Chlamydia trachomatis Tarp Harbors Distinct G and F Actin Binding Domains That Bundle Actin Filaments

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All species of Chlamydia undergo a unique developmental cycle that transitions between extracellular and intracellular environments and requires the capacity to invade new cells for dissemination. A chlamydial protein called Tarp has been shown to nucleate actin in vitro and is implicated in bacterial entry into human cells. Colocalization studies of ectopically expressed enhanced green fluorescent protein (EGFP)-Tarp indicate that actin filament recruitment is restricted to the C-terminal half of the effector protein. Actin filaments are presumably associated with Tarp via an actin binding alpha helix that is also required for actin nucleation in vitro, but this has not been investigated. Tarp orthologs from C. pneumoniae, C. muridarum, and C. caviae harbor between 1 and 4 actin binding domains located in the C-terminal half of the protein, but C. trachomatis serovar L2 has only one characterized domain. In this work, we examined the effects of domain-specific mutations on actin filament colocalization with EGFP-Tarp. We now demonstrate that actin filament colocalization with Tarp is dependent on two novel F-actin binding domains that endow the Tarp effector with actin-bundling activity. Furthermore, Tarp-mediated actin bundling did not require actin nucleation, as the ability to bundle actin filaments was observed in mutant Tarp proteins deficient in actin nucleation. These data shed molecular insight on the complex cytoskeletal rearrangements required for C. trachomatis entry into host cells.

The obligate intracellular bacterium Chlamydia trachomatis causes the most frequently reported sexually transmitted bacterial disease in the United States, with over 1 million cases reported to the Centers for Disease Control and Prevention annually (1). Worldwide, ocular infection with C. trachomatis (trachoma) is the leading cause of preventable blindness and is the subject of a global initiative led by the World Health Organization to eradicate trachoma by 2020 (2).

Species of Chlamydia utilize a unique developmental cycle in which bacteria transition from the infectious spore-like elementary body (EB) to the metabolically active reticulate body (RB) within the protective confines of a membrane-bound parasitophorous vacuole termed the inclusion (3). The invasive EB is formed in the middle to late stages of the intracellular development cycle as the RBs differentiate back to EBs and are packed with metabolites and proteins designed to facilitate extracellular survival and reinfection (4, 5). Additional infectious cycles arise from EBs that are released and disseminate from infected tissues (6).

C. trachomatis invasion is induced by cytoskeletal rearrangements initiated upon microbe contact with the host cell surface (7). Alterations of the host cytoskeleton are required for bacterial uptake, as drugs, such as cytochalasin D, that disrupt the cytoskeleton prevent C. trachomatis infections (7). A number of intracellular microorganisms harbor proteins that directly alter actin dynamics, which favor pathogen survival and propagation (8). These virulence factors can drive the formation of actin filaments and actin bundles or can lead to the disassembly of actin filaments. Cytoskeletal rearrangements initiated upon EB contact with the host cell surface may in part be triggered by the translocation of type III secreted effectors (9, 10). One of the effector proteins, called translocated actin recruiting protein (Tarp), is able to increase the rate of actin filament formation by directly nucleating actin (11). In addition, Tarp and the host cell Arp2-Arp3 actin-nucleating complex cooperate to increase the rate of actin filament formation, and both host- and bacterium-derived actin nucleators are implicated in C. trachomatis invasion (12–14).

