Domain Analyses Reveal That *Chlamydia trachomatis* CT694 Protein Belongs to the Membrane-localized Family of Type III Effector Proteins

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**Background:** The *Chlamydia trachomatis* secreted effector CT694 is deployed during invasion and exerts multiple effects on host cells.

**Results:** Residues 40–80 of CT694 contain a domain necessary and sufficient for peripheral localization to eukaryotic membranes.

**Conclusion:** CT694 employs membrane association to manifest effects on host cells.

**Significance:** Elucidating functional protein domains is essential to understand molecular mechanisms of infection employed by the pathogen *C. trachomatis.*

The *Chlamydia trachomatis* type three-secreted effector protein CT694 is expressed during late-cycle development yet is secreted by infectious particles during the invasion process. We have previously described the presence of at least two functional domains within CT694. CT694 was found to interact with the human protein Ahnak through a C-terminal domain and affect formation of host-cell actin stress fibers. Immunolocalization analyses of ectopically expressed pEGFP-CT694 also revealed plasma membrane localization for CT694 that was independent of Ahnak binding. Here we provide evidence that CT694 contains multiple functional domains. Plasma membrane localization and CT694-induced alterations in host cell morphology are dependent on an N-terminal domain. We demonstrate that membrane association of CT694 is dependent on a domain resembling a membrane localization domain (MLD) found in anti-host proteins from *Yersinia, Pseudomonas,* and *Salmonella* spp. This domain is necessary and sufficient for localization and morphology changes but is not required for Ahnak binding. Further, the CT694 MLD is able to complement ExoS ΔMLD when ectopically expressed. Taken together, our data indicate that CT694 is a multidomain protein with the potential to modulate multiple host cell processes.

*Chlamydia trachomatis* infection has been the most reported sexually transmitted disease in the United States because1994, with over 1.2 million cases reported in 2009 (1). However, it is believed that the true number of cases is much higher because of the potential for asymptomatic infections, particularly in males (1). Sequelae resulting from untreated or repeated *C. trachomatis* serovar D-K infections can include infertility, pelvic inflammatory disease, ectopic pregnancy, or pelvic pain (2).

Additionally, ocular infection with *C. trachomatis* serovars A–C causes blinding trachoma, the leading cause of preventable blindness worldwide, particularly in developing countries (3). An obligate intracellular bacterium, *C. trachomatis* exhibits a biphasic developmental cycle consisting of an extracellular, non-metabolic elementary body (EB) and an intracellular, replicative reticulate body (RB) (4). Both particle types possess a functional type-III secretion system (T3SS), which is essential for bacterial development (5). Contact with the host cell surface triggers secretion of effectors through the T3SS into the host cell (6). In *Chlamydia,* these immediate early effectors are packaged during the reticulate body-to-EB transition to be readily available immediately upon host cell contact (7, 8). One such effector is the conserved translocated actin recruiting phosphoprotein (TarP), which is translocated into host cells within minutes of host cell contact and induces actin cytoskeletal reorganization that is important for invasion (9). The ability to dynamically reorganize the host cytoskeleton is a common general theme among bacteria expressing T3SS, including *Yersinia* spp., *Salmonella,* *Shigella* spp., and *Pseudomonas aeruginosa* (10–12).

To achieve efficient anti-host function, some T3SS effectors must be targeted to the correct subcellular compartment (12). The presence of a membrane localization domain (MLD) is one mechanism employed to accomplish this goal. For example, discrete MLD domains within *Yersinia* spp. or ExoS of *P. aeruginosa* mediate association with host membranes (12). These MLDs lack the characteristic predicted hydrophobic α helix of a transmembrane domain (13) but contain a leucine-rich region that is essential for membrane association (12, 14). It has been proposed that interactions with membranes could be direct (15). Alternatively, there is evidence that these membrane-localized effectors do
so through interactions with membrane-associated proteins rather than direct interactions with the host cell membrane (14, 16, 17). Regardless of the mechanism, localization of effectors to cellular membranes allows a targeted response in which respective effector proteins manifest activities in a constrained microenvironment.

