An α-Helical Core Encodes the Dual Functions of the Chlamydial Protein IncA*

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Background: The inclusion protein IncA inhibits and activates membrane fusion events during infection.
Results: In vitro assays and dominant negative Chlamydia mutants show that a protease-resistant core of IncA forms dimers and encodes both functions of IncA.
Conclusion: IncA forms stable coiled-coils to manipulate membrane fusion.
Significance: This project provides the most detailed understanding of how a chlamydial inclusion protein operates to manipulate membrane fusion.

Chlamydia is an intracellular bacterium that establishes residence within parasitophorous compartments (inclusions) inside host cells. Chlamydial inclusions are uncoupled from the endolysosomal pathway and undergo fusion with cellular organelles and with each other. To do so, Chlamydia expresses proteins on the surface of the inclusion using a Type III secretion system. These proteins, termed Incs, are located at the interface between host and pathogen and carry out the functions necessary for Chlamydia survival. Among these Incs, IncA plays a critical role in both protecting the inclusion from lysis and manipulating the homotypic fusion of inclusions. Within IncA are two regions homologous to eukaryotic SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor) domains referred to as SNARE-like domain 1 (SLD1) and SNARE-like domain 2 (SLD2). Using a multidisciplinary approach, we have discovered the functional core of IncA that retains the ability to both inhibit SNARE-mediated fusion and promote the homotypic fusion of Chlamydia inclusions. Circular dichroism and analytical ultracentrifugation experiments show that this core region is composed almost entirely of α-helices and assembles into stable homodimers in solution. Altogether, we propose that both IncA functions are encoded in a structured core domain that encompasses SLD1 and part of SLD2.

Chlamydia trachomatis is the etiologic agent of human urogenital infection and of trachoma, the world’s leading cause of infectious blindness (1). Chlamydia infection is the most frequently reported bacterial sexually transmitted disease in the United States, with ~1.4 million new cases reported annually (Centers for Disease Control, United States). Failure to treat infections can result in tissue scarring, infertility, pelvic inflammatory disease, and ectopic pregnancy in females as well as permanent blindness in the case of ocular infections. Despite the availability of antibiotics to treat acute infections, C. trachomatis remains a significant medical and economic burden worldwide.

Members of the family Chlamydiaceae, including C. trachomatis, exhibit a characteristic two-stage developmental cycle as spore-like elementary bodies and as larger, metabolically active reticulate bodies. After internalization, elementary bodies differentiate into reticulate bodies, which proliferate inside a specialized membrane-bound compartment called an inclusion (2). The survival of Chlamydia depends on its ability to rapidly modify the nascent inclusion in order to (i) isolate it from the canonical endolysosomal pathway that normally leads to degradation and bacterial clearance, (ii) induce inclusion-inclusion (homotypic) fusion, and (iii) generate novel fusion events with host organelles to acquire nutrients (3–6). To accomplish these modifications, bacterial effectors known as inclusion membrane proteins (Incs) are shuttled to and embedded in the inclusion membrane via a putative Type III secretion system (7, 8), where they play essential roles in pathogenicity, tissue tropism, and maintenance of inclusion integrity (9, 10).

IncA was one of the first Incs for which a role in pathogenesis was established (11–13). IncA is a ~30-kDa transmembrane protein that contains two putative α-helical/SNARE-like domains, suggesting that this bacterial protein functionally mimics eukaryotic SNARE proteins (14, 15).

SNAREs are responsible for most membrane fusion events in eukaryotic cells. They are located on virtually every intracellular compartment and use specific α-helical domains (SNARE motifs) to assemble into stable multimeric complexes and cat-

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* The abbreviations used are: Inc, inclusion membrane protein; CTD, C-terminal domain; NBD, nitrobenzoxadiazole; SLD, SNARE-like domain; pk1, postinfection; Tet, anhydrotriacycline; TMD, transmembrane domain; VAMP, vesicle-associated membrane protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (systematic); MOI, multiplicity of infection; pk1 and pk2, peak 1 and 2, respectively.
analyze the fusion of the lipid bilayers on which they reside (16–18). In addition to their role in membrane fusion, certain SNARE proteins (known as inhibitory SNAREs, or i-SNAREs) are capable of suppressing certain fusion events to maintain organelle homeostasis (19). Thus, SNARE proteins constitute a universal membrane fusion machinery in eukaryotic cells and are prime targets for intracellular bacteria that need to establish residence inside host cells (20).

IncA has been shown to play a role in the homotypic fusion of inclusions (13). Microinjection of anti-IncA Fab fragments into C. trachomatis-infected cells and targeted mutation of the incA gene result in the accumulation of multiple, non-fused inclusions containing otherwise viable bacteria (13, 21). Recently, we have shown that IncA is also able to inhibit SNARE-mediated membrane fusion, likely to prevent fusion between the inclusion and the endocytic compartments (22, 23). Interestingly, clinical isolates of C. trachomatis lacking IncA display smaller inclusions and are significantly less virulent than IncA-positive strains (12, 24, 25).

In order to understand how IncA encodes both fusogenic and inhibitory functions, we previously studied the role of each SNARE-like domain contained in the protein (named SLD1 and SLD2; see diagram in Fig. 1A). We established that either SLD is capable of blocking host SNARE-mediated membrane fusion independent of the other, however, both domains are required to promote the homotypic fusion of inclusions (23).