Tarp contains a C-terminal actin binding and oligomerization domain required for actin nucleation and an N-terminal phosphorylation domain implicated in host cell signaling via association with host-derived proteins, such as phosphoinositide 3-kinase (PI3K) and Src homology 2 (SH2) domain-containing transforming protein 1 (SHC-1) (11, 15–17). Phosphorylated Tarp peptides have also been shown to immunoprecipitate a complex of proteins containing Sos1 and Vav2, two Rac guanine nucleotide exchange factors thought to participate in WAVE2 and Arp2-Arp3 complex recruitment (16). Colocalization studies of ectopically expressed enhanced green fluorescent protein (EGFP)-Tarp indicate that actin filament recruitment is restricted to the C-terminal half of the effector and is presumably associated with Tarp via the previously identified actin binding alpha helix required for actin nucleation in vitro (14, 15). Sequence and biochemical analyses of Tarp orthologs from C. pneumoniae, C. muridarum, C. caviae, and C. trachomatis serovars A, D, and L2 revealed the presence of between 1 and 4 actin binding sites (13). Although C. trachomatis Tarp (L2) appeared to harbor two putative actin binding domains (ABD), only one of the two alpha helices was found to associate with actin (13). In this work, we examined the effect of domain-specific mutations on actin filament colocalization with EGFP-Tarp. Here, we report that C. trachomatis L2 Tarp harbors two distinct filamentous-actin (F-actin) binding sites that allow the Tarp effector to bundle actin filaments.
Furthermore, Tarp-mediated actin bundling did not require actin nucleation, as the ability to bundle actin filaments was observed in mutant Tarp proteins deficient in actin nucleation. These findings attribute a novel activity to the critical Tarp protein and provide molecular insight into the complex cytoskeletal rearrangements required for *C. trachomatis* entry into host cells.

**MATERIALS AND METHODS**

Cloning and protein expression. In-frame amino-terminal glutathione S-transferase (GST) and carboxyl-terminal polyhistidine fusion proteins for full-length wild-type Tarp were generated by PCR by amplifying the corresponding coding regions from *C. trachomatis* serovar L2 LGV 434 genomic DNA (Qiagen genomic purification kit; Valencia, CA) as previously described (14). PCR was performed with custom synthesized oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) engineered with Sall, SacI, or NotI linkers. PCR products were purified (Qiagen), digested with restriction enzymes (New England BioLabs, Beverly, MA), and subcloned into linearized pGEX-6P-1 to generate translational fusions with GST and polyhistidine. Tarp domain deletion mutants—phosphorylation domain deletion (ΔphoD); deletion of D125 to Y424), proline-rich-domain (PRD) deletion (ΔPRD; deletion of S625 to N650); actin binding domain deletion (ΔABD); deletion of amino acids [aa] 748 to 758), F-actin binding domain deletion 1 (ΔFAB1; deletion of L871 to L882), and F-actin binding domain deletion 2 (ΔFAB2; deletion of N942 to 967)—were generated by inverse PCR by amplifying the pGEX-6P-1 plasmids encoding the wild-type Tarp fusion protein. Multiple domain deletions in a single *tar* gene (for example, ΔABD, ΔFAB1, and ΔFAB2) were generated sequentially by inverse PCR or by ligating individual deletion mutants together. The Tarp mutants described above were also cloned into pEGFP-C3 (BD Biosciences Clontech) to allow ectopic expression of EGFP-Tarp in HeLa cells. All pGEX-6P-1 plasmids were transformed into the BL21 strain of *Escherichia coli* (Novagen, Madison, WI). Protein expression and purification were performed according to the procedures outlined for Ni Sepharose 6 Fast Flow and glutathione Sepharose 4B in the Bulk GST Purification Module (GE Health Sciences, Piscataway, NY). In some experiments, the GST tag was removed prior to F-actin binding and bundling with PreScission Protease treatment according to the manufacturer’s recommendation (GE Health Sciences).

GST fusion pulldown experiments. GST fusion pulldown experiments were performed according to protocols previously described (11, 13). Briefly, HeLa 229 cells were suspended in 100 mM KCl, 10 mM HEPES (pH 7.7), 2 mM MgCl₂, and 2 mM ATP (buffer A) and disrupted by sonication delivered in four consecutive bursts at 20 s intervals on setting 4 (ultrasonic sonicator processor XL equipped with a microtip; Misonix Incorporated, Farmingdale, NY). Insoluble material was removed by centrifugation (12,000 × g, 25 min; 4°C). Glutathione-Sepharose beads were incubated with 10 μg of GST fusion proteins or GST for 1 h at 4°C in PBS (GE Health Sciences), GST fusion protein-coated beads were washed twice with PBS and once with buffer A prior to the addition of approximately 100 μg of HeLa extract. The extracts and beads were incubated together for 2 h at 4°C and washed three times with fresh buffer A, and bound proteins were eluted using sample buffer.

