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**Multipart Chaperone-Effector Recognition in the Type III Secretion System of Chlamydia trachomatis**

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**Background:** The type III secretion (T3S) chaperone Scc4 modulates Chlamydia RNA polymerase holoenzyme activity and is also required for secretion of the gatekeeper CopN.

**Results:** Interactions between the Scc4 and Scc1 chaperones and CopN are characterized.

**Conclusion:** Scc4 forms a ternary complex with Scc1 and CopN to promote CopN secretion during infection.

**Significance:** Scc4 is an important link between the T3S system and transcription.

Secretion of effector proteins into the eukaryotic host cell is required for *Chlamydia trachomatis* virulence. In the infection process, Scc1 and Scc4, two chaperones of the type III secretion (T3S) system, facilitate secretion of the important effector and plug protein, CopN, but little is known about the details of this event. Here we use biochemistry, mass spectrometry, nuclear magnetic resonance spectroscopy, and genetic analyses to characterize this trimolecular event. We find that Scc4 complexes with Scc1 and CopN in situ at the late developmental cycle of *C. trachomatis*. We show that Scc4 and Scc1 undergo dynamic interactions as part of the unique bacterial developmental cycle. Using alanine substitutions, we identify several amino acid residues in Scc4 that are critical for the Scc4-Scc1 interaction, which is required for forming the Scc4-Scc1-CopN ternary complex. These results, combined with our previous findings that Scc4 plays a role in transcription (Rao, X., Deighan, P., Hua, Z., Hu, X., Wang, J., Luo, M., Wang, J., Liang, Y., Zhong, G., Hochschild, A., and Shen, L. (2009) *Genes Dev.* 23, 1818–1829), reveal that the T3S process is linked to bacterial transcriptional processes, all of which are mediated by Scc4 and its interacting proteins. A model describing how the T3S process may affect gene expression is proposed.

*Chlamydia* spp. are obligate intracellular bacteria that cause a variety of diseases in humans. *Chlamydia pneumoniae* produces bronchitis and atypical pneumonia and has been linked to atherosclerosis (1). *Chlamydia trachomatis* serovars A–C are the leading causes of preventable blindness (trachoma), whereas serovars D–K and L1–L3 produce the most common bacterial sexually transmitted diseases. The deleterious effects of infection and the commensurate cost of treatment make these pathogens a significant public health concern worldwide (2). *Chlamydia* spp. have a unique developmental cycle, altering between infectious elementary bodies (EBs) and metabolically active reticulate bodies (RBs) (3, 4). The entire cycle takes approximately 48–72 h to complete in cultured epithelial cells. Shortly after invasion, EBs reside within a membrane-bound vacuole in the host cell, called an inclusion, and subsequently differentiate into RBs. RBs then undergo active replication and macromolecule synthesis. At ~24 h postinfection (hpi), RBs asynchronously differentiate into EBs. Finally, EBs exit the host cell to infect adjacent cells. The growth of *C. trachomatis* can be divided into early (EB-to-RB transition), middle (RB replication), and late stages (RB-to-EB transition and preparation for a subsequent infection cycle) of development (5, 6).

Numerous Gram-negative bacteria in the genera of *Yersinia*, *Salmonella*, *Escherichia coli*, *Salmonella*, *Shigella*, *Vibrio*, *Pseudomonas*, and *Chlamydia* employ a T3S system to inject effectors across host membrane barriers (7–11). Expression of the T3S system, its assembly into a highly ordered structural apparatus, and its activation are spatiotemporally regulated (12–15). The T3S apparatus assembles in the following order: a basal body, a “needle” extending from the bacterial surface, and the pore-forming translocon, which is a docking site for the needle.
tip on the host membrane (7). Once the T3S apparatus is assembled, translocators (translocon subunits) and effectors are then secreted. Many T3S effectors require cognate chaperones for proper secretion because they are translocated in an unfolded form (12, 14, 16). These chaperones serve to prevent untimely effector translocation. Several T3S chaperones have also been shown to regulate T3S gene expression (12, 13, 17–19). Despite conserved protein composition in the T3S apparatus, diverse effectors, chaperones, and regulators are found in various bacteria, reflecting the unique niche of each bacterial species. There has been little progress toward understanding exactly how specific effectors are paired with their chaperones and delivered to the T3S system apparatus for secretion, although this is a fundamental aspect of T3S system function.

The T3S system is a key pathogenic attribute of Chlamydia spp., in which ~80 different T3S effectors are predicted to be secreted into the host cytosol to modulate host function for survival and development (8, 20, 21). There are three classes of T3S chaperones in Chlamydia spp. (8, 22–27). The class I chaperones include class la chaperones (Scc1 and Scc4 or CT663), which bind to just one effector, and class lb chaperones (Scl1, MscC, and CT584), which bind to multiple effectors. Class II chaperones (Scc2 and Sc3) interact with translocators. The class III chaperones (CdsE and CdsG) interact with proteins comprising the needle. Whereas most T3S effectors require only one chaperone, the chlamydial CopN, which is homologous to the Yersinia gatekeeper or “plug” protein YopN/TyeA, binds to the Scc1, Scc4, and Scc3 chaperones (23, 27, 28). Through a central domain, CopN interferes with microtubule networks (29, 30). CopN protein also contains two chaperone binding domains at its opposite termini: the N-terminal chaperone binding domain binds with the Scc1 and Scc4 chaperones, and the C-terminal chaperone binding domain binds to a translocator-specific Scc3 chaperone (23, 27, 31). Biochemical studies and secretion assays in Yersinia indicate that the C. pneumoniae ScC4 and Sc1 chaperones promote CopN secretion, and the Scc3 chaperone represses its secretion (23). It remains unclear how such seemingly disparate chaperone-CopN interactions are utilized during chlamydial infection.

We have previously discovered that C. trachomatis CT663 (hereafter referred to as ScC4) serves as a transcriptional regulator (17) in addition to being a T3S chaperone. Unlike most previously described bacterial transcriptional regulators (with the exception of bacteriophage T4 AsIA protein) (32), C. trachomatis ScC4 targets region 4 of the RNA polymerase (RNAP) β subunit in the context of the RNAP holoenzyme. The RNAP transcribes the majority of the housekeeping genes, including T3S system genes, in C. trachomatis (33, 34). We hypothesize that ScC4, through specific interaction with the RNAP holoenzyme, plays a crucial role in facilitating a global change in gene expression involved in regulating the T3S process during C. trachomatis infection.

Understanding the dynamic interactions between T3S chaperones and their binding partners is a necessary step toward elucidating the regulated T3S process. Here, using biochemistry, quantitative mass spectrometry, NMR spectroscopy, and genetic approaches, we characterize the fundamental functionality of T3S chaperones using C. trachomatis ScC4, ScC1, and CopN as a model for chaperone-effector interactions. Combined with our previous results (17), we now demonstrate a dual role for C. trachomatis ScC4 as a necessary part of the ScC4-ScC1-CopN complex and also as a participant in RNA transcription through its binding to the RNAP holoenzyme. Our data extend the understanding of the role of ScC4 and ScC1 chaperones in CopN recognition and, importantly, provide a new paradigm for strict control of T3S system action that is mediated by ScC4 to ensure that the T3S system appropriately functions in response to developmental and environmental cues.