CT694 is a recently described C. trachomatis-specific T3SS effector (18). Aside from a predicted coiled-coil domain from residues 285–305, the primary sequence of CT694 does not contain additional domains identifiable via similarity searches or domain predictions (18). Like TarP (19), this protein is translocated during host cell invasion (18). CT694 protein levels decrease slightly around 1 h post-infection (hpi) but are then maintained until a robust increase during de novo CT694 synthesis at 18–24 hpi during late-cycle development (7, 18). Previous work (18) demonstrated that a GFP-CT694 chimera localizes to the plasma membrane, where it interacts with Ahnak, a large human protein involved in cytoskeleton maintenance and cell signaling (20, 21). Ectopic expression studies also revealed that deletion of the C terminus of CT694 precludes the interaction with Ahnak but does not affect membrane localization (18). Herein, we test the possibility that the N terminus of CT694 expresses an MLD that is necessary for localization of CT694 to host membranes.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—HeLa 229 epithelial cells (CCL 2.1, ATCC) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) FBS (Sigma-Aldrich, St. Louis, MO) at 37 °C in the presence of 5% CO2/95% humidified air. C. trachomatis serovar L2 (LGV 434, ATCC) was propagated in HeLa cells and purified through MD-76R (Mallinckrodt, St. Louis, MO) density gradients as described previously (22). For infections, HeLa monolayers were inoculated with C. trachomatis in Hank’s balanced salt solution (Invitrogen) and incubated at 37 °C for 1 h as described (22, 23). Inocula were replaced with RPMI 1640 + 10% FBS (v/v) and incubated for 24 h, unless otherwise indicated.

**DNA Methods**—C. trachomatis open reading frames were amplified from C. trachomatis serovar L2 genomic DNA using EconoTaq PLUS Green Master Mix (Lucigen, Middleton, WI) according to the guidelines of the manufacturer and using custom oligonucleotide primers containing engineered restriction sites, synthesized by Integrated DNA Technologies (Coralville, IA). Cloning was performed according to standard protocols (24). PCR products were ligated into vectors utilizing the appropriate restriction enzymes and ligated into pEGFP-C3. CT694-Δ40-80 was generated by Genewiz, Inc. (South Plainfield, NJ) using the GFP-CT694 backbone. GFP-ExoS/694-MLD was generated by insertion of restriction enzyme sites flanking the ExoS MLD on full-length GFP-ExoS by Quikchange Lightning (Agilent Technologies, Inc., Santa Clara, CA) according to the directions of the manufacturer and using primers ExoS-EcoRI-QC F/R and ExoS-KpnI-QC. The CT694 MLD insert was amplified using primers CT694 MLD EcoRI and CT694 MLD KpnI. Resulting PCR products were digested with the appropriate restriction enzymes and ligated to create GFP-ExoS/694 MLD. Primers CT694 MLD EcoRI 2 forward and reverse were used to correct out-of-frame ligation. All expression constructs were verified by DNA sequencing (Oncogenomics Core Facility, Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, FL).

**P. aeruginosa** open reading frames were amplified from either P. aeruginosa PA01 genomic DNA (Greg Plano, Miller School of Medicine, University of Miami Miller School of Medicine, Miami, FL) or P. aeruginosa PA103ΔUT expressing plasmid-encoded pUCP-ExoS mutants (Joan Olson, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV) using EconoTaq PLUS Green Master Mix (Lucigen) and primers ExoS F and ExoS R. Full-length ExoS and ExoS mutants were digested with the appropriate restriction enzymes and ligated into pEGFP-C3 (Clontech).

**Immunodetection**—For immunoblot analysis, samples were precipitated with either 10% trichloroacetic acid (sucrase gradients) or 50% acetone (Triton X-114 extractions), solubilized in 3× Laemmli buffer and resolved by SDS-PAGE 12% (v/v) homogenous polyacrylamide gels or SDS-PAGE 4–20% gradient polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), followed by transfer to Immobilon-P membranes (Millipore, Billerica, MA) in Tris-glycine buffer. Detection of specific proteins was accomplished using α-CT694 (18), α-MOMP (18), α-Tarp C terminus (19), α-Caveolin-1 (BD Biosciences), α-GAPDH (Millipore), or α-GFP (Sigma). After incubation with specific primary antibodies, immunoblots were probed with the appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma) followed by development with Amersham Biosciences ECL Plus (GE Healthcare UK Limited, Buckinghamshire, UK).