In order to define the mechanism of action of IncA, we have now identified and characterized a trypsin-resistant region of IncA (the so-called IncA-Core) that spans the entire length of SLD1 and about one-third of SLD2; see diagram in Fig. 1A). We established that either SLD is capable of blocking host SNARE-mediated membrane fusion independent of the other; however, both domains are required for the fusion of the lipid bilayers on which they reside (16–18). In addition to their role in membrane fusion, certain SNARE proteins (known as inhibitory SNAREs, or i-SNAREs) are capable of suppressing certain fusion events to maintain organelle homeostasis (19). Thus, SNARE proteins constitute a universal membrane fusion machinery in eukaryotic cells and are prime targets for intracellular bacteria that need to establish residence inside host cells (20).

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In order to define the mechanism of action of IncA, we have now identified and characterized a trypsin-resistant region of IncA (the so-called IncA-Core) that spans the entire length of SLD1 and about one-third of SLD2. Limited proteolysis and circular dichroism experiments suggest that this region is highly structured and very stable. In addition, biochemical and cellular assays demonstrate that this region encodes the dual functions of the full-length wild type protein. The physiological relevance of these data were confirmed using Chlamydia expressing inducible wild type and dominant-negative IncA as FLAG-tagged proteins. Interestingly, IncA-Core predominantly forms dimers in solution, as compared with WT-IncA, which is mostly monomeric. However, during infection, both WT and IncA-Core form oligomers. This suggests that the C-terminal peptide that is missing in IncA-Core controls the oligomeric state of IncA and that a cellular and/or bacterial factor releases this peptide during infection for IncA to become fully fusogenic. The identification of the functional core of IncA provides the basis for a more comprehensive understanding of its mechanisms of action and could aid in the development of novel therapeutics capable of interfering with its pathogenic function in vivo. Furthermore, such a structurally well defined region may be used to screen the proteomes of other intracellular bacteria, such as Salmonella typhi and Mycobacterium tuberculosis, for the presence of similar SNARE-like proteins.

### EXPERIMENTAL PROCEDURES

Recombinant DNA and Cloning—Tables 1 and 2 provide a summary of the primers and plasmids used in this study. The plasmid encoding the soluble C-terminal domain of IncA named ΔTMD-IncA (designated FD199) was described previously (23). The plasmid encoding IncA-Core was constructed by PCR-amplifying the region spanning Thr97–Leu237 using primers FO515 and FO516 and FD201 (the plasmid encoding wild type full-length IncA) as template DNA. The PCR product was digested with Ncol and Xhol and ligated into pET28a (Thermo Scientific) digested with the same enzymes. The resulting plasmid was designated FD577. His6-tagged TMD-IncA-Core (residues 1–237) was PCR-amplified using primers FO160 and FO527 and FD201 as template DNA. PCR product was digested with Ndel and BamHI (Thermo Scientific) and ligated into pET28a. This construct was designated FD582. DsRed-TMD-IncA-Core was constructed by PCR-amplifying the region spanning Met1–Leu237 using primers FO510 and FO511. The PCR product was digested with NotI and SalI and ligated into pDsRed-monomer-C1 kindly provided by Dr. Peter Antinozzi, Wake Forest School of Medicine). This construct was designated FD579. FD507 and FD509 (mammalian expression plasmids for IncA WT and SLD1, respectively) were described previously (23). pBOMB-IncA (also known as FD569) was constructed by amplifying the ORF of wild type IncA using primers FO510 and FO511. The PCR product was digested with NotI and Sall and ligated into the corresponding sites of pBOMB-tet-mCherry (26). pBOMB-SLD1 (also known as FD573) was constructed by amplifying residues 1–141 of wild type IncA using primers FO510 and FO512 and digesting and ligating as above. pBOMB-SLD2 (also known as FD576) was constructed by amplifying the ORF of IncA (mutant SLD1) using primers FO510 and FO511. FD472, described previously (23), was used as the DNA template. The mutations in this construct are I106D, F108A, F117A, F124A, T127D, V134D, F138A, and F145A. pBOMB-TMD-IncA-Core (FD588) was constructed by amplifying residues 1–237 of wild type IncA using primers FO510 and FO538 and digested and ligated into pBOMB-tet-
mCherry as described above. Plasmids were sequenced at the Nucleic Acids Facility at Thomas Jefferson University.

Protein Expression and Purification—Expression plasmids for all recombinant constructs were transformed into BL21(DE3) Escherichia coli and grown in Luria-Bertani (LB) medium to an optical density (at 600 nm) of 0.5. Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.2 mM and induced at 16 °C overnight. After centrifugation, ΔTMD-IncA and IncA-Core bacteria pellets were then resuspended in Buffer A (200 mM NaCl, 50 mM phosphate, 10% (w/v) glycerol, pH 7.4) and purified as described (23). Proteins were resolved on a HiLoad 16/60 Superdex preparation grade 200 column (Amersham Biosciences) equilibrated with Buffer A containing 1 mM dithiothreitol (DTT). Protein-containing fractions were collected and concentrated. ΔTMD-IncA was flash-frozen in liquid nitrogen and stored at −80 °C. IncA-Core peaks 1 and 2 were kept at 4 °C. The purity of each protein was assessed by SDS-PAGE and Coomassie Blue staining. Protein concentrations were determined by measuring the optical density at 280 nm.