Materials and Methods

Molecular Cloning—The pETDuet-1TEVSiteHis6 derived from pETDuet-1 (Novagen) encodes a hexahistidine (His6) tag that is removable with tobacco etch virus protease (TEV) cleavage. The Scc1 coding region was amplified from genomic DNA of C. trachomatis serovar F/IC-Cal-3 (ATCC VR-346) by PCR and cloned into the Ncol/Xhol sites of pETDuet-1TEVSiteHis6 to yield pDuetScc1-His6. The Pγ7-Scc1 fragment from pDuet-Scc1-His6 was then subcloned into pACYC184 to generate pACYCScc1-His6. The Scc4 sequence from pHis663 (17) was subcloned into the Ncol/Xhol sites of pET28a (Novagen) to obtain pET28-Scc4. Similarly, the Scc4 sequence was cloned into pCDFFlac (35) to yield pCDFScc4. A synthesized gBlock fragment (Integrated DNA Technologies) containing multiple mutations in Scc4 was cloned into pCDFScc4 at the Ncol/Xhol sites to generate pCDFScc4_6A. The BamHI/Sphl fragment from pCDFScc4_6A was subcloned into the BamHI/Sphl sites of pCDFScc4, resulting in pCDFScc4_4A. A PCR fragment containing a single point mutation in ScC4 was cloned into pCDFScc4 to yield pCDFScc4_95A. Plasmids encoding fusion proteins of the N-terminal domain of RNAP α subunit from E. coli (α-NTD) and ScC1, ScC4, or CopN were constructed by inserting the gene of interest into the NotI/Spel sites of pRBR (17). Plasmids encoding the fusion protein of a DNA binding domain (ACI) and ScC1 or ScC4 were created by inserting the gene of interest into the NotI/Spel sites of pRAC (17). The identity of all constructs was confirmed by restriction enzyme mapping and DNA sequencing.

C. trachomatis Infection and Microscopy Analysis—Human cervix adenocarcinoma HeLa cells (ATCC CCL-2.1) were cultured in Dulbecco’s modified Eagle’s medium with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum (Sigma) and 1-glutamine (2 mM) at 37 °C in a humidified incubator with 5% CO2. For the indirect immunofluorescence assay (IFA), a HeLa monolayer grown on 12-mm diameter glass coverslips in 24-well plates was inoculated with C. trachomatis serovar F/Cal-1-13 with a dose that results in infection of ~50% of cells. The plates were then centrifuged at 1600 × g for 40 min at 37 °C. Infected cells were cultured in medium containing 1 μg/ml cycloheximide at 37 °C for various times as indicated under “Results.” After fixing with 2% paraformaldehyde and permeabilizing with 4% saponin, cells were subjected to immunostaining with antibodies overnight at 4 °C. Cells were then incubated with Alexa 568-conjugated (red) or FITC-conjugated (green) secondary antibodies (Molecular Probes) for 45 min at 37 °C. Cells were counterstained with 4’,6-diamidino-2-
phenylindole dihydrochloride (DAPI). Images were obtained with a fluorescence microscope (Zeiss) and processed using AxioVision software.

**Antibodies**—A rabbit polyclonal antibody against ACI and a mouse polyclonal antibody against Scc4 were described previously (17). A mouse polyclonal antibody against Scc1 and a rabbit polyclonal anti-Scc4 antibody were kindly provided by Guangming Zhong (University of Texas, San Antonio, TX). Rabbit polyclonal antibodies against Scc1, Scc3, or CopN were kindly provided by Dr. Ken Fields (University of Kentucky) (28).

**Generation and Purification of Recombinant Proteins**—Recombinant Scc4 and Scc1 with a His\(_6\) tag at its C terminus (Scc1-His\(_6\)) were expressed in *E. coli* RosettaTM (DE3)pLysS cells harboring PET28-Scc4 or pCDFScc4 and/or pACYCScscl-His\(_6\). Cells were grown in LB broth at 37 °C to A\(_600\) = 0.8, and then protein expression was induced by the addition of isopropyl-1-thio-

β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM. 15N-Labeled proteins were expressed similarly induced by adding IPTG to a final concentration of 0.1 mM.

**Protein Extraction**—Cells harboring pET28-Scc4 or pCDFScc4 and/or pACYCScscl-His\(_6\) were expressed in *E. coli* RosettaTM (DE3)pLysSware (One Moon Scientific) (38). Refolded Scc1-His\(_6\) protein was refolded using a linear gradient from 6 to 0 M urea (30 ml total at 0.1 ml/min) and eluted with 20 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 300 mM imidazole.

**Analytical Gel Filtration**—Gel filtration analysis of N-terminal His\(_6\)-tagged Scc4 (His\(_6\)-Scc4) and the Scc4-Scc1-His\(_6\) complex was conducted under different buffer conditions as indicated under “Results.” Standard curves were produced using each buffer and a protein molecular weight standard mixture (bovine serum albumin (BSA), β-lactoglobulin, cytchrome c, and cytidine). Linear regression analysis was used to determine best-fit line equations, which were then used to calculate the apparent molecular weights of samples. For this study, either a HiPrep Sephacryl S-100 HR column (1 ml sample injection volume, 1.5 ml/min flow rate) or an ENrich 70 SEC column (50 μl competition injection volume, 1.0 ml/min flow rate) was used with a Bio-Rad Duoflow F10 work station.

**Protein Binding Assay and Quantitative Mass Spectrometry Analysis**—Purified [\(^{15}\)N]Scc4 was incubated with purified Scc4-Scc1-His\(_6\) complex in buffer containing 20 mM Tris (pH 7.6), 100 mM NaCl, and 5% glycerol at a ratio of 2:1 or 10:1 ([\(^{15}\)N]Scc4/Scc4-Scc1-His\(_6\) complex) for 0.5 and 2 h. Free Scc4 was then separated from the complex using a 5-ml HisTrap HP column. After extensive washing, the bound complex was eluted with 20 mM Tris (pH 8.0), 150 mM NaCl, 5% glycerol, and 300 mM imidazole. The eluate was dialyzed overnight against 20 mM Tris (pH 7.6), 100 mM NaCl, and 5% glycerol and then concentrated with a 5,000 molecular weight cut-off Amicon filter (Millipore). Concentrated proteins were then subjected to trypsin digestion and LC/MS analysis to determine the amount of [\(^{15}\)N]Scc4 that had incorporated into the Scc4-Scc1-His\(_6\) complex.

**NMR**—Purified [\(^{15}\)N]Scc4 was prepared for NMR analysis by buffer exchanging into 20 mM ammonium acetate (pH 5.5) using a 3,000 molecular weight cut-off centrifugal filter. This buffer was chosen based on screening conditions for gel filtration and NMR analysis. As shown under “Results,” the Scc4-Scc1 heterodimer is present in 20 mM sodium acetate buffer at pH 4.5 without higher order oligomers. Because Scc4 is unstable in this buffer, 20 mM ammonium acetate at pH 5.5 was screened and gave good NMR peak dispersion and uniformity, indicating that Scc4 was folded and stable in this buffer. [\(^{15}\)N]Scc4 (0.5 mM) was analyzed on a Varian 700-MHz spectrometer with 10% D\(_2\)O to provide a lock signal. A \(^{1}\)H-[\(^{15}\)N] heteronuclear single quantum coherence (HSQC) spectrum was collected by signal-averaging eight scans and collecting 128 increments in the indirect dimension. Data were processed with a sine bell weighting function in both dimensions using NMRPipe software (37) and visualized with NMRView software (One Moon Scientific) (38). Refolded Scc1-His\(_6\) protein was added to the [\(^{15}\)N]Scc4 sample in a 1:1 molar ratio. The sample volume was reduced using a centrifugal filter to obtain 0.5 mM [\(^{15}\)N]Scc4-Scc1-His\(_6\) in 10% D\(_2\)O for NMR analysis. A \(^{1}\)H-[\(^{15}\)N] HSQC spectrum was collected using the same conditions as described above.