SDS-PAGE and immunoblotting. Proteins were separated on SDS-10% polyacrylamide gels and transferred to 0.45-μm pure nitrocellulose transfer and immobilization membranes (Schleicher & Schuell, Keene, NH). Immunoblotting employed peroxidase-conjugated secondary antibodies (Chemicon International, Temecula, CA) and Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The anti-actin C4 monoclonal antibody was purchased from Chemicon International. The anti-phosphotyrosine 4G10 monoclonal antibody was purchased from Upstate (Millipore). Polyclonal rabbit antibodies directed toward *C. trachomatis* L2 LGV 434 Tarp (CT456) were developed at Rocky Mountain Laboratories as previously described (9). Peptide antibodies directed toward the Tarp actin binding domain and PRD were generated and purified by Sigma Genosys (Spring, TX) as previously described (13).

Transfection of HeLa cells and indirect immunofluorescence microscopy. HeLa cells (2 × 10⁵) were seeded in 6-well plates with coverslips and grown for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were then transfected with transfection mixture containing 8 μl of Fugene HD (Promega) and 2.5 μg of the respective plasmid. After 24 h, the cells were fixed by adding 4% paraformaldehyde and incubating them at 4°C for 15 min. The cells were then treated with ice-cold 0.4% Triton X for 10 min, followed by blocking with 5% bovine serum albumin (BSA) for 45 min. To visualize tyrosine-phosphorylated protein, cells were first incubated with anti-phosphotyrosine primary antibody 4G10 (Upstate) at 1:1,000 dilution in 0.5% BSA at room temperature (RT) for 45 min, followed by incubation with anti-mouse secondary antibody conjugated to Alexa 350 (Invitrogen). To simultaneously visualize actin, phallolidin conjugated to Alexa 568 (Invitrogen) was added to the above-mentioned mixture containing secondary antibodies. Coverslips were rinsed and mounted in Prolong Gold antifade reagent (Invitrogen). The cells were examined with a Zeiss Axios Observer A1 microscope equipped with phase-contrast and epifluorescence optics. Images were obtained using an AxioCam MRm camera controlled by AxioVision 4.8.2 and further processed using Adobe Photoshop CS2.

F-actin binding and bundling. Briefly, 5 μg of GST fusion proteins or control proteins (GST and α-actinin) was added to 40 μg of F actin (generated from adding 1/10 volume of polymerization buffer to globular actin (G actin) and incubating them at RT for 1 h) and allowed to incubate at RT for 30 min. F actin and the bound proteins were separated by differential sedimentation at 100,000 × g for 2 h at RT in a Beckman Optima TLX Ultracentrifuge using a TLA 55 or TLA 100.3 rotor (Beckman Coulter, Fullerton, CA). Proteins associated with the F-actin pellets were compared to unbound proteins that remained in the supernatant by resolving the proteins on 10% SDS-polyacrylamide gels, followed by Coomassie staining. Actin-bundling experiments were performed similarly to F-actin binding assays, except the actin bundles were isolated with a 15,000 × g spin.

Pyrene assay. Pyrene actin polymerization assays were performed as previously described (11, 13, 14). Briefly, monomeric pyrene-labeled actin was prepared by diluting 100 μg of lyophilized pyrene actin (cytoskeleton) in 2 ml of 5 mM Tris (pH 8.0)-0.2 mM CaCl₂-0.2 mM ATP (G buffer) and incubating for 1 h at RT, followed by 1 additional hour of incubation at 4°C. Monomeric pyrene actin was obtained by collecting the supernatant after a 2-h, 100,000 × g, 4°C spin in a Beckman Optima TLX Ultracentrifuge using a TLA 100.3 rotor (Beckman Coulter). Approximately 20 μg of pyrene-labeled actin was gently mixed with 5 μg of GST fusion proteins in a volume of 500 μl for 10 min before the addition of 1/20 volume of polymerization buffer (500 mM KCl, 20 mM MgCl₂, 10 mM ATP). The reaction was monitored for 1 h with an LS 55 Luminescence spectrophotometer directed by FL WinLab software version 4.0 (Perkin-Elmer, Beaconsfield, Bucks, United Kingdom) with 2.5-nm bandwidth at 365-nm excitation wavelength and 2.5-nm bandwidth at 407-nm emission wavelength.

