins was done with anti-SseA (murine monoclonal antibody #64) and immunodetection of SseB utilized anti-SseB murine polyclonal antibody (Zurawski & Stein, 2003). LexA fusion proteins were detected with a 1 : 3000 dilution of anti-LexA rabbit polyclonal (Invitrogen) and 1 : 1000 dilution of anti-rabbit alkaline phosphatase (Bio-Rad).

Recombinant protein expression, purification and co-

For the co-precipitation assays using truncated GST–SseB proteins, a fraction of the E. coli lysate was examined on Coomassie-stained SDS-PAGE gels. The lysates were adjusted to contain comparable amounts of recombinant protein and then incubated with the Salmonella fraction as before (Zurawski & Stein, 2003). The resulting co-precipitate was examined in Coomassie-stained SDS-PAGE gels to verify that similar amounts of recombinant were, in fact, present (e.g. Fig. 2c).

In co-precipitation experiments using Triton X-100, the recombinant proteins were added to the cleared lysate of ΔsseB. The lysates were incubated and precipitated as before. Identically processed samples were washed three times with wash buffer (Zurawski & Stein, 2003) or with wash buffer containing 1 % Triton X-100. The amount of SseA present in lysates before and after precipitation was estimated by refractive densitometry of immunoblots detailed previously (Zurawski & Stein, 2003) with a Fluor-S Multi-imager (Bio-Rad) and the Quantity One 4.4.0 software package.

Cell culture and Sif analysis. HeLa cells were grown in Earle’s minimal essential medium (ATCC 30-2003) containing 2 % glutamine and supplemented with 10 % fetal calf serum. HeLa cells were invaded using a standard gentamicin protection assay (Guy et al., 2000). Six hours after invasion, cells were fixed in 2.5 % formaldehyde in PBS. Salmonella were labelled with anti-CSA-1-FITC (Kirkegaard and Perry Labs), and Sifs were detected using anti-

Alignment and structural comparison. The alignment was generated by the BLAST algorithm (PAM 250 matrix) and depicted using Seq vu 5.0. The coiled-coil domains within SseA, SseB and EspA were identified using COILS (Lupas et al., 1991) and MultiCoils (Wolf et al., 1997) in window 21 and window 28 with weighting for positions ‘a’ and ‘d’ (both programs are available at www.expasy.ch). The SseB amphipathic helix was modelled using the HelixWheel Program (available at http://us.expasy.org/tools/).

RESULTS

SseA differentially binds to the SseB and SseD SPI2 translocon components

The binding of endogenous SseA to its partners is summarized in Fig. 1. SseA is co-precipitated when GST fused to SseB amino acids 2–196 (SseB2–196) is precipitated with glutathione Sepharose 4B. SseA is also precipitated by GST–SseD1–195 (Fig. 1). Using refractive densitometry we estimate that 30 % of the total SseA present in the lysate is precipitated by full-length SseB2–196 and SseD1–195 (data not shown). However, no binding to SseA was detected with representative SPI2 effector fusion proteins, GST–SifA1–336 or GST–SpiC1–133. This demonstrates the selective binding of SseA for the SseB and SseD SPI2 translocon components. In the course of optimizing the pull-down

![Fig. 1. SseA selectively co-precipitates SseB and SseD, and this association is differentially affected by Triton X-100. The S. typhimurium cytoplasmic/periplasmic fraction was mixed with whole-cell lysate from E. coli BL21(DE3)/pLysS expressing GST–
SseB2–196, GST–SifA1–336, GST–SpiC1–133 or GST–SseD1–195, as indicated above the lanes. Two samples were processed in parallel for each fusion protein and they differed only in the presence or absence of 1 % Triton X-100 in the glutathione Sepharose 4B precipitate wash buffer. Precipitates were analysed on a 10 % Tris-Tricine gel stained with Coomassie blue (Coomassie) or blotted with anti-SseA (monoclonal antibody #64). The location and apparent molecular mass of electrophoretic standards are indicated.](http://mic.sgmjournals.org)
assay, a condition was identified that allowed discrimination between SseA binding to SseB and to SseD. SseA binding to SseB is unaffected by inclusion of the non-ionic detergent Triton X-100 in the precipitate wash buffer (Fig. 1). In contrast, binding to SseD was abrogated in the presence of detergent.