**His\(_6\)-mediated Pull-down Assay**—Purified Scc4-Scc1-His\(_6\) complex bound to 100 μl of \(^{40}\)Ni\(^{2+}\)-NTA beads (Pierce) was
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added to purified GST-tagged CopN and/or GST-tagged Scc3 in a total volume of 0.3 ml in 20 mM Tris (pH 8.0), 150 mM NaCl, and 10% glycerol. Ni²⁺-NTA beads added with GST protein were used as a negative control. After overnight incubation at 4 °C, the beads were washed extensively using 20 mM Tris (pH 8.0), 150 mM NaCl, and 10% glycerol. The captured proteins were eluted with 10 mM glutathione. Proteins were separated by 12% SDS-PAGE and then transferred to Immobilon-P membranes. Immunoblotting was performed using antibodies against Scc4, Scc1, Scc3, and CopN, as described previously (39). Blots were developed using horseradish peroxidase-conjugated secondary antibodies (Sigma) and the SuperSignal chemiluminescent detection kit (Pierce).

Cross-linking, Immunoprecipitation, Co-immunoprecipitation, and Immunoblotting—A vapor glutaraldehyde cross-linking assay was performed to ascertain the oligomeric state of Scc4 in vitro using the method described previously (40). For in situ protein cross-linking, HeLa cells in four 162-cm² flasks were infected with C. trachomatis serovar F/Cal-1-13 using a dose that results in 95% of cells being infected. The cells were harvested at 36 hpi in PBS (pH 7.4) and cross-linked for 60 min at 4 °C using the cross-linker dithiobis(succinimidyl propionate) (DSP) (Pierce) at a final concentration of 2 mM (diluted from a 40 mM stock solution in dimethyl sulfoxide) as described previously (41). Cross-linking was quenched by adding Tris-HCl (pH 8.0) to a final concentration of 50 mM (at 25 °C). Cells were then solubilized on ice for 20 min in 1% Nonidet P-40, 0.1% SDS, 10 mM HEPES (pH 7.8), 10% glycerol, and 150 mM NaCl containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor mixture (Sigma). After centrifugation at 10,000 × g for 30 min at 4 °C, cleared lysates were incubated with a rabbit anti-Scc4 antibody (or with preimmune serum from the corresponding rabbit) overnight at 4 °C. The antigen-antibody complexes were then combined with protein A/G-Sepharose (Pierce) and incubated for 60 min at 4 °C. Following extensive washing, the captured complex was eluted in SDS sample buffer, separated by SDS-PAGE, and subjected to immunoblotting.

Limited Proteolysis—Purified Scc4-Scc1-His₆ complex or Scc4 protein at concentrations of 1 mg/ml were digested with trypsin (Promega) on ice and at 25 °C in 20 mM HEPES (pH 7.4) and 125 mM NaCl. The ratio of enzyme to substrate was 1:100 (by weight). At different time intervals, aliquots were taken and added immediately to an equal volume of 2X SDS sample buffer, boiled for 5 min to stop the proteolysis, and stored at −20 °C. The samples were then analyzed on 4–20% Tris-Tricine gels (Bio-Rad) and stained by Coomassie blue. Bands of interest were then further proteolyzed at 37 °C with the combination of Glu-C (New England Biolabs) and trypsin (in a 10:1 (w/w) ratio) prior to mass spectroscopic analysis.

Protein Structure Prediction—The protein homology/analogy recognition engine version 2.0 (Phyre2) (42) was used to build three-dimensional structural models of CopN, Scc4, and Scc1. Each protein was modeled individually using the Phyre2 intensive option. The quaternary structural model of CopN, Scc4, and Scc1 was made by aligning the individual models to their homologous proteins in the crystal structure of Yersinia pestis YopN-SycN-YscB complex (Protein Data Bank code 1XKP) (43). The alignments were performed using the UCSF Chimera software and the Match/Align function with an amino acid residue distance cut-off of 3.0 Å, at most 10 iterations, and superimposition across the entire alignment (44).

β-Galactosidase (β-gal) Assay—A plate β-gal assay (45) was utilized to measure β-gal activity with mid-log phase E. coli cultures.

Results

CopN Directly Complexes with Scc4 and Scc1 in Vitro and in Situ—Direct evidence of CopN dynamically binding with Scc4, Scc1, and/or Scc3 has not been previously reported for C. trachomatis. To investigate the details of these molecular interactions, we first determined which intermolecular associations would occur in vitro using a His₆-mediated pull-down assay. Purified Scc4-Scc1-His₆ protein complex was immobilized on Ni²⁺-NTA beads and then incubated with purified GST-tagged CopN and/or purified GST-tagged Scc3 as described under “Materials and Methods.” Bound proteins were eluted, separated by SDS-PAGE, and detected by immunoblot with specific antiserum. Fig. 1A indicates that CopN binds directly to the Scc4-Scc1 complex. There was no direct binding between the Scc4-Scc1 complex and Scc3. We conclude that the Scc4-Scc1 complex associates with Scc3 only in the presence of CopN.

We next monitored the appearance of Scc4 and Scc1 in C. trachomatis-infected HeLa cells using IFA with specific antibodies. Using the same culture model and IFA, we previously reported that C. trachomatis accumulated detectable amounts of CopN at and beyond 20 hpi (46). We observed an Scc4-specific signal associated with chlamydial organisms at 16 hpi. An Scc1-specific signal was not easily visualized prior to 20 hpi. However, at 24 hpi, anti-Scc1 antibodies clearly labeled chlamydial organisms (Fig. 1B). The Scc1-positive organisms were also reactive with anti-Scc4 antibodies. As inclusions expanded, both Scc4- and Scc1-specific signals continued to increase throughout the 44-h period of observation. These data indicate the co-existence of Scc1 and Scc4 proteins at the late stage of C. trachomatis infection in HeLa cells.

