

## NOTES

# SigE Is a Chaperone for the *Salmonella enterica* Serovar Typhimurium Invasion Protein SigD

K. HERAN DARWIN, LLOYD S. ROBINSON, AND VIRGINIA L. MILLER\*

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 2 October 2000/Accepted 16 November 2000

**SigD is translocated into eucaryotic cells by a type III secretion system. In this work, evidence that the putative chaperone SigE directly interacts with SigD is presented. A bacterial two-hybrid system demonstrated that SigE can interact with itself and SigD. In addition, SigD was specifically copurified with SigE-His<sub>6</sub> on a nickel column.**

Many gram-negative pathogenic bacteria produce type III secretion systems (TTSS) required for virulence in an animal model of infection (for a review, see reference 15). These systems secrete and frequently translocate effector proteins into eucaryotic cells such as epithelial cells and macrophages (3, 4, 8, 10, 11, 17, 24, 29). Once in the eucaryotic cytoplasm, these effectors can stimulate events such as cytoskeletal rearrangements, ion flux, or apoptosis (1, 2, 13, 19, 23, 29, 30). *Salmonella enterica* serovar Typhimurium has at least two TTSS, one of which is encoded on a large pathogenicity island called SPI1 (22). The SPI1 system is required for invasion of salmonellae into epithelial cells as well as processes leading to fluid secretion in intestinal models of infection (for a review, see reference 6). SigD (also known as SopB in *S. enterica* serovar Dublin) is secreted by this TTSS and was found to be required for efficient invasion into epithelial cells in vitro (14). SopB was shown to have an inositol phosphatase activity within eucaryotic cells (23) and cause fluid secretion in a calf model of intestinal infection (11). SigD, which is not encoded within SPI1, was identified in a screen for invasion genes (14); however, the original Tn10dTc mutation was actually in the downstream gene *sigE*. This mutation resulted in the absence of SigD, but not other proteins, in culture supernatants. Because TTSS effectors often require a cognate chaperone, we hypothesized that SigE is a specific chaperone for SigD.

Chaperones have functions ranging from preventing premature association of one effector with another (20) to preventing degradation of the effector within the bacterium prior to secretion (9, 27, 28). Although we hypothesized that SigE is a specific chaperone for the SigD polypeptide, it was also possible that SigE affected the transcription of *sigDE*. Previous work has shown that another chaperone, SicA, is autoregulated and is required for the transcription of *sipBCDA* and *sigDE* (7). Therefore, we wanted to determine if the *sigD* promoter, which is dependent on SicA for expression (7), was dependent on *sigE*

for transcription. A *sigD-lacZYA* reporter plasmid (pHD5) was previously constructed (5) and integrated into the chromosome of the wild-type and *sigE::Tn10dTc* strains. Transduction analysis was used to confirm the linkage of the reporter fusion (conferring chloramphenicol resistance) to the transposon insertion (conferring tetracycline resistance) (18). This reporter did not disrupt secretion of wild-type levels of SigD (7).  $\beta$ -Galactosidase activities from the wild-type and *sigE* serovar Typhimurium 14028s (American Type Culture Collection) strains were measured and found to be almost identical ( $57 \pm 1$  and  $60 \pm 2$  Miller units for wild type and *sigE* mutant, respectively). Therefore, *sigE* is not required for transcription from the *sigD* promoter.

To see if *sigE* was required for the stability of the SigD polypeptide in the cytoplasm of the bacteria, Western blotting was performed on whole-cell proteins from overnight cultures of wild-type and *sigE::Tn10dTc* strains. Proteins that were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (7.5% gel) were transferred to polyvinylidene difluoride (Immobilon) membranes and incubated with antibodies raised against the first 192 amino acids of SigD fused to maltose binding protein (5). SigD was undetectable in whole-cell preparations of the *sigE* mutant (Fig. 1, lane 2) and the *sigD* mutant (lane 5). SigD could be restored in the *sigE* strain when transformed with a plasmid encoding *sigE* (pHH26) (13) (lane 4). In addition, overexpression of *sigDE* on a medium-copy-number plasmid significantly increased the amount of SigD in whole cells (lane 6). When *sigE* was disrupted with transposon TnMax2 (12), less SigD was observed (lane 7). This result suggests that SigD can be translated in the absence of SigE and that SigE is probably required for the stability of the SigD protein. Nevertheless, we cannot absolutely rule out the possibility that SigE has a role in translation of the *sigDE* transcript.

A bacterial two-hybrid system using the *Bordetella pertussis* adenylate cyclase gene (*cya*) (16) was used to determine if SigE forms dimers as has been suggested for other type III secretion chaperones in vitro (28). In addition, this system was used to test if SigE interacts with SigD. The adenylate cyclase protein

\* Corresponding author. Mailing address: Washington University School of Medicine, Department of Pediatrics, 660 S. Euclid Ave., Campus Box 8208, St. Louis, MO 63110. Phone: (314) 286-2891. Fax: (314) 286-2896. E-mail: virginia@borcim.wustl.edu.