A C-terminal SseB and SseD domain is required for SseA binding

For the majority of effector, translocon pore and flagellar chaperones, a discrete chaperone-binding region within the partner has been identified. To determine this region for SseA interactions with SseB, we conducted a yeast
two-hybrid interaction screen (Gyuris et al., 1993; reviewed by Fashena et al., 2000). Amino acids 3–108 of SseA were fused to the ‘B42 acid blob activator domain’ (B42–SseA; bait), and amino acids 2–196 of SseB, or various truncations, were fused to LexA (LexA–SseB; prey). The bait and prey were co-transformed to a single strain by mating; interaction is proportional to the transcriptional activation of the LexA-responsive lacZ (Estojak et al., 1995; Fashena et al., 2000).

Progressive truncations of the 196 aa SseB from both the C-terminus and N-terminus were constructed. The truncated SseB constructs fused to LexA are referred to by the amino acids deleted (e.g. SseBcated SseB constructs fused to LexA are referred to by the C-terminus and N-terminus were constructed. The truncations SseB starting with SseB1–51 resulted in increased reporter activity. This trend continued until a full fourfold increase above wild-type was obtained with SseB1–136. It was unclear if this increased β-galactosidase reporter activity was an artifact of the yeast two-hybrid system. To address this, GST-fusions were constructed using the same SseB truncations assayed by yeast two-hybrid and assayed for the ability to co-precipitate native SseA. Lysates containing GST–SseB1–196 or truncations, adjusted to contain comparable amounts of the recombinant protein (Fig. 2c; Coomassie) were added to lysates of a ΔsseB strain grown to induce SPI2-encoded protein production. The glutathione Sepharose 4B precipitate was analysed for the presence of SseA; a representative co-precipitation result is depicted in Fig. 2c. Increased precipitation of native SseA was obtained with GST–SseB1–51, GST–SseB1–90 and GST–SseB1–136 relative to that obtained with GST–SseB1–196. In contrast, no SseA precipitate was detected with GST–SseB (Fig. 2c), which was also found to be negative by yeast two-hybrid analysis. Using refractive densitometry we estimate that at least 80 % of SseA found in lysates is precipitated with the N-terminal truncated SseB constructs, which is about a 2.5-fold increase over that obtained with GST–SseB1–196 (data not shown).

Collectively, the increase in yeast two-hybrid β-galactosidase reporter activity and the increase in the amount of SseA co-precipitated by the N-terminal variants of SseB indicate a potential increase in chaperone–partner association. While the underlying cause for this apparent increase is not established, it is possible that the chaperone-binding domain is more accessible within the truncations. Alternatively, the N-terminal truncations could provide a context for the binding domain that more closely resembles the conformation the chaperone recognizes in the bacterial cytoplasm.

Since SseA selectively binds two distinct partners, we used the yeast two-hybrid system to address the region where SseA bound SseD. For this domain-mapping analysis, a set of internal deletions of SseD (1–195) were constructed and expressed as LexA fusion proteins. The constructs were examined for expression in yeast (Fig. 3a), and those that

**Fig. 2.** Delineation of a discrete domain within SseB required for SseA binding. (a) Evaluation of the LexA–SseB truncations for stability within yeast. Yeast lysates expressing the LexA–SseB truncations indicated above the lanes were resolved on 12 % Tris-glycine SDS-PAGE and immunoblotted with anti-LexA antibody. The positive control expressing LexA–Cdc42G12V, C188S (encoded protein production. The glutathione Sepharose 4B precipitate was analysed for the presence of SseA; a representative co-precipitation result is depicted in Fig. 2c. Increased precipitation of native SseA was obtained with GST–SseB1–51, GST–SseB1–90 and GST–SseB1–136 relative to that obtained with GST–SseB1–196. In contrast, no SseA precipitate was detected with GST–SseB (Fig. 2c), which was also found to be negative by yeast two-hybrid analysis. Using refractive densitometry we estimate that at least 80 % of SseA found in lysates is precipitated with the N-terminal truncated SseB constructs, which is about a 2.5-fold increase over that obtained with GST–SseB1–196 (data not shown).