RESULTS

Mutant Tarp proteins exhibit unique actin binding and polymerization kinetics. The actin-nucleating activity of Tarp results from distinct actin binding and proline-rich oligomerization domains in *vitro* (11). *C. trachomatis* L2 Tarp is a large, 1,005-aa protein, and studies to date have primarily focused on recombiant Tarp truncation mutants or Tarp peptides to identify the domains of the protein responsible for Tarp-mediated actin polymerization (9, 11, 13). To confirm that the previously identified domains were sufficient for actin binding and actin nucleation in the entire Tarp effector, we generated a series of GST-His and EGFP recombinant full-length L2 Tarp deletion mutants that are missing the phosphorylation, the actin binding, and/or the pro-
line-rich oligomerization domain(s) to examine the contribution that each domain makes to actin kinetics biochemically and in HeLa cells (Fig. 1A). Wild-type Tarp and deletion mutant Tarp proteins with dual N-terminal GST and C-terminal polyhistidine affinity tags were purified and employed in actin binding and actin polymerization assays (Fig. 1B and C). All full-length Tarp mutants were able to associate with host cell actin in a GST pulldown assay, except for the ΔABD Tarp mutant harboring an 11-amino-acid deletion (aa 748 to 758) of the previously characterized actin binding domain (Fig. 1B) (11). The purified Tarp proteins also demonstrated distinct actin polymerization kinetics, as observed in vitro pyrene actin polymerization assays (Fig. 1C). An increase in the rate of actin polymerization was observed in wild-type Tarp and the Tarp effector harboring a deletion in the phosphorylation domain compared to actin-only controls, which is in agreement with reports localizing the Tarp actin-nucleating activity to the C-terminal half of the protein (9, 11, 13, 14). Consistent with previous studies, a short PRD of 25 amino acids, implicated
in Tarp oligomerization, was required for Tarp-mediated actin nucleation. Deletion of PRD resulted in the creation of an actin-sequestering protein (TarpΔPRD) that, in the pyrene actin polymerization assay, produced a curve that appeared below the actin-only and GST controls (Fig. 1C) (11). Surprisingly, when actin filament colocalization with EGFP-Tarp was examined in HeLa cells with Alexa-conjugated phalloidin, Tarp mutants lacking the actin binding alpha helix, amino acids 748 to 758 (TarpΔABD), known to be essential for Tarp-mediated actin nucleation (Fig. 1C) retained the ability to colocalize with actin filaments (Fig. 1D). A Tarp-EGFP fusion lacking amino acids 749 to 1005 (TarpΔ749-1005) did not colocalize with phalloidin and served as a negative control (Fig. 1D). EGFP-Tarp harboring mutations in both the phosphorylation and actin binding domains was also found to colocalize with actin filaments (data not shown). The actin pulldown and actin polymerization experiments differ from the actin filament colocalization experiments in that the first two experiments primarily use G actin, whereas the last primarily uses F actin. These data suggest that Tarp may harbor an as yet uncharacterized F-actin binding domain(s) distinct from the previously characterized G-actin binding domain that is essential for actin nucleation.