**Transfection and Microscopy**—Seminfluent HeLa monolayers were grown on 12-mm-diameter glass coverslips for immunofluorescence analysis. Monolayers were directly infected with C. trachomatis or transfected with endotoxin-free plasmid DNAs. Transfections were accomplished using Lipofectamine 2000 according to the directions of the manufacturer (Invitrogen). For indirect immunofluorescence, samples were fixed with methanol, blocked in 5% BSA (Sigma) in Tris-buffered saline plus Tween 20 (TBST) (137 mM NaCl, 2.68 mM KCl, 2.48 mM Tris base (pH 7.4), supplemented with 0.5% (v/v) Tween 20), and appropriate antibodies were diluted in 5% BSA in TBST. Proteins were visualized by direct fluorescence of GFP-containing proteins or, where indicated, with α-GFP (Sigma), and the appropriate secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Nuclear staining was achieved by staining with DAPI (Invitrogen). Images were acquired by epi-
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fluorescence microscopy using a ×60 apochromat objective plus ×1.5 intermediate magnification on a TE2000U inverted photomicroscope (Nikon, Melville, NY) equipped with a Retiga EXi 1394, 12-bit monochrome CCD camera (QImaging, Surrey, BC, Canada) and MetaMorph imaging software version 6.3r2 (Molecular Devices, Downington, PA). Images were processed using Adobe Photoshop CS2 version 9.0 (Adobe Systems, San Jose, CA).

Yeast Two-hybrid Assay—Yeast two-hybrid assays were performed as described (18). Briefly, specific plasmid constructs were transformed into Saccharomyces cerevisiae AH109 using the S. cerevisiae Easycomp transformation kit (Invitrogen) followed by selection on S.D. agar plates deficient in leucine (Leu) and tryptophan (Trp) (Clontech). CT694 (full-length and truncated) were cloned in-frame downstream of the gal4 binding domain (BD) and used as bait in the MATCHMAKER Two Hybrid System 3 (Clontech). Primers used are listed in supplemental Table S1. Interaction studies with Ahnak were performed with engineered Ahnak constructs cloned downstream of the gal4 activating domain (AD) of pGADT7 (Clontech). Colonies expressing interacting proteins were selected for on QDO x-a-gal. To rule out growth because of auto-activation on selective media, BD/CT694 was cotransformed with AD/T or pGADT7 (AD/Empty). In the same way, Ahnak constructs were cotransformed with BD/53 or pGBKTK7 (BD/Empty). Yeast protein expression was evaluated by immunoblot with α-AD, α-BD, α-HA (Santa Cruz Biotechnology, Santa Cruz, CA), α-c-myc (BD Biosciences), or α-CT694.

Membrane Fractionation—Semiclonef HeLa monolayers were mock-infected or inoculated with C. trachomatis at an MOI of 100 for 2 or 24 h, as indicated. The cultures were then scraped into 10 ml of ice-cold 0.25 m sucrose buffer (10 mM Tris (pH 7.5), 1 mM EDTA, 0.25 m sucrose) supplemented with protease inhibitors (complete mixture, Roche). Whole-cell material was homogenized for 20–25 strokes in a Dounce homogenizer followed by centrifugation to remove cell debris. The resulting supernatant was layered over a 24–44% sucrose gradient, centrifuged, and processed as described (25).

Triton X-114 extractions were performed as described previously (26). HeLa monolayers were transfected or infected with C. trachomatis serovar L2. Cultures were lysed in 1.5 ml of ice-cold 1% Triton lysis buffer (1% Triton X-114 (Sigma), 100 mM KCl, 50 mM Tris-HCl (pH 7.4)), rotated for 30 min at 4 °C, and clarified by centrifugation for 35 min (14,000 rpm at 4 °C) using an Eppendorf 5810 R centrifuge (Eppendorf AG, Hamburg, Germany). The resulting supernatant was incubated at 37 °C for 10 min, followed by centrifugation for 12 min (14,000 rpm at room temperature) to separate the detergent phase (bottom fraction) from the aqueous phase (top fraction). The aqueous phase was removed to a fresh tube containing 200 μl of 10% Triton X-114 buffer (10% Triton X-114, 100 mM KCl, 50 mM Tris-HCl (pH 7.4)), and the detergent phase was mixed with 1 ml of buffer 1 (50 mM Tris (pH 7.4), 100 mM KCl). Both phases were incubated on ice for 10 min, followed by 10 min of incubation at 37 °C, and finally centrifuged for 12 min (14,000 rpm at room temperature). The top fraction of the detergent phase was removed, and the detergent-rich phase was mixed with 1 ml of buffer 1. The detergent-depleted aqueous phase was again mixed with 200 μl of 10% Triton X-114 buffer, and each tube was subjected to the same incubations on ice, followed by 37 °C, followed by centrifugation, repeated a total of four times. The final detergent and aqueous phases were precipitated in 50% aceton (v/v) at −20 °C overnight.