Collectively, the increase in yeast two-hybrid β-galactosidase reporter activity and the increase in the amount of SseA co-precipitated by the N-terminal variants of SseB indicate a potential increase in chaperone–partner association. While the underlying cause for this apparent increase is not established, it is possible that the chaperone-binding domain is more accessible within the truncations. Alternatively, the N-terminal truncations could provide a context for the binding domain that more closely resembles the conformation the chaperone recognizes in the bacterial cytoplasm.

Since SseA selectively binds two distinct partners, we used the yeast two-hybrid system to address the region where SseA bound SseD. For this domain-mapping analysis, a set of internal deletions of SseD (1–195) were constructed and expressed as LexA fusion proteins. The constructs were examined for expression in yeast (Fig. 3a), and those that...
yielded comparable amounts of stable fusion proteins were assayed for interaction with SseA. The results of the interaction screen are presented in Fig. 3b. Bracketing the C-terminal region that resulted in the loss of yeast two-hybrid β-galactosidase reporter activity with the nearest positives indicates that the essential SseD binding region is within amino acids 138–194. Disruption of a central region spanning amino acids 32–82 reduced the yeast two-hybrid reporter activity by about 50%; this region may also provide contacts that contribute to SseA association. In addition, similar to SseB, an increase in reporter activity was observed with two of the constructs assayed: SseD32–82 yielded over three- and sixfold more signal, respectively, than that obtained with SseD1–195 (Fig. 3b). Stable B42–SseA N- or C-terminal truncations were generated and evaluated for binding to LexA–SseD using the yeast-two hybrid system. SseAΔ1–31 still binds SseB, as represented in Fig. 4a, indicating that the N-terminus of SseA is dispensable for interaction with SseB. However, loss of the region containing the SseA C-terminal amphipathic helix (68–98) renders SseAΔ67–108 unable to bind its partner. These results were confirmed by GST co-precipitation assays (Fig. 4b). GST–SseAΔ1–31 precipitated a comparable

<table>
<thead>
<tr>
<th>Construct</th>
<th>Binding region</th>
<th>Filter lift activity</th>
<th>Miller U test/ Miller U neg. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>SseD2–195</td>
<td>C</td>
<td>++</td>
<td>12.0±2.00</td>
</tr>
<tr>
<td>SseD32–31</td>
<td>N</td>
<td>+</td>
<td>42.5±3.71</td>
</tr>
<tr>
<td>SseD32–82</td>
<td>N</td>
<td>++</td>
<td>5.16±0.62</td>
</tr>
<tr>
<td>SseD83–110</td>
<td>C</td>
<td>++</td>
<td>9.98±0.30</td>
</tr>
<tr>
<td>SseD111–137</td>
<td>C</td>
<td>+++</td>
<td>1.69±0.09</td>
</tr>
<tr>
<td>SseD138–163</td>
<td>C</td>
<td>-</td>
<td>1.25±0.21</td>
</tr>
<tr>
<td>SseD164–186</td>
<td>C</td>
<td>-</td>
<td>1.25±0.21</td>
</tr>
</tbody>
</table>

**Fig. 3.** Delineation of a discrete domain within SseD required for SseA binding. (a) Evaluation of LexA–SseD internal deletion constructs for stability. Lysates of yeast expressing the LexA–SseD internal deletions indicated above the lanes were resolved on 12% Tris-glycine SDS-PAGE and immunoblotted with anti-LexA antibody. (b) The region of SseD contained in the internal deletion constructs used for the yeast two-hybrid interaction screen. The reporter activity is shown in the columns on the right (see Fig. 2b for explanation). The region required for interaction with SseA is shaded.