Limited Proteolysis and N-terminal Sequencing—10 μg of ΔTMD-IncA in Buffer A supplemented with 1 mM DTT was incubated with varying mass ratios of trypsin in a total reaction volume of 15 μl. Reactions were stopped by adding PMSF to a final concentration of 1 mM and protein sample buffer to a final SDS concentration of 2% (w/v) and boiling at 95 °C for 10 min. Reaction products were resolved by SDS-PAGE using 12% bisacrylamide glycine gels or commercial Tris-Tricine 10–20% acrylamide gels (Bio-Rad). Gels were revealed with Coomassie Blue staining.

For N-terminal sequencing, trypsin fragments were resolved by SDS-PAGE and transferred onto PVDF membranes using a standard transfer protocol (23). Regions of the PVDF membrane corresponding to the different fragments were excised and sent for sequencing at the Tufts University Core Facility (Boston, MA).

Homology Modeling—The amino acid sequence of C. trachomatis IncA spanning Ser76–Ser273 was used as the query sequence in PHYRE2 (27). The Protein Data Bank file of the homology model was analyzed using Chimera (University of California, San Francisco).

MARCOIL Analysis—The entire amino acid sequence of C. trachomatis IncA was used as the query sequence in MARCOIL (28). The sequence was scored against the 9FAM matrix with default settings.

Circular Dichroism—Proteins were diluted to 10 μM in Buffer A supplemented with 1 mM DTT and analyzed in a 0.1-cm quartz cuvette (Starna) using a Jasco-810 spectropolarimeter. Wavelength scans were taken between 196 and 250 nm at 20 °C in the X-Ray Crystallography and Protein Characterization Core Facility at Thomas Jefferson University. For each replicate, five accumulations were taken and averaged. At least three replicate scans for each protein were performed. Raw data were normalized to the mean residue ellipticity (Φ) for each protein using the following equations,

$$\text{Mean residue weight (MRW)} = \left( \frac{\text{molecular weight}}{\text{no. of residues} - 1} \right)$$ (Eq. 1)

$$\text{Mean residue ellipticity (Φ)} = \left( \frac{\text{mdeg} \times \text{MRW}}{\text{path length} \times \text{concentration}} \right)$$ (Eq. 2)

where the molecular mass is expressed in daltons, mdeg is the raw output data from the spectropolarimeter, the path length is expressed in mm, and protein concentration is expressed in mg/ml. The percentage secondary structure in each construct was calculated using the K2D3 algorithm (29).

Analytical Ultracentrifugation—ΔTMD-IncA, IncA-Core peak 1, and IncA-Core peak 2 obtained after size exclusion chromatography were diluted in Buffer A supplemented with 1 mM DTT to concentrations between 25 and 130 μM and subjected to analytical ultracentrifugation analysis using a Beckman Coulter ProteomeLab XL-I analytical ultracentrifuge equipped with absorbance optics and an eight-hole An-50 Ti analytical rotor. Sedimentation equilibrium experiments were carried out at 4 °C by collecting absorption scans at a fixed wavelength of 278 nm and spinning samples at four different velocities of 12,000, 18,000, 24,000, and 30,000 revolutions/min. The program SEDPHAT version 1.09 was used to correct the experimental s value to standard conditions at 20 °C in water ($s_{20,w}$) and to calculate the partial specific volume of each protein, solvent density, and relative viscosity values along with the theoretical molecular mass (30). Data were fit to a "species analysis" model available in the SEDPHAT program (31, 32).

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**TABLE 2**

Plasmids used in this study

All plasmids carry the gene cassette encoding resistance to kanamycin unless stated otherwise.

| Plasmid name | Relevant characteristics | Reference/Source |
|--------------|--------------------------|------------------|
| pET28a       | IPTG-inducible expression of His-tagged proteins in E. coli | Novagen |
| FD199        | pET28 expression vector for IncA lacking N-terminal tail and transmembrane domain (ΔTMD-IncA) | Ref. 23 |
| FD201        | pET28 expression vector for wild type IncA (IncA WT) | Ref. 22 |
| FD577        | pET28 expression vector for IncA-Core (residues 87–237) | This study |
| FD582        | pET28 expression vector for TMD-IncA-Core (residues 1–237) | This study |
| DsRed-monomer–C1 | Mammalian expression vector with N-terminal DsRed-monomer tag | Dr. P. Antinozzi (Wake Forest School of Medicine) |
| FD507        | Mammalian expression vector for DsRed-tagged IncA WT (residues 1–273) | Ref. 23 |
| FD509        | Mammalian expression vector for DsRed-tagged SLD1 (residues 1–141) | Ref. 23 |
| FD579        | Mammalian expression vector for DsRed-tagged TMD-IncA-Core (residues 1–237) | This study |
| pBOMB-tet-mCherry | C. trachomatis vector expressing tetracycline-inducible-mCherry; ampicillin-resistant | Ref. 26 |
| FD569        | pBOMB vector expressing wild type IncA-FLAG; ampicillin-resistant | This study |
| FD573        | pBOMB vector expressing FLAG-IncA SLD1; ampicillin-resistant | This study |
| FD576        | pBOMB vector expressing FLAG-IncA SLD2; ampicillin-resistant | This study |
| FD588        | pBOMB vector expressing FLAG-TMD-IncA-Core; ampicillin-resistant | This study |
Functional IncA Core Region

Analysis was performed for each protein concentration separately, and the molecular masses were determined from the average obtained from the analyses of three protein concentrations.