We asked whether, at the late growth stage, CopN associated with Scc4 and Scc1 in C. trachomatis. Cross-linking with DSP and co-immunoprecipitation assays were performed with C. trachomatis-infected cells harvested at 36 hpi. In control experiments, uninfected cells and ampicillin-exposed cells were used. It is known that exposure of C. trachomatis to inhibitors of peptidoglycan synthesis, such as ampicillin, leads to the formation of aberrant RBs that cannot differentiate to EBs (the infectious late developmental form). Ampicillin exposure is also known to down-regulate CopN expression (46). The cleared lysates from the cross-linked cells were subjected to immunoprecipitation with anti-Scc4 antibodies and then immunoblotted with antibodies specific to Scc4, CopN, or Scc1. No Scc4, Scc1, or CopN was immunoprecipitated by pre-immune sera (not shown). As expected, we observed that anti-Scc4 antibodies immunoprecipitated Scc4 from the lysate of C. trachomatis culture unexposed to ampicillin (Fig. 1C). CopN and Scc1 were also co-precipitated with Scc4. However, CopN appeared somewhat smeary, which is probably due to the heterogeneous labeling by the DSP cross-linking reagent. DSP is a
reversible cross-linking reagent but not a cleavable reagent. A 3-mercaptopropionamide moiety remains at each cross-linked lysine residue; CopN has 27 lysines, giving numerous possible compositions. Little Scc4 was obtained from the cells exposed to ampicillin, suggesting that ampicillin exposure reduced Scc4 expression similar to CopN (46). Neither CopN nor Scc1 was immunoprecipitated from the ampicillin-exposed samples. Together with the IFA data (Fig. 1B), these data indicate that Scc4 binds to both Scc1 and CopN in the late stages of infection. Cells were fixed at 16, 20, 24, 36, and 44 h postinfection and processed for IFA. Shown are cells fixed at 24 h postinfection. Scc1 and Scc4 proteins were probed with specific polyclonal antibodies and then visualized with fluorescent secondary antibodies. Scc1 and Scc4 were visualized as green and red, respectively. Host and bacterial DNA were visualized with DAPI staining (blue). Inclusions are indicated by arrows. The co-localization of the two proteins (orange) within a selected inclusion (inset) is indicated by arrowheads. Scale bar, 10 μm. C, results of cross-linking; immunoprecipitation; and immunoblotting of Scc4, Scc1, and CopN. Representative blots from three individual experiments are shown. DSP cross-linked cultures harvested at 36 hpi were subjected to immunoprecipitation with anti-Scc4 antibodies. The resultant protein complexes were subjected to immunoblotting with antibodies specific to each protein. Mock infection HeLa cells and cells exposed to ampicillin were used as controls.

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FIGURE 1. CopN directly binds the Scc1-Scc4 complex or isolated Scc3. A, analysis of Scc4-Scc1-bound proteins using a His$_6$-mediated pull-down assay. Protein inputs are shown at the top (+), and pulled-down proteins (outputs) were probed with appropriate antibodies shown on the left. B, Scc4 and Scc1 co-localize during later stages of infection. Cells were fixed at 16, 20, 24, 36, and 44 h postinfection and processed for IFA. Shown are cells fixed at 24 h postinfection. Scc1 and Scc4 proteins were probed with specific polyclonal antibodies and then visualized with fluorescent secondary antibodies. Scc1 and Scc4 were visualized as green and red, respectively. Host and bacterial DNA were visualized with DAPI staining (blue). Inclusions are indicated by arrows. The co-localization of the two proteins (orange) within a selected inclusion (inset) is indicated by arrowheads. Scale bar, 10 μm. C, results of cross-linking; immunoprecipitation; and immunoblotting of Scc4, Scc1, and CopN. Representative blots from three individual experiments are shown. DSP cross-linked cultures harvested at 36 hpi were subjected to immunoprecipitation with anti-Scc4 antibodies. The resultant protein-antibody complexes were subjected to immunoblotting with antibodies specific to each protein. Mock infection HeLa cells and cells exposed to ampicillin were used as controls.

Co-expression of Scc4 Promotes Scc1 Solubility in E. coli—To facilitate the study of protein-protein interactions between Scc4 and Scc1, either tag-free Scc4 alone or Scc4 with Scc1-His$_6$ were expressed in E. coli BL21(DE3) cells. The levels and solubility of Scc4, Scc1-His$_6$, or both proteins were examined (Fig. 2). Expression of Scc4 alone resulted in a highly soluble Scc4 protein, but expression of Scc1-His$_6$ alone resulted in very low levels of soluble protein (Fig. 2A). In contrast, co-expression of Scc4 and Scc1-His$_6$ led to a significant increase in soluble Scc1. Using Ni$^{2+}$ affinity chromatography, the Scc4 and Scc1-His$_6$ remained together and co-eluted (data not shown). Further purification by gel filtration chromatography demonstrated that the Scc4-Scc1-His$_6$ complex was obtained as a highly pure protein species, as evidenced by SDS-PAGE and Coomassie Blue staining (Fig. 2, B and C). The Scc4-Scc1-His$_6$ complex displayed protein bands of apparently equal relative intensities, implying a stoichiometry of 1:1 and suggesting a heterodimer complex. Identical results were obtained when N-terminal His$_6$-tagged Scc4 (His$_6$-Scc4) and the N-terminal FLAG-tagged Scc1 were co-expressed. Neither the expression of Scc1 and Scc4 nor the stoichiometry of Scc4-Scc1 complex was affected by the location of affinity tags (N-terminal or C-terminal). These data indicate that the solubility of Scc1 is dependent upon co-expression of and binding to the Scc4 protein in E. coli.

Oligomeric States of Scc4 and the Scc4-Scc1 Complex in Solution—Despite the observation of the Scc4-Scc1 heterodimer, most class I chaperones form homodimers for targeting the effectors (12). To determine whether Scc4 does form homo-oligomers, we conducted a vapor glutaraldehyde cross-linking assay with purified Scc4 protein in vitro. Indeed, Scc4 does self-associate, as evidenced by SDS-PAGE analysis (data not shown). We then sought to identify buffer conditions that might influence the aggregation state of Scc4 in the presence or absence of Scc1 to address the possibility that Scc4 self-aggregation might influence in vitro binding experiments with Scc1. We also experimented with buffer conditions to determine those appropriate for NMR analysis. Analytical gel filtration chromatography was performed to study the aggregation states of His$_6$-Scc4 and Scc4-Scc1-His$_6$ at various protein concentrations and under different buffer conditions (Fig. 3 and Table 1). An apparent 1:1 molar ratio of eluting Scc4-Scc1-His$_6$ species was confirmed by SDS-PAGE analysis (data not shown). In Tris-buffered saline at pH 8.0, His$_6$-Scc4 (solid black line) pre-dominantly exists as a homodimer with some higher order oligomers (>300 kDa) evident, whereas the Scc4-Scc1-His$_6$ complex (dashed black line) mainly elutes as a stable tetramer (Fig. 3A), which is consistent with the tendency of Scc4 to dimerize under these conditions. In acetate buffer at pH 4.5, both His$_6$-
Scc4 and the Scc4/H18528 Scc1-His6 complex were unstable. Under these conditions (pH 4.5), His6-Scc4 alone was prone to precipitation. However, the Scc4/H18528 Scc1-His6 complex appeared to be somewhat more stable, with some soluble Scc4-Scc1-His6 complex evident as a heterodimer (solid gray line). In phosphate buffer at pH 7.3, His6-Scc4 formed a homodimer (solid black line).

**FIGURE 2.** Protein expression, solubility analysis, and stoichiometry. A, expression levels and solubility analysis for Scc4, Scc1, and Scc4 + Scc1 co-expression in E. coli cells. Protein expression was induced with IPTG (+). Proteins from different fractions were analyzed by SDS-PAGE and Coomassie Blue staining. B, gel filtration chromatography of the Scc4-Scc1-His6 complex. The Ni²⁺ column purified Scc4-Scc1-His6 complex was concentrated to 30 mg/ml. Sequential dilutions were performed on the samples and subsequently loaded on a 26/60 Superdex 75 column equilibrated with buffer N (20 mM Tris (pH 7.6), 100 mM NaCl, and 5% glycerol). The column was calibrated in buffer N using albumin (66 kDa), chicken ovalbumin (44 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and vitamin B₁₂ (1.35 kDa) as standards. C, analysis of selected 4-ml protein fractions on 12% SDS-PAGE with Coomassie Blue staining. Note that the stoichiometry of the purified Scc4 and Scc1 protein complex appears to be 1:1.