Stabilization and secretion of SseB require distinct domains within SseA

SseA and SseB synthesis is induced in pH 7–0 MgM medium (MgM 7–0), but export of SseB does not occur unless the pH is further reduced to pH 5–0. SseA serves to stabilize SseB, as only ~20% of the wild-type level accumulated in a Δssea strain grown in pH 5–0 MgM (MgM 5–0; Zurawski & Stein, 2003). A contribution of SseA to export was also observed, since SseB export to the bacterial surface was not detectable when the Δssea strain was grown in MgM 5–0. It was unclear if the apparent instability of the protein indirectly resulted in the export defect or if the chaperone plays a direct role in promoting SseB export under appropriate environmental conditions. To gain further insight into this question, we determined if the stabilization and secretion roles served by SseA could be uncoupled. To this end, we assayed for SseA mutants that stabilized SseB, like the native chaperone, but failed to restore secretion.

Stable B42–SseA N- or C-terminal truncations were generated and evaluated for binding to LexA–SseB using the yeast-two hybrid system. SseAΔ1–31 still binds SseB, as represented in Fig. 4a, indicating that the N-terminus of SseA is dispensable for interaction with SseB. However, loss of the region containing the SseA C-terminal amphipathic helix (68–98) renders SseAΔ67–108 unable to bind its partner. These results were confirmed by GST co-precipitation assays (Fig. 4b). GST–SseAΔ1–31 precipitated a comparable
amount of SseB as GST–SseA3_108. In contrast, GST–SseA67_108 failed to co-precipitate SseB, just as this truncation had failed to support yeast two-hybrid interactions.

The finding that a third of SseA is dispensable for binding the SseB partner was extended to address the physiological function of this N-terminal SseA domain within Salmonella. To this end, we determined if the SseAΔ1–31 truncation was able to restore physiological functions within a ΔsseA isogenic strain by inducible complementation (ΔsseA/pSA; Zurawski & Stein, 2003). SseAΔ1–31 was cloned in front of the arabinose-activated araBAD promoter (pSAAΔ1–31; Fig. 5a) and compared with pSA for its ability to restore stabilization and export of SseB in the ΔsseA strain. Whole bacterial lysates contain the total amount of SseB present (i.e. intracellular and extracellular), while the hexadecane fraction contains only surface-associated SseB (i.e. extracellular). When sseAΔ1–108 transcription in a ΔsseA/pSA strain was induced in the presence of arabinose, significantly more SseB was detectable in whole-cell lysates compared with the uninduced control (Fig. 5a; Zurawski & Stein, 2003). In addition, a portion of the SseB, comparable to that exported by wild-type strains and estimated to be about 5% of bacterial SseB, was now associated with the cell surface (Fig. 5a). Similarly, pSAAΔ1–31, when induced with arabinose, allowed similar stabilization of SseB in ΔsseA (Fig. 5a; ΔsseA/pSAAΔ1–31 + Ara). However, no surface-associated SseB (hexadecane; Fig. 5a) was detected. The result suggests that SseA partner binding correlates to its ability to allow SseB stabilization, but that the N-terminus participates in export-associated functions.

To confirm the finding that loss of the SseA N-terminus uncouples SseB stabilization from SseB export, site-directed mutations within the N-terminus of SseA were generated in pSA to create pSAQ17D,Q31D. These particular residues were altered to disrupt a predicted secondary structure. The N-terminus of SseA (amino acids 1–42) likely forms a coiled-coil (>62% score) based on the COILS predictive algorithm (see Methods). The site-directed mutagenesis of glutamine 17 and 31 to aspartic acid lowered this predicted score to 48%. Therefore, potential protein–protein interactions involving this coiled-coil should be adversely affected.

Similar amounts of SseA and SseAQ17D,Q31D are produced within Salmonella (Fig. 5b). SseAQ17D,Q31D and SseA similarly increase the total amount of SseB (Fig. 5b) detected in ΔsseA. SseB on the ΔsseA/pSA surface was readily detectable following arabinose induction. In contrast, the production of SseAQ17D,Q31D resulted in a very small amount of SseB on the bacterial surface (Fig. 5b). This result is consistent with the results obtained with the sseAΔ1–31 strain and supports the conclusion that the N-terminus of SseA participates in events associated with SseB export.