HeLa Transfection—HeLa cells were cultured as described previously (23). Twenty-four hours prior to transfection, HeLa cells were seeded into either 6-well or 10-cm tissue culture plates in complete DMEM (DMEM supplemented with 2 mM l-glutamine, 10% fetal bovine serum (FBS), and 10 μg/ml gentamicin) and grown to 80% confluence. Cells were transiently transfected in DMEM without antibiotics (DMEM supplemented with 2 mM l-glutamine and 10% FBS) with Lipofectamine 2000 according to the manufacturer’s instructions using 0.5 μg (6-well plate) and 3 μg (10-cm dish) of DNA with a Lipofectamine/DNA ratio of 3.5:1. These conditions consistently led to an average of 60% transfection. Six hours after the addition of Lipofectamine-DNA complexes, cells were reseeded into 24-well plates containing coverslips (1 × 10⁵ cells/well) or into 10-cm dishes (1.75 × 10⁶ cells/plate) in DMEM without antibiotics and incubated for an additional 18 h before infection. For immunofluorescence experiments, each construct was reseeded in duplicate.

Transformation of Chlamydia—C. trachomatis serovar L2 expressing FLAG-IncA (FD569), FLAG-SLD1 (FD573), FLAG-SLD2 (FD576), or FLAG-TMD-IncA-Core (FD588) under the control of the tetracycline promoter were generated as described (26).

Immunoprecipitation—HeLa cells were infected at an MOI of 10 with Chlamydia expressing different forms of FLAG-tagged IncA. 8 h postinfection, FLAG proteins were induced with 10 ng/ml anhydrotetracycline (Tet). The cells were lysed 24 h postinfection with lysis buffer (25 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10% glycerol, pH 7.4) containing protease inhibitors. Equal amounts of lysate were incubated with Protein G Plus-agarose beads prebound with anti-FLAG antibody (Pierce) overnight at 4 °C. The beads were thoroughly washed with lysis buffer by centrifugation and then boiled in SDS-containing buffer. The samples were then analyzed by Western blot using anti-IncA antibody as described (33).

Analysis of Homotypic Fusion—HeLa cells were seeded onto coverslips in 24-well plates and infected with C. trachomatis serovar L2 or transformed Chlamydia strains at an MOI of 10. The infection was synchronized by centrifugation at 900 × g for 15 min at 22 °C. To induce FLAG-tagged protein expression, Tet was added to a final concentration of 10 ng/ml 8 h postinfection. DMSO was used as a control. Twenty-four hours postinfection, the cells were fixed with 2% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) at room temperature and stained with Hoechst for 30 min before being mounted on glass slides. For cells infected with Chlamydia expressing FLAG-tagged proteins, the cells were permeabilized with permeabilization buffer (10% goat serum, 0.1% saponin, 0.1% BSA in PBS) for 30 min and then stained with anti-FLAG (Santa Cruz Biotechnology) and AlexaFluor647 (Invitrogen) antibodies prior to being mounted. The coverslips were then analyzed using a Nikon TiE inverted microscope with a 60 × oil immersion objective, and images were acquired using NIS Elements (Nikon). For each condition, fields were selected at random, and 100–200 infected cells were counted and scored for the presence of multiple non-fused inclusions. For cells transfected with DsRed fusion proteins, only transfected and infected cells in the randomly chosen fields were scored.

Liposome Fusion Assay—Liposome fusion assays were performed as described (18, 22, 23). Briefly, t-SNARE acceptor liposomes were reconstituted with the late endocytic SNARE complex (His₄₋VAMP8 with or without His₄₋TMD-IncA-Core or His₄₋IncA WT. Following density gradient centrifugation, 45 μl of acceptor liposome were incubated with 5 μl of donor liposome at 37 °C for 2 h. NBD fluorescence was recorded every 2 min. Raw data were normalized to maximal fluorescence as described (34).

Statistics—Student’s two-tailed t test was used to compare averages. Significant differences were designated as p values of <0.05: *, p < 0.05; **, p < 0.01; ***, p < 0.0001.

RESULTS

The C-Terminal Domain (CTD) of IncA Is Enriched in α-Helix/Coiled-coil Motifs—IncA encodes two SNARE-like domains, SNARE-like domain 1 (SLD1) and SNARE-like domain 2 (SLD2) (23). Based on their homology with eukaryotic SNAREs, we expected that either SLD would be able to both block and induce fusion. However, we previously demonstrated that neither SLD is capable of carrying out the dual functions of IncA (23). This observation prompted us to search for the functional core responsible for both functions of IncA. The MARCOIL program was first used to determine the presence of coiled-coil motifs throughout the protein (28). Full-length IncA, including the N-terminal tail region (residues 1–34), the large hydrophobic TMD (residues 35–75), and the CTD (residues 76–273), was tested against the 9FAM matrix, which compares the sequence with a data set derived from nine families of coiled-coil-containing proteins. Consistent with previous observations, only the CTD contained coiled-coil motifs (Fig. 1A) (14, 15). No significant coiled-coil propensity was found in either the N-terminal tail or the TMD.

