Structural basis for the homotypic fusion of chlamydial inclusions by the SNARE-like protein IncA

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Many intracellular bacteria, including Chlamydia, establish a parasitic membrane-bound organelle inside the host cell that is essential for the bacteria’s survival. Chlamydia trachomatis forms inclusions that are decorated with poorly characterized membrane proteins known as Incs. The prototypical Inc, called IncA, enhances Chlamydia pathogenicity by promoting the homotypic fusion of inclusions and shares structural and functional similarity to eukaryotic SNAREs. Here, we present the atomic structure of the cytoplasmic domain of IncA, which reveals a non-canonical four-helix bundle. Structure-based mutagenesis, molecular dynamics simulation, and functional cellular assays identify an intramolecular clamp that is essential for IncA-mediated homotypic membrane fusion during infection.
The obligate intracellular pathogen *Chlamydia trachomatis* is the most frequent cause of bacterial sexually transmitted disease and infectious blindness worldwide, yet it is still considered a neglected disease pathogen by the World Health Organization. *Chlamydia*’s life cycle depends on the establishment of a fast-growing parasitic organelle inside the host cell called the “inclusion”, the development of which is poorly understood. Inclusion membranes are decorated with ~60 transmembrane Inc proteins that are known to directly interact with host cell components and play a critical role in sustaining *Chlamydia*’s life cycle. Despite their importance, Inc proteins have remained relatively uncharacterized and little is known about their function at the molecular level. The low sequence conservation and minimal similarity that Incs share with other proteins limits the usefulness of conventional bioinformatic tools to predict their structure and function. The best-characterized chlamydial Inc is IncA. It contains two extended 3,4-hydrophobic helax repeat segments similar to the coiled-coil regions of the eukaryotic “SNAREs” (soluble N-ethylmaleimide-sensitive factor attachment receptors), which are proteins involved in cellular transport and membrane fusion. IncA is involved in the homotypic fusion of chlamydial inclusions and is the best-characterized bacterial SNARE-like protein to date. During infection, each *Chlamydia* bacterium establishes its own inclusion inside a host cell. At high multiplicities of infection (MOI), cells contain multiple inclusions that ultimately fuse together to form one large inclusion per cell. This homotypic fusion event is important for the pathogenicity of *C. trachomatis* because natural non-fusogenic IncA mutants are replication-defective in humans and cause significantly milder disease compared with patients infected with normal fusogenic strains.

Measurements of relative chlamydial rRNA quantities in the multiplying organisms have also revealed that non-fusogenic strains grow more slowly than fusogenic strains of *Chlamydia*, which is supported by a reduced rate of protein synthesis and decreased multiplication efficiency. A direct role for IncA in membrane fusion was demonstrated using microinjection of anti-IncA antibody during infection, which resulted in multiple inclusions that were unable to undergo homotypic fusion. Cells infected with an IncA-deficient strain of *C. trachomatis* similarly displayed multiple inclusions at a high multiplicity of infection, further establishing that IncA is required for the homotypic fusion of inclusions.

IncA localizes to the inclusion membrane where it can potentially interact with host and inclusion-associated proteins. Yeast two-hybrid analysis demonstrated that IncA can bind itself, while immunoprecipitation also showed that IncA associates in trans when present on opposite membranes in a cell. In *Chlamydia*-infected HeLa cells, the expression of ectopic IncA on the endoplasmic reticulum (ER) membrane impacts inclusion integrity and ER morphology, suggesting that IncA present on the ER interacts homotypically with IncA expressed on the inclusion and induces the fusion of both compartments. While it is well-established that IncA is involved in the homotypic fusion of inclusions, the molecular mechanism of its fusogenic activity remains unknown.

Here, we describe the crystal structure of IncA, which we probed using biophysical, computational, and functional methods. We demonstrate that IncA folds into a stable, non-canonical four-helix bundle that is maintained as a monomer by intramolecular interactions. We also show that the monomeric conformation of IncA is critical for its activity during membrane fusion. Our work sheds light on a class of bacterial transmembrane proteins that control membrane fusion during infection, which is critical for *Chlamydia* pathogenicity.

**Results**

**IncA folds into a non-canonical four-helix bundle.** IncA is a protein of 273 amino acids composed of a short, cytoplasmic N-terminal moiety (residues 1–34), a bilobed transmembrane domain (residues 35–84), and a long cytoplasmic C-terminal domain that ends in a tail with low complexity (residues 247–273) (Fig. 1a). The bacterial SNARE-like domains are found in the cytoplasmic C-terminal domain. To better understand the mechanism of IncA-mediated membrane fusion, we generated high-quality crystals of a chymotryptic fragment of IncA spanning residues 87–246 (IncA<sub>87–246</sub>) (Supplementary Fig. 1b) and determined a crystal structure of IncA<sub>87–246</sub> to an R<sub>work</sub>/R<sub>free</sub> of 14.1/16.8% at 1.12 Å resolution (Fig. 1b and Table 1). The electron density for IncA<sub>87–246</sub> including the six N-terminal histidines of the affinity tag, is exceptionally clear (Supplementary Fig. 2), consistent with the low B-factor of the crystal structure (~10.8 Å<sup>2</sup>). We found that IncA<sub>87–246</sub> adopts an asymmetric and slightly blocky conformation, somewhat similar to a four-helix bundle (Fig. 1b). Both in crystal and in solution, IncA<sub>87–246</sub> exists as a monomer (Supplementary Fig. 3a, b and Supplementary Table 1), as previously observed for the full-length cytosolic domain of IncA (ΔTMD-IncA - Supplementary Fig. 1a), that sediments at equilibrium as a single species of 23.9 ± 0.8 kDa. Altogether, these results suggest that the C-terminal protease-sensitive tail