To confirm that similar events occur within host cells, we compared the ability of pSA and pSAQ17D,Q31D (Fig. 5b) to restore SPI2 effector translocation via inducible intracellular complementation (Stein et al., 1996; Guy et al., 2000). The indicator of ex vivo effector translocation was the Sif phenotype, which requires translocation of at least three effectors (sifA, sseF and sseG) by the SPI2 TTS system (Stein et al., 1996; Guy et al., 2000). Loss of SseA prevents Sif formation, but the phenotype is nearly fully restored when SseA is provided in trans (see below). HeLa cells were infected with ΔsseA/pSA or ΔsseA/pSAQ17D,Q31D and induced by addition of arabinose to the extracellular media (Guy et al., 2000). The negative control, ΔsseA, failed to generate Sif. In contrast, arabinose-induced ΔsseA/pSA formed Sif within HeLa, relative to wild-type Salmonella, at 89.9 ± 2.7% (mean ± SD). Sif formation by arabinose-induced ΔsseAQ17D,Q31D was 96.6 ± 3.0% that of wild-type. Collectively, the point mutations within the N-terminus of SseA restore SseB stability, but significantly reduce in vitro SseB export and the ex vivo Sif phenotype, corroborating the involvement of the N-terminus in partner export.

![Fig. 4](https://www.microbiologyresearch.org/content/fig/4.png)

Fig. 4. The N-terminal region of SseA is dispensable for binding to SseB and SseD. (a) Yeast two-hybrid analysis of truncated B42–SseA variants co-expressed with LexA–SseB2–196. Constructs depicted were all stably expressed within yeast (data not shown). The result of filter lift assays are shown on the right. (b) Co-precipitation assays using selected SseA truncations. Lysates containing comparable amounts of the GST–SseA fusion proteins or GST alone were added to S. typhimurium Sarkosyl-soluble fraction and precipitated with glutathione Sepharose 4B. The precipitate samples were resolved on a 16.5% Tris-Tricine SDS-PAGE gel and Coomassie blue stained (Coomassie) or immunoblotted with anti-SseB polyclonal antibody. Two bands are present in the immunoblot: the upper band is SseB and the lower band is a commonly observed breakdown product *SseB* (Zurawski & Stein, 2003).
DISCUSSION

The 108 aa SseA, encoded within the SPI2 effector region, was previously demonstrated to be required for effector translocation (Coombes et al., 2003) and fulfils sufficient functional and physiological criteria to classify it as a TTS chaperone for SseB and SseD (Zurawski & Stein, 2003; Ruiz-Albert et al., 2003). TTS chaperones are an expanding class of virulence factors that transiently associate with one or more partners. These chaperones are all less than 20 kDa (11–15 kDa), generally have an acidic pI, are cytosolic, and contain a C-terminal amphipathic helix required for partner binding. A range of documented functions for chaperones have been reported, including stabilizing the partner before secretion and acting as a secretion pilot during effector tranlocation (Parsot et al., 2003).

Classification of the SseA chaperone

Presently, three distinct classes are recognized. Class I chaperones are the best characterized, and all bind a discrete domain within the N-terminus of their partner (Parsot et al., 2003). They dimerize and share remarkable structural similarity, despite the lack of significant sequence similarity. Class II chaperones associate with a pair of translocon pore proteins. No consensus for a binding domain has emerged: Shigella spp. IgpC binds within the N-terminus of both pore proteins (Page et al., 2001), while the Yersinia spp. LcrH (SycD) recognizes multiple sites on YopB and requires both a central domain and a 30 aa C-terminal domain for wild-type binding to YopD (Neyt & Cornelis, 1999; Francis et al., 2000). Class III chaperones participate in flagellar assembly and recognize a C-terminal domain (reviewed by Bennett & Hughes, 2000; Parsot et al., 2003).
SseA, at the time this study was initiated, was not readily placed into any of the existing chaperone classes. This is because the set of partners it binds, a putative translocon sheath protein and a single translocon pore protein, was distinct from the partner sets bound by classical chaperones. In addition, SseA is atypical by virtue of its predicted basic pI and a portion being localized to the Sarkosyl-soluble, cytosolic membrane fraction (Zurawski & Stein, 2003). Also atypical of the SseA chaperone is the prediction of coiled-coil domains involved in protein–protein interactions (Delahay & Frankel, 2002). Our analysis indicates that the C-terminal amphipathic helix is strongly predicted to form a coiled-coil (>95%; COILS, see Methods) while another coiled-coil (>62%; COILS) may be present in the N-terminus.