**FIGURE 3.** Gel filtration reveals various oligomeric states of Scc4 and Scc4-Scc1 influenced by buffer conditions. A, HiPrep Sephacryl S-100 HR column using 1-ml sample volumes. B, Enrich SEC 70 analytical column with 50-μl sample volumes. Injected protein concentrations were in the range of 81–152 μM with the exception of the Scc4-Scc1 complex at pH 4.5 (27 μM) due to its low solubility in this buffer (sample conditions are described in detail in Table 1). AU, absorbance units.
mine the amount of [15N]Scc4 that had exchanged with unla-

gel and subjected to quantitative LC/MS analysis to deter-

of 50 mM phosphate buffer, whereas the pH of the two buffers is

ated 8.7% of prebound Scc4 was displaced when [15N]Scc4

10-fold molar excess for a 2-h incubation period. An esti-

prebound to Scc1-His6. Approximately 12.4% of Scc4 was

dispersed by an excess of [15N]Scc4 when it was present at a

TABLE 1

| Sample        | Sample volume | Running buffer                     | Column            | Results        |
|---------------|---------------|------------------------------------|-------------------|----------------|
| Scc4 (152 μM) | 1 ml          | 20 mM Tris, 150 mM NaCl, pH 8.0     | HiPrep Sephacryl S-100 HR | Oligomer, dimer |
| Scc4-Scc1 (133 μM) | 1 ml          | 20 mM Tris, 150 mM NaCl, pH 8.0     | HiPrep Sephacryl S-100 HR | Heterotetramer |
| Scc4-Scc1 (27 μM) | 1 ml          | 20 mM sodium acetate, pH 4.5        | HiPrep Sephacryl S-100 HR | Heterodimer    |
| Scc4 (81 μM)  | 50 μl         | 50 mM sodium phosphate, pH 7.3      | ENrich 70 SEC      | Dimer          |
| Scc4 (81 μM)  | 50 μl         | 20 mM Tris, pH 7.4                   | ENRich 70 SEC      | Trimer         |

line); however, in Tris buffer at pH 7.4 (dashed black line), His$_{Scc4}$-

Scc4 surprisingly formed homotrimers (Fig. 3B). Because the

ionic strength of the 20 mM Tris buffer is about one-tenth that

of 50 mM phosphate buffer, whereas the pH of the two buffers is

nearly identical, it is likely that the homotrimers seen in the Tris

buffer are utilizing polar contacts to construct the trimers, and

these polar interactions are significantly weakened by the

higher ionic strength of the phosphate buffer. These results

indicate that at very close to physiological pH, Scc4 can exist as

a dimer or a trimer, depending on the ionic strength and per-

haps the particular ion species.

We also observed the Scc4-Scc1-His$_{Scc4}$ complex eluting as a

heterotetramer in 20 mM Tris, 150 mM NaCl at pH 8.0 and as a

heterodimer in 20 mM sodium acetate at pH 4.5. In these

experiments, there is a large difference in pH as well as differences in

ionic strength. Differences in pH will affect the protonation

state of surface amino acid residues involved in oligomeri-

zation, and ionic strength differences can affect polar

interactions.

Collectively, these data indicate that the buffer conditions

influence the oligomeric states of Scc4 and Scc4-Scc1 in vitro,

and because in general, hydrophobic interactions are less sen-

tive to ionic strength than polar interactions, variations in

oligomeric species seen here are modulated by polar interac-

tions through ionic strength differences and the protonation

state of key amino acid side chains.

**Slow Scc4 Exchange Occurs with the Scc4-Scc1 Heterodimer—** To gain insight into the binding parameters of Scc4 and Scc1, we conducted a competition protein binding assay. Purified Scc4-Scc1-His$_{Scc4}$ complex was incubated with an excess of [15N]Scc4 at two different molar ratios, 2:1 and 10:1 ([15N]Scc4/$\text{Scc4-His}_{Scc4}$), in Tris buffer (pH 7.6) for either 0.5- or 2-h time periods (Fig. 4). The resultant Scc4-Scc1-His$_{Scc4}$ complex was then isolated from free Scc4 protein using Ni$^{2+}$ affinity chromatography and subsequently subjected to SDS-PAGE isolation. The proper protein bands were excised from the gel and subjected to quantitative LC/MS analysis to deter-

mine the amount of [15N]Scc4 that had exchanged with unla-

belled Scc4 protein from the Scc4-Scc1-His$_{Scc4}$ complex. Com-

parison of Coomassie Blue-stained bands on SDS-PAGE con-

sponding to Scc4 and Scc1 indicated an apparent 1:1 stoichiometry (data not shown), showing that incubation with excess Scc4 protein does not alter the stoichiometry of the Scc4-Scc1 complex. Fig. 4, B and C, show that low levels of [15N]Scc4 were exchanged with unlabeled Scc4 that was prebound to Scc1-His$_{Scc1}$. Approximately 12.4% of Scc4 was displaced by an excess of [15N]Scc4 when it was present at a 10-fold molar excess for a 2-h incubation period. An estimated 8.7% of prebound Scc4 was displaced when [15N]Scc4 was present at a 2-fold molar excess during the same 2-h incubation period. Other Scc4 peptides showed similar incorporation ratios. These data verify a slow exchange between Scc4-Scc1-His$_{Scc4}$ and the Scc4.

**Chemical Shift Analysis of the Scc4-Scc1 Interaction—** 1H-15N HSQC NMR spectroscopy was used to monitor the competition between Scc4 homodimer formation and Scc4-Scc1-His$_{Scc4}$ heterodimer formation (Fig. 5). To this end, purified [15N]Scc4 (0.5 mM) in 20 mM ammonium acetate (pH 5.5) was analyzed before and after the addition of unlabeled, purified Scc1-His$_{Scc1}$ added at a 1:1 molar ratio (see "Materials and Methods"). Fig. 5 shows an overlay of [15N]Scc4 alone and [15N]Scc4 in complex with the Scc1-His$_{Scc1}$. The peak widths in both spectra are characteristic of a dimer (~30 kDa) rather than a monomer (~15 kDa), indicating that Scc4 alone forms a homodimer, and Scc4 in the presence of Scc1-His$_{Scc1}$ forms a heterodimer under these conditions. The shifts of the NMR peaks correspond to changes in the backbone structure of [15N]Scc4 upon binding Scc1-His$_{Scc1}$. Of the 13 amide signals shown in the inset of Fig. 5, four show little or no change in chemical shifts, and nine show varying degrees of shifts, indicating local structural changes upon heterodimer formation. Because the peak intensities of Scc4 with and without Scc1-His$_{Scc1}$ are approximately the same, and no residual peaks occur at the Scc4 homodimer chemical shifts, the Scc4-Scc1-His$_{Scc4}$ complex must have a significantly lower dissociation constant ($K_d$) compared with the Scc4 homodimer complex, favoring formation of the Scc4-Scc1-His$_{Scc4}$ complex. This observation is consistent with the low level of exchange between Scc4-Scc1 and Scc4 observed in the protein competition assays (Fig. 4). Therefore, homo-oligomers of Scc4, which presumably have a distinct function in the *C. trachomatis* developmental cycle, can be disrupted by the presence of Scc1, driving the formation of Scc4-Scc1 heterodimers, which presumably elicit their own unique effects.