Endogenous CT694 Is Membrane-associated During Early Cycle Development—We have demonstrated previously that ectopically expressed CT694 is capable of colocalization with the plasma membrane of transfected HeLa cells (18). These data raised the possibility that CT694 membrane localization correlates with effector function(s). We first sought to investigate the subcellular partitioning of endogenous CT694 to confirm the relevance of interaction with host cell membranes. HeLa cells were infected with C. trachomatis serovar L2 at an MOI of 100, disrupted 2 hpi, and lysates were subjected to a sucrose gradient fractionation. Mock-infected parallel cultures were similarly processed as an immunoblot control for antibody specificity (data not shown). Proteins in subsequent gradient fractions were probed in immunoblot analyses with antibodies specific for CT694 or TarP. Immunoblot analyses were probed with C. trachomatis major outer membrane protein (MOMP) antibodies or human Caveolin-1 as controls for intact bacteria and host membranes, respectively (Fig. 1). Both CT694 and TarP were detected in fractions 1–5 as well as fraction 14. Conversely, MOMP was detected only in the lower column fractions, indicating migration of intact chlamydiae. As expected, the host transmembrane protein Caveolin-1 was present in the top fractions, indicating that these fractions contained the majority of host cell membrane material (Fig. 1). These results are consistent with membrane association of both CT694 and TarP during C. trachomatis infections. This localization was specific for early infection time points because CT694 was detected solely in fractions containing intact bacteria when cultures were harvested during late-cycle development (supplemental Fig. S1).

We examined phase separation of CT694 in detergent extracts to further assess the subcellular localization of endog-
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A domain within residues 40–80 is necessary for membrane localization but not for interaction with Ahnak. Consistent with previous studies, CT694-transfected HeLa cells also routinely displayed aberrant cell morphology in which cellular edges had a feathered appearance (Fig. 3B). This phenotype correlated with membrane localization because versions of CT694 (694–Δ243, Δ42–694) that showed decreased abundance in membrane localization also mediated less overt alterations in HeLa morphology. In addition, HeLa cells transfected with 694–Δ40–80 were comparable with the GFP control with respect to gross morphology (Fig. 3C).

Residues 40–80 of CT694 were expressed as a GFP-fusion protein to further assess the ability of this segment to act as a membrane localization domain (Fig. 4A). Similar to full-length 694, the signal for GFP +40–80 was detectable in the plasma membrane of transfected HeLa cells. Interestingly, the cellular morphology appeared comparable with the GFP-only control. Triton X-114 extraction was also performed on HeLa cells expressing GFP, 694-FL, 694–Δ40–80, or GFP +40–80 to confirm and quantify membrane localization of chimeric proteins. Immunoblot analyses confirmed that ectopically expressed CT694, like endogenous CT694, partitioned into both the detergent and aqueous fractions (Fig. 4B). Densitometry analysis of immunoblots revealed nearly equal distribution of protein (Fig. 4C). Partitioning of 694–Δ40–80 appeared similar to the GFP-only control and was detected predominantly in the aqueous fraction. Conversely, GFP +40–80 was detected most abundantly in the detergent phase, indicating that these residues were both necessary and sufficient for membrane localization of CT694.

Residues 40–80 Constitute a Functional Membrane Localization Domain—The mechanism for membrane association was unclear given the lack of a predicted transmembrane domain in CT694. Interestingly, T3SS effector proteins in other systems have been shown to possess a functional MLD that is essential for these proteins to express respective anti-host functions. The best characterized effectors of this family include the *Yersinia* T3SS effector YopE and ExoS of *P. aeruginosa* (12). Both YopE and ExoS are targeted to the host cell plasma membrane upon translocation via a discrete MLD domain, and such localization is necessary to exert RhoGAP activity (12, 28–30). Consistent with the divergent nature of amino acids among the MLD family of proteins, there was no significant similarity between CT694 and the MLD domains of either YopE or ExoS. Likewise, there is no significant overall sequence similarity between full-length CT694 and either YopE or ExoS. This is in contrast to direct comparisons of YopE and ExoS, which share significant N-terminal homology (12, 31). However, all three domains are predicted to contain an α helix of approximately 20 residues (residues 51–72 of ExoS and 54–75 of YopE) (data not shown, 12). Because the YopE and ExoS MLD domains can be functionally exchanged (12), we tested whether replacing the MLD of ExoS with amino acids 40–80 of CT694 would result in functional complementation.