In this report, sufficient information about how SseA interacts with SseBD was obtained to allow comparison with similarly characterized chaperones. Our results indicate that SseA interactions with both partners absolutely require a C-terminal domain, narrowed to amino acids 137–182 in SseB and 138–194 in SseD. However, loss of a central domain within SseD also reduced binding. Therefore, this central region may play a role in SseA–SseD association analogous to the central region of YopD (Francis et al., 2000). The C-terminal binding aspect of SseA is atypical for characterized virulence chaperones, with the LcrH/YopD pair being the only other instance where loss of the C-terminus impacts chaperone binding (Francis et al., 2000). However, the determination that SseB shares a common binding theme with flagellar chaperones is not counterintuitive since the only structurally characterized Esc group translocon sheath (EspA) shares striking structural similarity with flagella (Daniell et al., 2003). Collectively, our results suggest that the C-terminus of both partners is critical, and that SseA is most comparable to the class III flagellar chaperones.

**A model for SseA function**

A function provided by some virulence chaperones (Neyt & Cornelis, 1999; reviewed by Parsot et al., 2003) and by C-terminal binding flagellar chaperones (Auvray et al., 2001; Ozin et al., 2003) is to control association of subunits before export. It was anticipated that this is the role provided by SseA binding to SseB (Zurawski & Stein, 2003), and the location of the binding region supports this hypothesis. A C-terminal coiled-coil domain is involved in the multimerization of the corresponding EspA (Delahay et al., 1999). The C-terminal regions of SseB and EspA are 60% similar, and the predicted SseB coiled-coil aligns with a portion of the EspA coiled-coil domain (Fig. 6). SseB 162–182 is modelled to form an amphipathic helix (Fig. 6b) which is predicted by the COILS and MultiCoils algorithms to serve as a coiled-coil with a confidence of ~40% and 70%, respectively. The SseA-binding region of SseB (aa 137–183) is inclusive of the SseB coiled-coil region (162–182), and binding is prevented when deletions impinge on this area (e.g. SseBΔ169–196). This suggests that at least part of the domain occupied by SseA involves the putative coiled-coil. Occupancy of a self-association domain, as demonstrated for flagellar subunit chaperones (Auvray et al., 2001; Ozin et al., 2003), is predicted to interfere with SseB assembly into a translocon sheath.

**Fig. 6.** Alignment of SseB to the EspA translocon sheath subunit and modelling of the putative SseB coiled-coil domain. (a) EspA and SseB were aligned using the PAM 250 matrix, and similar (grey) and identical (boxed) regions are indicated. The 46 aa SseA binding region is surrounded by a thick lined box. Analysis by COILS and MultiCoils (2-5 weight for ‘a’ and ‘d’ residues) identified an amphipathic helix predicted to participate in coiled-coil type protein–protein interactions. The hydrophobic residues, ‘a’ and ‘d’, of the heptad repeats (Window 21) are indicated above SseB and below EspA. The mean predictive confidence for the SseB domain is >70% by COILS and 40% by MultiCoils, while the EspA domain is assigned >90% confidence. The location of the ΔsseB169–196 deletion that abrogates SseA binding within the putative SseB coiled-coil spanning residues 162–182 (ΔsseB169–196) is depicted with a vertical dashed line. (b) A portion of the putative SseB coiled-coil (162–179) was modelled with HelixWheel and is predicted to be an amphipathic helix consistent with a coiled-coil forming domain. The hydrophobic amino acids within the amphipathic helix are denoted by a thick circle.
In addition, heterologous protein–protein interactions occurring with SseB are also likely, as suggested by the EPEC paradigm for Esc TTS phylogenetic class. EspA associates with the EspB translocon pore protein (Hartland et al., 2000), with the EscF secretion needle component (Ide et al., 2001), and interaction with the EspD translocon pore protein appears to occur during EspA export (Sekiya et al., 2001). Therefore, SseB is likely to interact with the analogous SPI2 proteins SseD, SsaG, SscC, respectively. The possibility that chaperones in general, and SseA in particular, participate in controlling heterologous association has not been eliminated. Studies are ongoing to define the effect of SseA binding upon SseB homologous interactions and upon relevant heterologous protein–protein interactions.