Next, we determined the α-helical content within the CTD using circular dichroism. We purified His₄-tagged recombinant IncA-CTD (referred to as ΔTMD-IncA) spanning residues 76–273 (23). ΔTMD-IncA was diluted to a final concentration of 10 μM in Buffer A, and circular dichroism was measured (Fig. 1B). ΔTMD-IncA showed local minima around 207 and 220 nm, demonstrating strong α-helix formation. Quantification of secondary structure using the K2D3 algorithm showed that ΔTMD-IncA is 60.55 ± 0.22% helical. β-Sheet structure was negligible (<4%). Moreover, the ratio of the mean residue ellipticities at 220 and 207 nm (θ₂₂₀nm/θ₂₀₇nm) was 1.090 ± 0.002. Ratios of ≥1.0 correlate with the presence of interacting α-helices or coiled-coils, whereas non-interacting helices are associated with ratios <1.0 (35–40), suggesting that IncA has a propensity to oligomerize.

We then generated a structural model of ΔTMD-IncA using the PHyre2 server (27). This algorithm compares input sequences against a large data set of established protein struct-
The functional IncA core region was identified through analysis of the MARCOIL program to identify putative coiled-coil domains. A schematic of wild type IncA and ΔTMD-IncA is shown, indicating the protected regions (green ribbons) and unstructured regions (purple ribbons) within the C-terminal domain of IncA. The predicted coiled-coil regions within IncA were mapped by limited proteolysis, revealing a protected region. Double minima at 207 and 220 nm indicate high helical content. The Amino Acid Number (horizontal axis) and wavelength (nm) are shown on the graph. The results demonstrated the strong presence of α-helices and coiled-coils throughout the C-terminal domain of IncA.
Functional IncA Core Region

FIGURE 2. Limited proteolysis of ∆TMD-IncA. A, ∆TMD-IncA was incubated with increasing mass ratios of trypsin for 30 min at room temperature (∼22 °C). Digestion products were resolved on SDS-PAGE and stained with Coomassie Blue. Three distinct bands appear after incubation with high concentrations of trypsin (1:270 trypsin/IncA mass ratio and above). *, undigested protein. In parallel, ∆TMD-IncA was incubated overnight in the presence of trypsin at the mass ratios listed. The presence of protected regions demonstrates the existence of a highly stable, structured core region within ∆TMD-IncA. B, the molecular weights (MW) of the protected fragments were determined by their relative mobilities in SDS-PAGE by comparison against standards of known molecular weight (Ladder lane). The apparent molecular weights as well as the N-terminal sequencing results are shown beside the corresponding bands in the SDS-PAGE image (right). C, map of trypsin-susceptible residues in ∆TMD-IncA. Sequencing results reveal Lys86 and Arg237 are targets of trypsin. Based on the molecular weight determination in B, Lys235 is also a target for trypsin.

Analysis of the primary sequence revealed a trypsin cut site at position Lys235 within SLD2 (Fig. 2C). Furthermore, the region spanning Thr97–Lys235 has a theoretical molecular mass of 17.5 kDa, consistent with the calculated and apparent masses of the large fragment (Fig. 2, A and B). Thus, the large fragment generated by trypsin cleavage probably extends from Thr97 well into SLD2 (Fig. 2C). Overall, limited trypsin digestion revealed a conformationally stable 16.8 kDa core consisting of a short, flexible linker region (N’-RKLFSQ-C’) flanked by SLD1 and one-third of SLD2.

Ectopic Expression of IncA-Core Interferes with the Homotypic Fusion of Inclusions—In vivo, IncA is involved in the homotypic fusion of inclusions (13). IncA likely mediates homotypic fusion via the formation of complexes that physically connect apposing inclusion membranes. In support of this hypothesis, it has been shown that ectopically expressed IncA, which embeds in the endoplasmic reticulum in HeLa cells, inhibits the fusion of developing inclusions by forming cross-membrane interactions and titrating away endogenous IncA located on the inclusion (14). Thus, functional IncA that induces stable cross-membrane interactions (e.g. wild type IncA) will inhibit subsequent inclusion fusion when expressed ectopically by the host cell (14, 23, 44, 45). As such, if IncA-Core is functional during homotypic fusion, it should mediate cross-membrane interactions and recapitulate the function of wild type IncA. To assess IncA-Core functionality, HeLa cells were transfected with plasmids expressing either TMD-IncA-Core (IncA(1–237)), IncA WT (full-length IncA, positive control), or empty vector (negative control) prior to being infected at an MOI of 10 with C. trachomatis str. L2. Twenty-four hours postinfection, cells were analyzed for the presence of multiple non-fused inclusions using fluorescence microscopy. Compared with the empty vector (Fig. 3A, top row), the expression of TMD-IncA-Core (Fig. 3A, bottom row) results in the formation of multiple, non-fused inclusions. The frequency of these non-fused inclusions was significantly higher compared with the vector control (37% versus 12.8%) (Fig. 3A, graph). There is a difference in effect between IncA WT and TMD-IncA-Core, which could be due to different expression levels of the transfected IncA constructs. Alternatively, this difference may mean that residues between Leu237 and Ser273, absent in TMD-IncA-Core but present in IncA WT, contribute to maximal IncA function or stability in vivo.