C. trachomatis L2 Tarp harbors a distinct F-actin binding domain. Tarp orthologs contain between one and four actin binding domains, according to GST pulldown assays performed with HeLa extracts (13). Previous reports have indicated that C. trachomatis L2 Tarp harbors one actin binding domain, which is consistent with the data presented in Fig. 1B (11, 13). Deletion of 11 amino acids contained within the L2 Tarp actin binding domain was sufficient to prevent actin binding in the GST pulldown assay with the mutant protein compared to the wild-type control. Interestingly, bioinformatics analysis of the entire L2 Tarp protein sequence identified a putative second actin binding domain with sequence similarity to the experimentally characterized L2 Tarp actin binding domain; however, this sequence lacked the ability to associate with host cell actin in a GST pulldown assay (13). This finding further supports the prediction that Tarp may harbor protein domains that differentiate between monomeric (globular) actin, found predominately in the HeLa-generated protein lysates, and filamentous actin, detected by fluorescent phalloidin in the transfected host cells. In order to examine whether the second actin binding-like domain found in the C. trachomatis L2 Tarp sequence (amino acids 871 to 883) was able to differentially associate with globular versus filamentous actin, GST-Tarp fusions to the domain were tested for their ability to associate with actin generated from HeLa lysates in a GST pulldown assay and an F-actin cosedimentation binding assay (Fig. 2). Similar to our previous findings, the 100-amino-acid peptide harboring the original actin binding domain was able to associate with actin generated from a HeLa lysate; however, the alternate putative actin binding sequence did not associate with actin generated from the same lysate (Fig. 2A) (11, 13). Interestingly, the two domains did cosediment with filamentous actin, indicating that the second domain preferentially associates with filamentous actin, while the original actin binding domain is able to associate with both monomeric and filamentous actin, as previously described (Fig. 2B) (11). Since the second actin binding sequence prefers F actin, we have called this site F-actin binding domain 1 (FAB1) to differentiate it from the originally characterized ABD, which associates with both G and F actin. Previous studies examining the conserved domains within Tarp orthologs demonstrated that, like C. trachomatis L2 Tarp, C. trachomatis serovar A and D Tarps each harbored a domain that surprisingly did not associate with G actin in GST pulldown experiments (13). In order to determine if C. trachomatis serovar A and D Tarps harbored FAB1, GST-Tarp fusions harboring approximately 100 amino acids of each domain (C. trachomatis serovar A T940-D1040 and C. trachomatis serovar D Q820-K940) were tested in F-actin cosedimentation experiments (Fig. 2C). Similar to C. trachomatis L2 Tarp, the comparable protein domains within C. trachomatis serovar A and D Tarps were able to associate with filamentous actin (Fig. 2C).
4C). Tarp proteins lacking all three actin binding sites showed the least F-actin binding (Fig. 4C).

**Tarp bundles actin filaments.** Tarp has previously been shown to function as an actin nucleator (11). The actin-nucleating activity was localized to a 200-amino-acid region of the Tarp protein sequence that was found to contain a proline-rich region responsible for protein oligomerization and a solitary actin binding domain (11). This actin binding domain was able to associate with monomeric and filamentous actin (11). In light of the identification of two additional F-actin binding domains, we sought to examine whether the Tarp protein was capable of bundling actin filaments (Fig. 5). Actin bundles sediment at a higher rate than actin filaments and monomeric actin. Therefore, proteins capable of bundling actin filaments will appear in the pellet upon low-speed centrifugation. Interestingly, Tarp functioned to bundle actin filaments (Fig. 5A); however, actin bundling was not dependent on Tarp-mediated actin nucleation, as the Tarp ΔPRD mutant, which fails to nucleate actin in vitro (Fig. 1), retained actin-bundling activity (Fig. 5B). The Tarp triple mutant lacking the ABD, FAB1, and FAB2 alpha helices was unable to bundle actin filaments, which is consistent with both the EGFP colocalization and F-actin cosedimentation results (Fig. 5A).

**DISCUSSION**

The Tarp effector is a multifunctional protein that primes the host cell for bacterial entry and residence. We now demonstrate that, in
addition to the previously characterized G-actin binding/nucleating domain (11), the Tarp protein harbors two distinct F-actin binding/bundling domains (FAB1 and -2). All three domains are similar in that they mediate a direct link to the host cytoskeleton, yet biochemically, they are discrete sites that specifically associate with globular or filamentous actin. A comparison of Tarp orthologs indicates that the FAB1 and FAB2 sites are conserved among serovars of *C. trachomatis* and may also be present in *C. caviae* and *C. muridarum*. F-actin-specific binding sites might also be found in other species, such as *C. pneumoniae*, but will have to be located biochemically, as sequences of some Tarp orthologs are more divergent, making domain comparisons more difficult to predict.