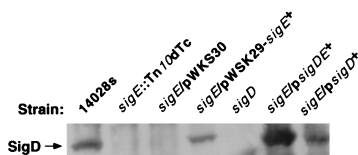


FIG. 1. Immunoblot analysis using polyclonal antibodies directed against SigD (amino acids 1 to 192) of proteins from 14028s (wild type), SVM167 (*sigE::Tn10dTc*), SVM167 pWKS30 (vector), SVM167 pHH26 (pWSK29-*sigE*), SVM255 (*sigD*), SVM167 pHH10 (*sigDE*<sup>+</sup>), and pHH10-23 (*sigD*<sup>+</sup>) strains.

(Cya) can be separated into two domains (designated T25 for the N-terminal domain and T18 for the C-terminal domain) which cannot function independently. When fused to interacting proteins, the Cya domains can potentially interact and function, resulting in the production of cyclic AMP (cAMP). cAMP production can be indirectly measured by maltose or lactose metabolism in an *Escherichia coli* *cya* mutant (DHP1) (16). The N-terminal region of SigD (SigD') and the entire SigE protein were each fused to Cya domains T25 and T18 encoded in plasmids pT25 and pT18, respectively. The first 101 codons of SigD were amplified using *Pfu* polymerase (Stratagene) with primers SigDfKpnI (5'-TTACGGTACCTATGC AAATACAGAGCTTCTATCAC-3') and SigDr102KpnI (5'-TTGAGGTACCATTGACGTTAGAACCGGGTCTTG-3') (Life Technologies). The primers used to amplify SigE were SigEfKpnI (5'-TTGAGGTACCTATGGAAAGTCTATTAA ATCG-3') and SigErKpnI (5'-ATTAGGTACCGCATAATG CTCTTTCAATTG-3'). *KpnI*-digested fragments were cloned into the *KpnI* sites of pT18 and pT25. Clones were sequenced to check for the correct orientation of the inserts and any mutations that may have been incorporated during the amplification or cloning process. *E. coli* strain DHP1 was transformed with these plasmids, and  $\beta$ -galactosidase activity from each strain was measured.

When SigE was fused to both domains of Cya, a high level of  $\beta$ -galactosidase activity (21) was measured in liquid overnight cultures grown at 26°C (Table 1). No activity was detected when a SigE fusion was combined with a SicA fusion. When an N-terminal portion (amino acids 1 to 100) of SigD was fused to the T18 domain of Cya (SigD'-T18) and combined with T25-SigE, the level of  $\beta$ -galactosidase activity measured was higher

TABLE 1. Complementation of *cya* in *E. coli* DHP1 using *sigE* and *sigD* fusions to *B. pertussis* *cya* domains

Test plasmid pair	Mean $\beta$ -galactosidase activity (Miller units) $\pm$ SD <sup>a</sup>
pT18 + pT25 (vectors) .....	81 $\pm$ 2
pT18 + pT25-SigD' .....	55 $\pm$ 2
pSigD'-T18 + pT25 .....	61 $\pm$ 0
pSigE-T18 + pT25 .....	61 $\pm$ 5
pSigE-T18 + pT25-SigE .....	2,405 $\pm$ 220
pSigE-T18 + pT25-SicA .....	62 $\pm$ 5
pSigD'-T18 + pT25-SigE .....	439 $\pm$ 43
pSigD'-T18 + pT25-SicA .....	68 $\pm$ 4
pSigE-T18 + pT25-SigD' .....	93 $\pm$ 3

<sup>a</sup> *E. coli* strain DHP1 containing the indicated plasmids was grown overnight (18 h) in Luria-Bertani broth supplemented with chloramphenicol (25  $\mu$ g/ml) and ampicillin (100  $\mu$ g/ml) in 13-cm test tubes at 26°C on a roller drum.  $\beta$ -Galactosidase activity was measured as described in the text.