We expect that the ectopic expression of IncA and TMD-IncA-Core block homotypic fusion through the formation of oligomers between the ER and the inclusion membrane. Therefore, both of these proteins should be capable of forming highly structured dimers and/or oligomers. Using biophysical approaches, we then assessed the properties of both of these constructs. First, we cloned and expressed IncA-Core (residues Thr87–Leu237) as a C-terminal His6-tagged recombinant protein. Interestingly, size exclusion chromatography of ∆TMD-IncA and IncA-Core revealed small yet consistent differences in the apparent hydrodynamic radii of the two proteins, with the smaller construct, IncA-Core (theoretical monomeric molecular mass, 17.4 kDa; Fig. 3B, inset), eluting as a major peak (pk1, 82 ml) followed by a minor peak (pk2, 94 ml) overlapping with the elution profile of ∆TMD-IncA, which has a theoretical monomeric molecular mass of 23.7 kDa and elutes at 86 ml (Fig. 3B). We then analyzed the secondary and tertiary structures of IncA-Core.

We measured the circular dichroism (CD) of IncA-Core to determine the degree of helicity. Because this peptide is extremely protease-resistant, we expected that it would be highly structured. As shown in Fig. 3C, IncA-Core formed α-helices in solution as determined by the presence of double minima around 207 and 220 nm. The lower overall mean residue ellipticity of IncA-Core compared with ∆TMD-IncA suggested that the protein contained a higher proportion of helical residues than ∆TMD-IncA (compare Figs. 3C and 1B). In fact, K2D3 analysis revealed that IncA-Core was 86.71 ± 0.47% helical versus 60.55 ± 0.22% for ∆TMD-IncA. Not surprisingly, the percentage β-sheet structure in IncA-Core was very low (<1%). The increase in percentage helicity seen in IncA-Core is con-
sistent with the removal of unstructured regions from ∆TMD-IncA.

We next investigated the oligomerization state of each construct. Analytical size exclusion chromatography is heavily biased by the shape of the protein. Therefore, we resorted to analytical ultracentrifugation as an alternative means of determining the oligomerization state of each protein.

Sedimentation equilibrium was used to analyze the oligomerization of ∆TMD-IncA, IncA-Core pk1, and IncA-Core pk2 (Fig. 3D). Sedimentation equilibrium data at four different rotor speeds were analyzed globally using the “species analysis” model in SEDPHAT. The resultant fit for ∆TMD-IncA suggested a molecular mass of 23.9 ± 0.8 kDa, consistent with a monomer (23.7 kDa). Interestingly, analysis of IncA-Core pk1 yielded a molecular mass of 35.6 ± 0.7 kDa, consistent with a dimer (34.8 kDa), whereas IncA-Core pk2 resulted in a molecular mass of 18.2 ± 0.8 kDa, consistent with a monomer (17.4 kDa). Even at relatively high concentrations (127, 130, and 100 μM, respectively), the data fit to a “single-species” rather than a “two-species” model, thus ruling out any concentration-depen-
dent monomer/dimer equilibrium. This suggests that the monomeric form of IncA-Core could be a product of cotranslational protein misfolding, which does not undergo equilibrium with correctly folded IncA-Core dimer species. Nonetheless, given the predominant monomeric nature of ΔTMD-IncA in solution, these data suggest that the dimerization of IncA may be regulated by the fragment of SLD2 that is present in ΔTMD-IncA but missing in IncA-Core. Furthermore, through the formation of stable cross-membrane interactions, both IncA and IncA-Core are able to inhibit homotypic fusion when ectopically expressed in HeLa cells.

**IncA-Core Recapitulates the Dual Functions of IncA**—Due to the intractability of *Chlamydia* to genetic manipulation, the only approach to assess the function of IncA and its mutants during infection has been through ectopic expression in HeLa cells and indirect biochemical assays. However, recent advances in genetic tools now allow for the stable transformation of *Chlamydia* and the overexpression of secreted proteins, such as Incs (26). To determine whether IncA WT forms dimers/oligomers during infection and to assess the function of IncA-Core during infection, we generated *Chlamydia* clones that express FLAG-tagged TMD-IncA-Core under the control of the Tet promoter, which permits temporal regulation of effector protein expression. This strategy allows for different FLAG-IncA constructs to be overexpressed in parallel with endogenous IncA so as to not interfere with invasion or the early stages of infection due to aberrant IncA expression. These clones also constitutively express GFP, which enhances visualization. As controls, we generated FLAG-IncA WT, which is competent to mediate homotypic fusion, as well as FLAG-SLD1 and FLAG-SLD2 clones, which are unable to interfere with homotypic fusion when expressed ectopically in HeLa cells (23).