EB attachment to the surface of an epithelial cell ultimately results in the formation of an actin-rich pedestal at the site of contact and is associated with bacterial invasion (7). Actin filament-destabilizing drugs, such as cytochalasin D, inhibit the formation of these projections and subsequent uptake of *C. trachomatis* (7). The arrangement of the actin filaments within the pedestal is unknown, but presumably, the actin filaments form polarized actin bundles (actin filaments sharing the same orientation with respect to their barbed (+) and pointed (−) ends) similar to those characterized in microvilli and filopodia (18). Actin-bundling proteins, such as fascin 1, colocalize with filopodia on the leading edge of the growth cones of developing nerve cells and are implicated in the formation of actin bundles (19). Similarly, Tarp may play a role in the formation of actin bundles located directly beneath the invading microbe. *C. trachomatis* entry into...
host cells in vitro is temperature dependent and involves the recruitment of actin to the site of EB attachment (7). Once internalized, the recruited actin quickly disseminates. The molecular details of actin disassembly are not well defined, but the process may involve the translocation of the chlamydial effector CT694 (10). CT694 associates with the human AHNAK protein, and ectopic expression of CT694 in HeLa cells is associated with a reduction in stress fibers (10). It is interesting to speculate that actin depolymerization may drive EB entry, and the recent examination of chlamydial invasion in the presence of actin filament-stabilizing drugs, such as jasplakinolide (Jas), supports this hypothesis, as Jas was found to inhibit EB entry (14, 20). However, the effects of Jas on actin filaments in vivo is controversial, as changes in cell morphology that are consistent with a reduction in filamentous actin are observed in some Jas-treated cells (21).

Similar to Tarp, the Salmonella enterica serovar Typhimurium SipC effector is able to nucleate actin and to bundle actin filaments (22). Recently, mutant bacteria lacking the C-terminal region of SipC responsible for F-actin binding and bundling were found to be less invasive than wild-type Salmonella, suggesting that the bundling activity of SipC plays a role in pathogen entry into HeLa cells (23). Whether Tarp’s ability to bundle actin filaments also contributes to pathogen entry is unknown but worthy of investigation, as new molecular tools continue to be developed to examine the genetic requirements of C. trachomatis pathogenicity (24, 25).

Actin bundles are tightly controlled by a variety of actin binding proteins (ABPs) that drive specific cytoskeletal processes and result in actin assemblies of defined thickness, length, and organization. The architecture of Tarp-mediated actin bundles has not
that favor host cell survival during early chlamydial development (17). The significance of Tarp’s association with PI3K still requires further investigation, although *C. trachomatis* infection of host cells has been found to lead to PI3K activation, and this activation is thought to contribute to host cell survival (30). We have previously reported that Tarp phosphorylation *in vitro* does not alter actin polymerization kinetics (14). The data presented in this report further corroborate the model in which Tarp appears to be divided into two separate functional parts that reside within each half of the Tarp polypeptide. Tarp phosphorylation and subsequent association with host cell signaling and adapter proteins is restricted to the amino-terminal half of the protein, whereas Tarp-mediated actin nucleation and actin filament binding is restricted to the carboxyl-terminal half of the Tarp protein. The report further defines the biochemical bridge between the Tarp effector and the cytoskeleton of the host cell by examining the two newly characterized FAB domains. These findings attribute a novel activity to the critical Tarp protein and provide molecular insight into the complex cytoskeletal rearrangements required for *C. trachomatis* entry into host cells.

**ACKNOWLEDGMENTS**

We thank Mollie Jewett and Ted Hackstadt for careful review of the manuscript and acknowledge the technical assistance of Talia Chavez.

This work was supported by the NIAID, NIH, K award 5K22AI81729-2 and a University of Central Florida grant to T.J.J.

**REFERENCES**