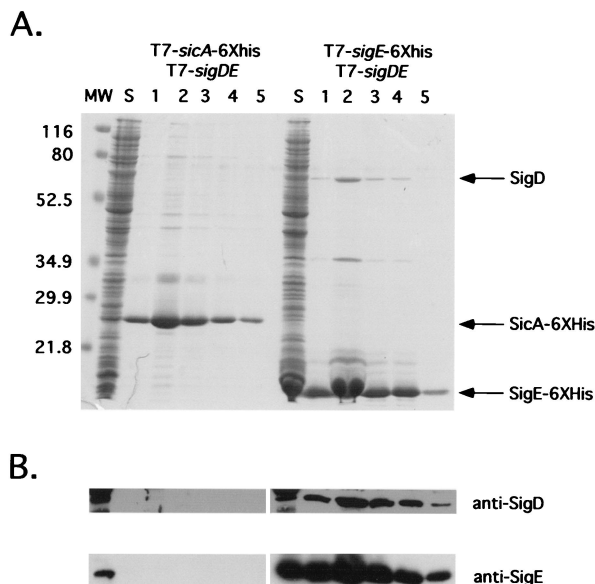


FIG. 2. SigD copurifies with SigE-His<sub>6</sub>. (A) Coomassie brilliant blue-stained 12.5% polyacrylamide gel of fractions eluted from a nickel-agarose column. Positions of molecular mass standards (MW) are indicated on the left in kilodaltons. Lane S, soluble fraction after sonication of cell pellets and removal of insoluble debris by centrifugation; lanes 1 to 5, imidazole-eluted fractions. (B) Immunoblot analysis of the same fractions using antibodies to SigD or SigE. The major band labeled SicA-6XHis in panel A was confirmed to be SicA by antibodies specific to SicA (data not shown).

than background activity (Table 1). The same SigD'-T18 construct yielded no activity when combined with T25 fused to the chaperone SicA. Interestingly, when SigD' was fused to the T25 domain of Cya (T25-SigD'), no activity was measured. It was possible that the SigE binding domain on SigD was occluded by the T25 domain in this construct or that this fusion was not stable, producing no T25-SigD'.

In addition to the two-hybrid system, we used a biochemical approach to determine if SigD could interact with SigE. Hexahistidine (His<sub>6</sub>) fusions to the C termini of chaperones SicA and SigE were constructed in the expression vector pET24(+) (Novagen) and transformed into the *E. coli* strain BL21(DE3), which encodes an inducible T7 polymerase gene (25). In addition, these strains were transformed with plasmid pHH2, which contains *sigDE* downstream of a T7 promoter (14). One-liter cultures of each strain were induced with 100  $\mu$ M isopropyl- $\beta$ -D-thiogalactopyranoside during exponential phase (optical density at 600 nm of 0.6), and cell lysates were prepared under native conditions according to the QIAexpressionist manual (Qiagen). When *sigDE* was coexpressed with SicA-His<sub>6</sub>, SigD was not coeluted along with SicA-His<sub>6</sub> from the nickel column (Fig. 2, lanes 3 to 7). The *sicA*-His<sub>6</sub> construct should have produced an active protein because it was able to activate transcription of a *sicA-lacZYA* reporter plasmid when coexpressed with *invF* in *E. coli* (data not shown). When *sigE*-His<sub>6</sub> and *sigDE* were coexpressed, SigD copurified with SigE-His<sub>6</sub>, as seen in either a 12.5% Coomassie-stained SDS-polyacrylamide gel or Western blot (separated on a 7.5% gel and transferred to 0.2- $\mu$ m-pore-size Schleicher & Schuell nitrocel-

lulose) using antibodies to SigD (Fig. 2A, lanes 9 to 13, and 2B).

Many putative type III secretion chaperones have been identified in other gram-negative bacteria but have yet to be shown to multimerize or specifically interact with effector molecules (15). Type III chaperones share several characteristics: small size (around 12 to 20 kDa), acidic isoelectric point (~4), and the presence of an amphipathic alpha helix near the C terminus. The results of this work show that SigE appears to be a chaperone for SigD, a type III secreted effector molecule. The inability of SigD to interact with SicA suggests the interaction with SigD is specific for SigE rather than a general interaction with type III chaperones. In addition, SigE appears to form dimers or other higher-order multimers. SigE is not required for transcription of the *sigDE* operon. In one case in *Salmonella*, a mutation in the *sicA* chaperone results in reduced expression of genes encoding several effector molecules (7). It is notable that a mutation in *sicA* is likely to also have post-translational effects on two effectors, SipB and SipC (7, 26). The *sigDE* operon, however, is more similar to other known chaperone-effector gene pairs, like *sicP-sptP* (9) from *Salmonella* and *ipgC-ipaBC* from *Shigella* (20), that do not appear to be dependent on a chaperone for transcription activation. Rather than affect transcription, SigE is likely to affect either the translation or stability of SigD. The transcriptional organization of *sigDE* suggests that *sigD* and *sigE* are cotranslated. Moreover, overexpression of *sigD* in a *sigE* mutant results in the production, albeit in reduced amounts, of SigD. Together, these results suggest that SigE enhances the stability of SigD in the cytoplasm, but we cannot rule out the possibility that SigE also has a role in *sigD* translation. Previous studies on other chaperone-effector pairs have also shown that chaperones can directly interact with their cognate effector molecules (9, 20, 26). In this work, both genetic (bacterial two-hybrid) and biochemical (affinity purification) approaches indicate that SigD and SigE can specifically interact with each other. In addition, similar to other chaperone-effector pairs, the N-terminal region of SigD contains at least a part of the SigE chaperone binding domain.

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