First, we infected HeLa cells with *Chlamydia* transformed with a plasmid encoding FLAG-IncA WT in the presence or absence of Tet. We then scored the cells for the presence of multiple, non-fused inclusions by immunofluorescence microscopy. As anticipated, FLAG-IncA WT is expressed on the surface of the inclusion in the presence of Tet (Fig. 4A, *IncA, DMSO* and *Tet*). Similar to the DMSO control, the overexpression of FLAG-IncA WT results in mostly single large fused inclusions (Fig. 4, A and B, *IncA WT*), whereas the overexpression of FLAG-SLD1 or FLAG-SLD2 blocks homotypic fusion (Fig. 4, A and B, *SLD1* and *SLD2*). Note that despite the expression of endogenous IncA (Fig. 4C, *A*), we observed a significant increase in the percentage of cells with multiple non-fused inclusions when infected with FLAG-SLD1-expressing (50.3 versus 8.9%) and FLAG-SLD2-expressing (60 versus 14.5%) *Chlamydia*. This demonstrates that the overexpression of SLD1 and SLD2 acts in a dominant negative manner to alter inclusion morphology. Altogether, these data confirm that neither SLD is sufficient to mediate homotypic fusion.

Next, we infected HeLa cells with FLAG-TMD-IncA-Core *Chlamydia* in the presence or absence of Tet and assessed its impact on homotypic fusion. Similar to FLAG-IncA WT, ~80% of cells infected with FLAG-TMD-IncA-Core *Chlamydia* possess single large fused inclusions (Fig. 4, A and B, *TMD-IncA-Core*), although a slight increase in the number of cells with multiple inclusions in the presence of Tet (10 versus 19%) is observed. This increase could be due to the enhanced dimerization and/or stability of IncA-Core (Fig. 3D). In total, these data show that IncA-Core retains its ability to mediate homotypic fusion and introduces a novel approach to dissecting Inc function during *Chlamydia* infection. Note that because both IncA WT and TMD-IncA-Core induce homotypic fusion to a similar extent, it is likely that the difference observed in the DsRed HeLa assay (Fig. 3A) is due to differences in expression level and or stability of the constructs.

Using these *Chlamydia* strains, we then assessed IncA oligomerization during infection by conducting a series of immunoprecipitation experiments. To do so, HeLa cells were infected with FLAG-TMD-IncA-Core, FLAG-SLD1, and FLAG-SLD2 expressing *Chlamydia*, and the binding of endogenous IncA to these FLAG-tagged proteins was assessed by immunoprecipitation and Western blot. As shown in Fig. 4D (left) endogenous IncA co-precipitated with FLAG-TMD-Core. Differences in molecular weight between endogenous IncA and these FLAG-IncA constructs enabled us to discriminate between these two proteins, which was not possible with endogenous IncA and FLAG-IncA WT. To assess IncA-IncA WT binding, we transfected HeLa cells with DsRed-IncA WT prior to infection with FLAG-IncA WT expressing *Chlamydia*. As controls, we also transfected cells with DsRed-TMD-IncA-Core (positive control) or DsRed (negative control). Twenty-four hours postinfection, FLAG-IncA WT was immunoprecipitated and the binding of DsRed proteins was analyzed by Western blot using anti-IncA antibody. As shown in Fig. 4D (right), both DsRed-IncA WT and DsRed-TMD-Core co-precipitated with FLAG-IncA, demonstrating that although IncA WT is mostly a monomer in solution, it readily multimerizes during infection. Surprisingly, we also observed binding between SLD1 and IncA as well as between SLD2 and IncA, although neither of these two constructs is capable of inducing homotypic fusion. This suggests that similar to SNARE proteins, binding alone is not sufficient to generate membrane fusion (46–49).

In addition to mediating homotypic fusion, IncA has been shown to inhibit SNARE-mediated membrane fusion (22, 23). The inhibitory function of IncA is encoded entirely within the C-terminal domain and requires at least one intact SNARE-like domain (23). We validated the inhibitory capacity of IncA-Core using a well established liposome fusion assay (18). Recombinant human VAMP8 was reconstituted with or without IncA WT or TMD-IncA-Core into donor liposomes containing the FRET pair NBD (nitrobenzoxadiazole) and rhodamine (Fig. 5, lanes 2–4 of inset). In parallel, recombinant human t-SNAREs syntaxin 7 (Stx7), syntaxin 8 (Stx8), and Vti1b were reconstituted into non-fluorescent acceptor liposomes (Fig. 5, lane 1 of inset). Following density gradient centrifugation, the two liposome populations were mixed at a 9:1 acceptor/donor volumetric ratio (total volume 50 μl) and incubated at 37 °C for 2 h. Membrane fusion was measured as an increase in NBD fluorescence over time (34). Compared with the control (Fig. 5, black line), both IncA WT and TMD-IncA-Core significantly inhibited fusion, demonstrating that IncA-Core retains its ability to inhibit SNARE-mediated membrane fusion. Altogether, these results demonstrate that IncA-Core recapitulates the
dual functions of IncA and identify, for the first time, the functional core of a SNARE-like bacterial protein.

DISCUSSION

*C. trachomatis*-containing compartments avoid lysosomal fusion and undergo homotypic fusion to enhance infection. Mounting evidence suggests that the inclusion-bound protein IncA is at the intersection of these two processes, although it is unclear exactly how IncA accomplishes these tasks. Elucidating the structure of IncA will ultimately increase our understanding of how this protein performs its functions as well as aid in the identification of homologous effectors utilized by other intracellular bacteria, such as *Salmonella* species and *M. tuberculosis*, which also manipulate host membrane fusion.