**Identification of a Region of Importance for Scc4-Scc1 Complex Formation Using Proteolysis—** To define regions of each protein that are involved in binding, the Scc4-Scc1 complex was subjected to limited proteolysis coupled to LC/MS analysis (Fig. 6). Regions of the proteins that are involved in tight binding should be resistant to proteolysis, whereas exposed, extraneous sequences should be more susceptible to proteolytic cleavage. In pilot experiments, the purified Scc4-Scc1 complex remained relatively undigested by the narrow specificity protease, trypsin. However, Scc4 protein alone was susceptible to proteolysis by trypsin and was almost completely degraded over time. We next examined Scc4-Scc1 complex peptides liberated by limited digestion with trypsin. All tryptic products smaller than the Scc4 molecular weight that were clearly visualized on Coomasie-stained Tricine-SDS-PAGE were subjected to digestion.
with Glu-C protease and trypsin. The peptide bands marked by arrows (Fig. 6B), which probably resulted from cleavage at the most vulnerable regions, were subjected to LC/MS analysis. Band A, stable through several time points, contained mostly Scc1 with peptides arising from amino acid residues Arg31–Leu146, implying that the Scc1 region bound by Scc4 was located between Arg31 and the C terminus. Band B comprised amino acid residues Arg40–Lys129 from Scc4. This result implies that Scc1 protects Scc4 amino acid residues Arg40–Lys129 from trypsin attack and that these amino acid residues (Arg40–Lys129) may be heavily involved in formation of the Scc4–Scc1 heterodimer. Band C contained mostly Scc1 peptides probably arising from degraded Scc1 released from partially digested, destabilized Scc4–Scc1 complex. Similar treatment of Scc4 with trypsin led to rapid, complete degradation of Scc4, suggesting that Scc4 or its homo-oligomers may not be as stable as the Scc4–Scc1 heterodimer under these experimental conditions. These data support the notion that Scc4–Scc1 is a stable heterocomplex that is resistant to proteolysis. Fig. 6C illustrates the Scc1 Arg31–Leu146 and Scc4 Arg40–Lys129 regions mapped.
onto a homolog-based structural model of the Scc4-Scc1 heterodimer.

Role of Scc4 in Forming the C. trachomatis Scc4-Scc1-CopN Complex—Although Scc4 and Scc1 are predicted to share structural similarity to Yersinia SycN and YscB (23, 43), the amino acid residues at the interface of this Yersinia heterodimer (that complexes with Yersinia YopN (homologous to CopN)) are poorly conserved. We hypothesize that either Scc4 or Scc1 predominantly contacts CopN in the Scc4-Scc1-CopN complex. To test this, we used a modified version of the bacterial two-hybrid (BTH) assay, referred to as a “bridge” BTH assay (47). In this assay, an unfused bridging protein is expressed with two fusion protein moieties that do not interact directly with each other (Fig. 7A). One of these protein moieties is fused to the CI protein (a sequence-specific DNA-binding protein), and the other one is fused to the N-terminal domain of the subunit of RNAP (α-NTD). Interaction of the bridging protein with the two fused moieties results in the recruitment of RNAP and activation of transcription from a test promoter (here p lac-OL2-62), which drives the expression of a linked lacZ gene in the reporter strain, FW102 OL2-62 (48) (Fig. 7A). Thus, tripartite protein-protein interactions can be detected by performing the -gal assays.

Attempts to make full-length CopN fused to the α-NTD or to λCI were unsuccessful. However, we were able to obtain a truncated version of CopN (missing 50 amino acid residues from its C terminus) fused to the α-NTD, which we call α-CopNΔC. Various combinations of expression constructs (each directed by an IPTG-induced promoter) were expressed in the reporter strain. We found that the expression of Scc4 in the presence of CI-Scc1 and α-CopNΔC activated transcription from the test promoter with an increase of up to 4.2-fold compared with the non-induced control (Fig. 7B, lane 5). No significant increase in β-gal activity was seen for the control strain carrying empty vectors (Fig. 7B, lane 1). These results imply that Scc4 makes simultaneous contact with both Scc1 and CopN in the context of a Scc4-Scc1-CopN complex. Interestingly, expression of unfused Scc1 did not enhance transcription in the presence of α-CopNΔC and CI-Scc4 (Fig. 7B, lane 6). This suggests that Scc1 is unable to bridge interactions between Scc4 and CopN in the context of a Scc4-Scc1-CopNΔC complex or that Scc1 may not be stable. The latter possibility was ruled out because immunoblotting showed that stable Scc1 was present in the reporter strain co-expressing Scc1, α-CopNΔC, and CI-Scc4 (Fig. 7C). Moreover, α-Scc1 interacted with CI-Scc4 (Fig. 7B, lane 2). Individually, neither the Scc4 as the CI-Scc4 fusion protein nor the Scc1 as the CI-Scc1 fusion protein appeared to interact with α-CopNΔC (Fig. 7B, lanes 3 and 4). The individual chaperones may not provide enough surface area to form a sufficiently stable chaperone-CopN complex (to promote transcription from the test promoter). Alternatively, when Scc4 and Scc1 bind each other, there may be a significant conformational change and/or a significant stabilization of structural elements required for tight binding to CopN.
Type III Secretion Effector-Chaperone Recognition

In an attempt to visualize structural details required for the stable ternary complex, a homology model of the Scc4-Scc1-CopN complex was produced using the Phyre2 protein fold recognition server (42) (Fig. 8). The Scc4 and Scc1 structural models were made by Phyre2 using several homologous structures, including 1K3E chain A (E. coli CesT, Uniprot P58233) (49), 1TTW chain A (Y. pestis SynC, Uniprot Q7BTX0) (50), 1YJO chain A (S. enterica SicP, Uniprot P0CL16) (51), 4G6T chain A (Pseudomonas syringae ScaA, Uniprot Q87UE6) (52), and 3EPU chain B (S. enterica STM2138, Uniprot Q8ZNP3) (53). 99% of the structure for amino acid residues 1–132 of Scc4 was modeled with >90% confidence. 93% of the Scc1 structure corresponding to amino acid residues 1–136 was modeled with >90% confidence. The homology model of CopN was made using two template structures: 1XKP chain A (Y. pestis YopN amino acid residues Gln33–Thr77 and Glu65–Gly269, UniProt P68640) (43) and 4P40 chain A (C. pneumoniae CopN, amino acid residues Leu96–Glu383, UniProt Q9Z8L4) (30). 81% of CopN amino acid residues 37–60 and 77–381 were modeled with >90% confidence. Protein Data Bank entry 1XKP is a complex of three proteins (the Y. pestis plug protein YopN and two T3S class I chaperones, SycN and YscB), and these were used to arrange the

C. trachomatis models with respect to each other. Alignment of the CopN structure on YopN (from 1XKP) yielded two overlapping regions for the two structures corresponding to 18 CopN amino acid residues (within amino acid residues Gln37–Ile60) and 102 CopN amino acid residues (within amino acid residues Thr77–Asn367). The RMSD for Ca atoms after alignment of the CopN Gln37–Ile60 region is 1.18 Å (18 amino acid residues), and for the Thr77–Asn367 region it is 1.57 Å (102 amino acid residues). Scc4 and Scc1 were aligned with their counterparts from the YopN-YscB-SycN complex. Scc4 aligned with YscB (110 amino acid residues, Ca RMSD of 1.40 Å), and Scc1 aligned with YscN (86 amino acid residues, Ca RMSD of 1.51 Å). Note that nearly all contacts with CopN in this model are provided by one chaperone that is serving as the "bridging" chaperone. We have modeled Scc4 as the bridging chaperone based on the bridge BTH results (Fig. 7), but it should be noted that there is no significant difference in the structure quality when the chaperone positions are reversed (Fig. 8). With the Scc4 structure superimposed on SycN, 87 Ca atoms align with an RMSD of 1.46 Å. Similarly, 104 Scc1 Ca atoms superimpose on YscB with an RMSD of 1.48 Å.