The association of SseA with SseD may exert control over interactions required for translocon assembly. The most likely association influenced is the anticipated association of SseD with SseB. This interaction has been documented for the related EspB with EspA (Hartland et al., 2000). It is also likely that SseD is also influenced by other, currently uncharacterized, chaperones. For example, export of EspB requires the CesD chaperone (Wainwright & Kaper, 1998), which also stabilizes the other translocon pore protein, EspD. Since it is anticipated that SscA serves as the analogous chaperone for the SscCD translocon pore proteins (Nikolaus et al., 2001; Holden, 2002), our current working model is that SscA binds to SscD to prevent its premature association with SseB, while a CesD paralogue (sscA) influences its association with the other translocon component, SscC.

SseA-like chaperones as a common feature of Esc-TTS systems

A recent report characterizing associations between LEE-encoded proteins suggests that a basic pl sheath/pore chaperone may be a conserved feature of Esc TTS systems. Specifically, a 107 aa protein encoded by cesAB of the Esc TTS system of EPEC was reported to selectively bind the EspA sheath and the EspB pore protein that are related to the putative Salmonella SseB sheath and SseD pore protein, respectively (Creasey et al., 2003). While SseA and the CesAB chaperone share no primary sequence similarity, both have a similar predicted basic pi (9·8 and 9·5 respectively). Anecdotal evidence suggests that EspA aggregation occurs in the absence of CesAB, and an anti-polymerization role for this binding partner is also anticipated (Creasey et al., 2003).

Evidence for participation of the SseA N-terminus in the export of SseB

The N-terminus of SseA (amino acids 1–31) was found to be dispensable for its interaction with SseB when evaluated by both the yeast two-hybrid system and GST precipitation assays (Fig. 4). Subsequently, we found that expression of SseA32–108 within Salmonella resulted in the restoration of SseB stabilization, but failed to promote secretion of SseB. Similarly, it was reported that the alteration of the SycE chaperone, by addition of GST to either end, prevented YopE export, despite wild-type binding and stabilization (Cheng & Schneewind, 1999). These findings suggest that domains of a chaperone which are essential for partner export may be distinct from those that mediate binding and stabilization. Specifically, the N-terminal domain of SseA is predicted to be a coiled-coil, which could be involved in protein–protein interactions other than binding and stabilization. For example, the N-terminus of SseA may be required for an interaction that targets the chaperone–partner complex to the cytoplasmic face of the SPI2 TTS apparatus. In fact, it has been reported that chaperone–partner complexes can strongly associate with the ATPase of the apparatus in two related secretion systems (Gauthier & Finlay, 2003; Thomas et al., 2004).

Conclusions

The flagellar filament and the EspA translocon sheath were observed to share structural similarities (Daniell et al., 2003). Collectively, our study provides another example of similarities between the flagellar system and SseB, a putative translocon sheath. It now appears that characterized flagellar chaperones and SseA bind within the C-terminus of its partners. In the case of SseB, it appears that the chaperone impinges upon domains (Fig. 6) likely to participate in subunit assembly, as demonstrated for the flagellar chaperone, FlIS (Auvray et al., 2001; Ozin et al., 2003). In addition, a second instance where the stabilization and secretion functions of a chaperone can be uncoupled has been reported (Cheng & Schneewind, 1999; Fig. 5). Additionally, we provide the first example where different functions were ascribed to a specific region within these small, significant virulence factors.

ACKNOWLEDGEMENTS

We would like to kindly thank Doug Johnson and members of his laboratory, Alysa vandenBerg and Kurt Toenjes, for providing the yeast strains and plasmids for the yeast two-hybrid system. We would also like to thank Mark Rould for help with the coiled-coil modelling and scientific discussion, and Akamol Survarnapunya and Keith Mintz for scientific discussion and critical reading of this manuscript.

REFERENCES