Here, we have identified a stable, protease-resistant core within IncA that encodes its dual functions. Our biophysical characterization of ΔTMD-IncA and IncA-Core revealed that, although both constructs are clearly structured and enriched in α-helices, ΔTMD-IncA is mostly monomeric, whereas IncA-Core is mostly dimeric (Fig. 3). These data suggest that residues present in ΔTMD-IncA that are absent in IncA-Core keep ΔTMD-IncA in a monomeric conformation and that the loss of these residues results in IncA-Core dimerization (see model in Fig. 6).
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ΔTMD-IncA encompasses SLD1 and SLD2 (residues 87–273), which are separated by a long linker region. Our limited proteolysis mapping experiment (Fig. 2) revealed that two-thirds of SLD2 (residues 236–273; Fig. 6A, striped area) are removed following exposure to trypsin. We hypothesize that residues within this region are involved in keeping ΔTMD-IncA in its monomeric state (Fig. 6B, left) and that removal of this region permits the resulting protein (IncA-Core) to homodimerize (Fig. 6B, right).

Interestingly, intramolecular protein autoregulation has been demonstrated for SNARE proteins. In particular, the N-terminal region of the syntaxin family plays a role in the autoinhibition of SNARE complex formation, thus impacting the fusogenic state of the SNARE complex. For instance, Stx1A and Stx7 contain N termini that organize into three-helix bundles that fold back onto the SNARE domains of their respective syntaxin and inhibit ternary complex formation and membrane fusion (50, 51). Upon stimulation, the N-terminal domain is released from the SNARE domain, and SNARE complex formation and membrane fusion proceed (50, 51). In the case of IncA, autoinhibition may act to keep IncA from prematurely forming dimers on the inclusion membrane. The removal or unfolding of the autoinhibitory region would then stimulate cross-membrane dimer formation, leading to the homotypic fusion of inclusions. The transition of IncA from a monomeric state into a dimeric state is likely regulated by host or bacterial proteins since the presence of ΔTMD-IncA dimers in solution was minimal (Fig. 3), yet IncA-IncA binding was readily detectable during infection (Fig. 4D). Consistent with IncA dimerization/oligomerization in cells, chemical cross-linking of ectopically expressed IncA in HeLa cells followed by Western blotting revealed protein bands with apparent molecular weights consistent with IncA dimers and tetramers (14, 15). Our use of analytical ultracentrifugation allowed for the determination of the oligomeric state of IncA independent of chemical modifications and in isolation from other cellular proteins whose non-specific cross-linking to IncA could affect migration on SDS-PAGE. The fact that IncA-Core formed dimers in solution suggests that the tetramers seen by Western blot could be the result of over cross-linking of the dimeric form (14); however, we cannot exclude the possibility that high local concentrations of IncA on biological membranes lead to higher order oligomer formation or that regions of IncA outside of the CTD (e.g. in the N-terminal tail region) promote tetramerization in vivo.

The core region includes the majority of SLD1 and thus should inhibit fusion, as observed previously (22, 23). However, unlike ΔTMD-IncA, which is predominantly monomeric, IncA-Core mostly forms a dimer in solution. Interestingly, the oligomeric state of IncA did not influence its inhibitory capacity, and TMD-IncA-Core is still able to block SNARE-mediated membrane fusion (Fig. 5). One possible mechanism is that binding to VAMP8 disrupts the IncA-IncA dimer, resulting in IncA-VAMP8 complex formation and blockage of the fusion event. Alternatively, IncA oligomers may directly bind to VAMP8, resulting in the formation of a stable four-helix bundle, structurally similar to SNARE complexes (17, 52). Additional studies will be necessary to discriminate between both of these possibilities.
Using both a well established HeLa assay and dominant negative IncA mutants, we showed that TMD-IncA-Core is fully competent to mediate the homotypic fusion of inclusions. The creation of Chlamydia strains expressing dominant-negative IncA constructs represents a novel and powerful tool to study the function of chlamydial effector proteins. Using this new technology, we demonstrated that SLD1 or SLD2 alone was not sufficient to induce homotypic fusion, whereas IncA-Core, which displays an additional 94 amino acids compared with SLD1, is fully fusogenic. Homology modeling and circular dichroism analysis showed that IncA-Core consists almost exclusively of α-helices, consistent with its homology to SNARE proteins. It is interesting to note that SLD1 and SLD2 are both able to bind IncA WT, although this binding does not translate into fusion. Altogether, these data suggest that either (i) a minimum size and a proper amino acid sequence is required to generate enough energy to induce membrane fusion, or (ii) the truncation and mutation of IncA resulted in the loss of one or several binding partner(s) necessary for IncA to enter into a fusion-competent complex.

In sum, the detailed structural analysis of IncA provides evidence that a highly structured core region of this bacterial effector (i) mediates cross-membrane interactions and homotypic fusion by forming stable coiled-coil multimers and (ii) retains its capacity to inhibit late endocytic SNARE-mediated membrane fusion. These data set the stage for future structural studies of IncA and other Inc proteins. If Inc SNARE-like proteins are key proteins for Chlamydia survival, it is possible that modulation or inhibition of Inc protein function by small molecules or drugs could represent a viable therapeutic option in the fight against infection. Indeed, our investigation into the ectopic expression of IncA mutants in infected HeLa cells as well as the generation of dominant negative IncA Chlamydia strains demonstrates the feasibility of engineering peptides that interact with endogenous IncA in vivo.

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