The Scc4-Scc1-CopN Complex Requires Tight Interactions between Scc4 and Scc1—In the Scc4-Scc1-CopN model, six Scc4 amino acid residues (Glu65, Met70, Glu71, Phe75, Val85, and
mapped on the model of CopN with the degree of sequence homology to modeled interface is poorly conserved for all proteins. When our models relative to one another. Note that the sequence homology at the Phyre models were in hand, we used the 1XKP ternary structure to position “Results”). Similar Phyre2 results gave us the CopN model. Once these three low

The predicted structures of CopN, Scc4, and Scc1 proteins and interface conservation. A, the predicted quaternary structure of Scc1-Scc4-CopN aligned with the YopN, YscB, and SycN quaternary structure (brown, Protein Data Bank code 1XKP). Two segments of CopN were aligned with YopN: the N-terminal fragment Glu63–Ile60 (purple) and Thr7–Asn77 (blue). Scc4 (green) is aligned with YscB as the bridging chaperone, and Scc1 (pink) is aligned with SycN. B, the predicted chaperone-effector interface mapped on the model of CopN with the degree of sequence homology to YopN mapped by color: identical residues (green), highly similar residues (yellow), and different residues (red). The Scc4 and Scc1 models were each made by Phyre2 using several related bacterial protein structures (as detailed under “Results”). Similar Phyre2 results gave us the CopN model. Once these three Phyre models were in hand, we used the 1XKP ternary structure to position our models relative to one another. Note that the sequence homology at the modeled interface is poorly conserved for all proteins.

Arg97 lie within 5 Å of Scc1, defining a potential intermolecular interface for these chaperones (Fig. 9A). Whereas Glu63, Met70, Glu71, and Phe75 are positioned in the second α helix, Val95 and Arg97 are located in the third β strand in the Scc4 model. All of these amino acid residues fall within the Scc4-Scc1 core region that is protected from proteolysis (Fig. 6). Taking advantage of our ability to quantify specific interactions between Scc4, Scc1, and CopN through the use of our bridge BTH assay, we introduced alanine substitutions at these amino acid residue positions, singly or in combination, as an initial step toward defining structural elements in Scc4 vital to Scc1 binding (Fig. 9B). If the alanine substitutions negatively affect binding, the Scc4 and Scc1 interaction will diminish and in turn, reduce binding to CopN with a resulting decrease in transcription in the bridge BTH assay.

We were able to obtain three Scc4 mutants (Scc4_6A, Scc4_4A, and Scc4_95A) containing six, four, and one alanine substitution, respectively (Fig. 9B). We observed that the Scc4_6A mutant abolished interactions between λCI-Scc1 and α-CopNΔC, whereas Scc4_4A had a less dramatic effect in the bridge BTH assays (Fig. 9C). Interestingly, a low level of Scc4_95A induction appears to disproportionately enhance transcription from the test promoter, whereas higher levels of Scc4_95A induction do not proportionately increase transcription from the test promoter as compared with induction of wild-type Scc4 (Fig. 9C). One possible explanation for this observation is that both the Scc4-Scc1 and the Scc4-CopN interactions are strengthened by the V95A mutation. At low protein levels, Scc4_95A finds and binds to Scc1 and vigorously promotes transcription, whereas at higher induction levels, formation of the Scc4_95A homodimer is favored, and there is little net increase of “free” Scc4_95A protein that can bind to Scc1. The alanine substitutions do not have a profound effect on protein stability because immunoblotting indicated that the Scc4_95A protein was produced in an IPTG dose-dependent manner and was apparently stable, similar to the wild-type Scc4 and other Scc4 alanine mutants (Fig. 9D).

To determine whether the alanine substitutions directly affected the Scc4–Scc1 interaction, we assessed the affinity of Scc4 mutants for Scc1 in the absence of CopN. The Scc1 and His6-Scc4 or its derivatives were co-expressed in E. coli cells at high levels, and the soluble Scc4-Scc1 complexes were obtained using Ni2+-NTA affinity chromatography and analyzed by SDS-PAGE. Unlike the wild-type Scc4-Scc1 complex that displayed an apparent stoichiometry of 1:1, the ratio of Scc1 to Scc4_6A, Scc4_4A, or Scc4_95A was notably decreased (<1). These results confirm decreased affinity of these Scc4 mutants to bind with Scc1 (Fig. 9E). Because the Scc4 mutant proteins were expressed at levels comparable with the wild-type Scc4 (Fig. 9E), the observed decrease is not a result of Scc4 protein poor expression. The unbound Scc1 might not be soluble, as observed above (Fig. 2A). The weakened Scc4_95A-Scc1 interaction provides an explanation for the decreased β-gal transcription observed in the bridge BTH assay (Fig. 9D). With two independent experimental approaches, these data reveal the importance of Scc4 amino acid residues Glu63, Met70, Glu71, Phe75, Val95, and Arg97, for mediating Scc4 and Scc1 interactions.

Discussion

In this current study, we examined the dynamic Scc4, Scc1, and CopN interactions as well as the sequence and structural requirements governing these events. We have previously demonstrated a role for Scc4 in transcriptional regulation through its interaction with the σ28RNAP holoenzyme (17), and we now expand the role of this protein in the T3S process by confirming its requirement (along with Scc1), as a T3S chaperone, for CopN binding. Our study extends the role of the chlamydial Scc4-Scc1 interchaperone interaction for CopN recognition (23). Thus, we provide a new paradigm for tight control of T3S system function mediated by Scc4 and its associated proteins in C. trachomatis.

Unlike many other bacteria that have their T3S system genes clustered in dedicated pathogenicity islands in plasmids or chromosome (7, 10, 54), C. trachomatis T3S system genes are scattered throughout the chromosome (55). Whereas the Scc1 and CopN genes are adjacent to each other and co-transcribed, the Scc4 gene is located in a distinct operon encoding T3S needle proteins, chaperones, and energetic CdsN ATPase. How Scc4, Scc1, and CopN function in conjunction with the T3S protein expression, apparatus assembly, and effector secretion is unknown. Nevertheless, evidence that the Scc4-Scc1-CopN complex is formed during the late developmental cycle suggests
that this complex may play a role in an early step of the infection process. For example, this complex may act to ensure that translocators are secreted prior to effectors, so that effectors will be exported directly into the targeted host cells to promote invasion, similar to that seen with their *Yersinia* spp. counterparts (16, 56).