Transient expression of GFP-ExoS in mammalian cells results in cell rounding, and mutation of the MLD is sufficient to abrogate this phenotype because maintained membrane localization is required for optimal RhoGAP activity of ExoS.
**FIGURE 3.** CT694 truncation analyses. A, CT694 truncations were engineered and expressed either in HeLa cells as GFP fusion proteins to examine membrane localization or in *S. cerevisiae* for yeast-two hybrid (Y2H) screens to assess interaction with Ahnak. A "+/−" for Ahnak-binding indicates growth on stringent selection medium and is indicative of interaction between Ahnak and the respective version of CT694. A "+" for membrane localization indicates fluorescence microscopy detection of the recombinant protein in a pattern consistent with plasma membrane localization. B, representative images demonstrating aberrant cell morphology of HeLa cells expressing GFP-694-FL compared with cells expressing GFP empty vector. Insets represent digitally zoomed areas indicated by arrows. C, representative images for membrane localization and morphology changes for the indicated GFP-CT694 mutants. Scale bar = 10 μm.
We tested whether complementation of ExoS/H9004MLD with the CT694 MLD (ExoS/694MLD) would restore ExoS-mediated HeLa cell rounding. HeLa cells were transfected with 400 ng of the indicated DNA, and GFP or DAPI staining were subsequently used to assess subcellular localization and cell rounding (Fig. 5). As published previously, GFP-ExoS was detected in perinuclear concentrations, whereas GFP-ExoS/H9004MLD was localized diffusely in the cytosol (15). CT694 MLD was able to complement both the perinuclear localization and the rounded cell phenotype in cells expressing GFP-ExoS/694MLD (Fig. 5A). In addition, rounded cells were quantified for each construct, with GFP-ExoS/694MLD displaying a similar percentage of rounded cells compared with GFP-ExoS WT, whereas vector-only and GFP-ExoS ΔMLD had significantly fewer rounded cells (Fig. 5B). These data are therefore consistent with residues 40–80 containing a functional MLD that targets the chlamydial effector to the plasma membrane of infected host cells, similar to the MLD family of T3S effector proteins.

**DISCUSSION**

The data presented here support a model in which the *C. trachomatis* T3SS effector CT694 contains multiple functional domains. These domains include the previously described Ahnak binding domain (18), an undefined toxicity domain that manifests as altered cell morphology in transfected HeLa cells, and a membrane localization domain residing within amino acids 40 to 80. The CT694 MLD is necessary to target CT694 to host cell membranes. Our transfection studies show that this localization is independent of the Ahnak binding domain, and the MLD alone is sufficient to localize GFP to the plasma membrane. However, the MLD alone is not sufficient to induce host cell morphology changes, suggesting that although the Ahnak-binding activity is independent of localization, toxicity is not. In the absence of a tractable genetic system, we cannot directly establish the role of this domain during *C. trachomatis* infection. However, endogenous CT694 also localizes to cellular membranes as early as immediately post-infection and stays associated with membranes for up to 6 h post-infection. These data provide evidence that secreted CT694 does associate with host membranes and is consistent with our model in which CT694 plays a role in host cell invasion or establishment of a subcellular niche for *C. trachomatis* development.
Immediate deployment of effectors concomitant with host cell contact is a common theme among bacteria expressing a T3SS. For example, *Shigella* spp., *Salmonella* typhimurium, *Yersinia* spp., and *P. aeruginosa* each express unique sets of T3SS effectors that are translocated upon host cell contact (33). In *C. trachomatis*, the immediate-early T3SS effectors that have thus far been identified are the effectors TarP (19) and CT694 (18) and the translocator proteins CopB and CopB2 (25, 34). Of the described T3SS effectors in other systems, several associate with host cell membranes after translocation. *Shigella* effector IpaC integrates into host cell membranes, where it is involved in inducing actin polymerization to mediate invasion (9). *Yersinia* spp. encode genes for at least two effectors that are targeted to host cell membranes after translocation. YpkA/YopO is targeted to the plasma membrane, whereas YopE localizes to the perinuclear region (12, 35). *Salmonella* SptP (16) and *Pseu-
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