Our study reveals that the Scc4/H18528Scc1 complex is a tight interaction, which can form higher order oligomers under certain buffer conditions. Scc4 also forms various oligomeric species that are influenced by pH and ionic strength. Because the Scc4/H18528Scc1 complex is very stable, if both chaperones are present at equal abundance, this species will prevail. Therefore, in order for Scc4 to conduct its specific function, either it will need to be expressed at a different time in the developmental cycle of *C. trachomatis*, or Scc4 will need to be expressed at higher levels relative to Scc1. Evidence of the differential expression of the Scc4, Scc1, and CopN genes or proteins during the developmental cycle has been documented (6, 17, 28, 46, 57). Despite the technical challenges associated with tracking early protein expression in *C. trachomatis*, we detected the accumulation of Scc4 protein in HeLa cells at 16 hpi, which is significantly earlier than the 20 hpi required to detect CopN and Scc1 using IFA (46). Note that 20 hpi coincides with the start of the RB-to-EB transition and the increasing accumulation of EBs.

In the absence of Scc1 and CopN, Scc4 performs at least one independent function in *C. trachomatis* as a transcriptional regulator via its interaction with σ54 region 4 and the β-flap of the RNAP (17). The current study is unable to directly answer whether the Scc4-Scc1-CopN complex forms after Scc4 dissociates from the σ54RNAP. Presumably, when Scc1 and CopN are synthesized, Scc4 is available and binds with Scc1, and the two serve as chaperones for CopN. Based on the high affinity of the complex, Scc1 may extract Scc4 protein from σ54RNAP, thereby suddenly halting its effect on transcription. It is also possible that Scc4 is produced constitutively during the developmental cycle, early for transcriptional regulation activities and later to bind and stabilize Scc1. However, if at the time of Scc1 and CopN expression there was a significant excess of Scc4 protein, free Scc4 protein might still function in transcriptional regulation while the majority of Scc4 protein was involved in heteromer formation with Scc1. In *C. trachomatis*, Scc4 seems to stably express as determined previously by immunoblotting (17). The dual role of Scc4 (as a T3S chaperone and as a transcriptional regulator) is mediated by the expression and stability of Scc1. Clarifying the expression pattern and degradation of Scc1 may be critical to understanding the temporal function of Scc4.

Our experimental results are consistent with a predicted structure of the Scc4-Scc1-CopN complex (Fig. 8). In this model, Scc4, like YscB, is positioned as the bridging protein, making simultaneous contacts with both Scc1 and CopN. We note that there is no significant difference in the predicted structure quality when the Scc1 and Scc4 chaperone positions are reversed, based on computational comparison of Scc4 with SycN and Scc1 with YscB. This is because Scc1, Scc4, and the *Yersinia* chaperones, SycN and YscB, are all structurally homo-

![FIGURE 9. Key alanine substitutions in Scc4 affect its interaction with Scc1 and CopN.](image-url)
logous, albeit demonstrating quite low sequence conservation. However, our bridge BTH assay (Fig. 7) indicates that Scc4 bridges interactions between Scc1 and CopN and therefore should occupy the position in the complex analogous to that occupied by YscB in the IXP structure. Moreover, the limited sequence homology present apparently involves amino acid residues that comprise the hydrophobic core of the proteins and not surface amino acid residues that would mediate quaternary interactions. Another explanation for the observed lack of sequence homology at the protein interfaces is the fact that chaperones are typically very specific, and an equivalent protein from one species cannot bind the T3S system substrate effector of another species (12). For instance, the expression of C. pneumoniae Scc4, but not C. trachomatis Scc4, enhances C. pneumoniae CopN secretion in Yersinia.\(^5\)

The Scc3 from Chlamydia psittaci binds the CopN equivalent from C. pneumoniae but not C. trachomatis CopN (27).

Using limited proteolysis, we found that the Scc4 C terminus and the central region of Scc1 were required for Scc4-Scc1 interaction. Moreover, Scc4 amino acid residues important for the Scc4-Scc1-CopN interaction were identified using alanine substitutions, the bridge BTH assays, and affinity chromatography. Our data indicate that acidic amino acid residues (Glu\(^{143}\) and Glu\(^{152}\)) and hydrophobic amino acid residues (Met\(^{70}\), Phe\(^{75}\), and Val\(^{89}\)) in Scc4 are required for tight binding to Scc1. Hydrophobic and electronegative surfaces have been implicated in T3S chaperone function in effector recognition, synthesis, and stability as reported previously (12, 43, 58). Because CopN only binds the Scc1-Scc4 complex and not the individual Scc1 or Scc4 proteins, formation of the Scc1-Scc4 complex is required prior to binding of CopN. It is possible that, rather than simply increasing the size of the CopN binding surface, the Scc4 and Scc1 interaction triggers a conformational change in the chaperones that alters the quality of the bimolecular surface presented to CopN. Our data suggest that in the presence of Scc1, Scc4 will form the Scc4-Scc1 heterodimer; in the absence of Scc1, Scc4 can interact with \(\sigma^{66}\)RNAP and may do so as a homodimer. To better understand the details of its interactions with \(\sigma^{66}\) and the \(\beta\)-flap of RNAP, further mutational studies of Scc4 are warranted. These will provide a more complete understanding of how Scc4 exerts distinct functions in both transcription and the T3S process.

Our findings that Scc4 acts as both a chaperone for CopN and a transcriptional regulator via direct interaction with \(\sigma^{66}\) and the \(\beta\)-flap of RNAP suggest that the T3S system activity is intimately linked to gene expression events in C. trachomatis. We have developed a working model that depicts how T3S system activation or effector secretion might influence gene expression (Fig. 10). *Chlamydia*-host cell contact induces CopN dissociation from Scc4 and Scc1 through an as yet unidentified mechanism, followed by CopN secretion. The liberated Scc4 and/or host cell contact-induced *de novo* synthesis of Scc4 would then allow Scc4 to interact with the \(\sigma^{66}\)RNAP. In turn, the Scc4-\(\sigma^{66}\)RNAP holoenzyme selectively modulates transcription in a promoter-specific manner. This hypothetical model gives a rationale for future studies to clarify the relationship between T3S system activation and gene expression during C. trachomatis infection.

The detailed configuration of the binding interfaces involved in forming the Scc4-Scc1-CopN complex or the Scc4-\(\sigma^{66}\)-\(\beta\)-flap (of RNAP) complex will remain a puzzle until structural data is provided. Meanwhile, our results provide a foundation for further investigations of the mechanisms that triggers T3S system activation essential for infection progression. Recently, rapid progress has been made toward the development of genetic tools for studying the expression and localization of proteins from C. trachomatis (59–62). These and other advances may ultimately promote the elucidation of the mechanisms underlying the regulation of the T3S system in C. trachomatis.

**Author Contributions**—L. S. and M. A. M. designed the study. L. S., M. A. M., K. M. F., C. C., and D. K. W. wrote the paper. L. S., K. M. F., C. C., Y. C., K. K., M. L., L. L., and D. K. W. performed biochemical and genetic experiments. M. A. M., K. M. F., C. C., and D. K. W. wrote the paper. L. S., and M. A. M. designed the study. L. S., M. A. M., K. M. F., C. C., and D. K. W. wrote the paper.

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\(^5\) G. Plano, personal communication.
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