![Fig 1](https://example.com/fig1.png)

**Fig 1** The cytosolic domain of IncA folds into a non-canonical α-helical bundle. a Schematic diagram of *C. trachomatis* IncA domain organization. Transmembrane helices (TMH) were predicted using the HHM motifs server. The stable core encompassing residues 87–246 (IncA<sub>87–246</sub>) was identified by limited proteolysis of the cytosolic domain as described. b Ribbon diagram of IncA<sub>87–246</sub>. With α-helices and random coiled linkers colored in cyan and red, respectively. c, d Two rotated views of IncA<sub>87–246</sub> orthogonal to the representation in b, with α-helices shown as cylinders. c highlights the segmented structure of the helix H<sub>β</sub>, while d shows the relative angle between helices H<sub>C</sub> and H<sub>D</sub>.
IncA87 identifies four-helix bundles with high structural similarities to HA, HB throughout their entire length (Fig. 1c), the longest helix HD. down-up antiparallel α-helix, the loop connecting helices HB and HC contains a short insertion helix (res. 165–169), which we termed the 'clamp' helix (Hclamp) (Fig. 1b, c). The clamp makes numerous contacts with HA, HB, HC and HD that account for a total of eight hydrogen bonds and 94 non-bonded interactions (Supplementary Fig. 4 and Supplementary Table 2). Third, while helices HA and HC run parallel to each other making an acute angle of ~10° and bonding throughout their entire length (Fig. 1c), the longest helix HD makes a 40° angle from helix HC pointing away from the Hclamp (Fig. 1d) and gives an asymmetric appearance to the helical bundle. Consistent with the unique four-helix-composition of IncA87–246 a search for structural relatives using DALI17 did not identify four-helix bundles with high structural similarities to IncA87–246 despite the abundance of this fold in nature. Instead, DALI found IncA*–246 bears structural similarity (Z-score = 9.0) to the talin-hippi/f12p actin-tethering C-terminal homology (THATCH) domain core of the Huntingtin Interacting Protein 12, (PDB ID 1R0D) (Supplementary Fig. 5), which superimposes to IncA87–246 with a Ca root-mean-square deviation (RMSD) ~ 3.6 Å. This helical bundle, involved in the association between actin and clathrin-coated structures at the plasma membrane and trans-Golgi network18, also contains a clamp helix between HB and HC but, unlike IncA87–246, its C-terminal helix HD is split into two α-helices, HD and HD, thus, the high-resolution crystal structure of the cytosolic domain of IncA reveals a non-canonical helical bundle.

Table 1 Crystallographic data collection, phasing, and refinement statistics

| Data collection                  | IncA87-246 | IncA87-246NaI | IncA87-246(G144A) |
|---------------------------------|------------|--------------|------------------|
| X-ray source                    | SSRL 9-2   | MicroMax-007 HF | MicroMax-007 HF  |
| Detector                        | Pilatus 6M PAD | Pilatus3 R 200K | Pilatus3 R 200K  |
| Space group                     | P2_1       | P2_1          | P1               |
| Cell dimensions                 | 38.4, 48.8, 41.7 | 38.4, 48.7, 41.7 | 41.0, 43.5, 45.8 |
| α β γ (°)                       | 90.0, 103.7, 90.0 | 90.0, 103.8, 90.0 | 92.9, 95.9, 93.9 |
| Wavelength (Å)                  | 0.98       | 1.54          | 1.54             |
| Resolution (Å)                  | 15-12 Å (1.12-1.12) | 15-1.80 (1.83-1.80) | 15-1.95 (2.02-1.95) |
| No. reflections (tot/unique)    | 875,396/52,606 | 1,788,976/13,917 | 282,344/20,042   |
| Rsym                           | 5.5 (29.8) | 7.6 (22.4)    | 4.8 (13.1)       |
| Rsym                           | 2.3 (14.8) | 1.9 (2.2)     | 4.8 (13.0)       |
| Meanl / σl                     | 47.9 (4.7) | 104.5 (23.9)  | 20.4 (5.8)       |
| CCI/2                          | 0.963      | 0.997         | 0.950            |
| Completeness (%)               | 91.3 (55.0) | 99.5 (99.4)   | 88.1 (66.3)      |
| Redundancy                     | 7.3 (4.5)  | 16.0 (12.4)   | 1.5 (1.5)        |
| Wilson B-factor (Å²)           | 10.8       | 13.3          | 13.4             |
| SAD phasing                    | 16         | 0.37          |                 |
| Number iodine sites            | 11         | 0.37          |                 |
| Corr. of local RMS density     | 0.61       | 0.61          |                 |
| Refinement                     |            |               |                 |
| PDB ID                         | 6E7E       | 6E6A          |                 |
| Resolution (Å)                 | 15-112 Å  | 15-195 Å     |                 |
| No. reflections                | 50,533     | 20,013        |                 |
| Rsym                          | 14.1/16.8 | 16.9/21.2     |                 |
| No. of complexes in AU         | 1          | 2             |                 |
| No. of protein atoms           | 1375       | 2650          |                 |
| Ramachandar (favored/allowed/outliers) | 100/0.0/0.0 | 99.7/0.3/0.0 |                 |
| R.M.S.D. from ideality         |            |               |                 |
| Bond lengths (Å)               | 0.012      | 0.005         |                 |
| Bond angles (°)                | 1.649      | 0.860         |                 |
| MolProbity Score/rankingb     | 115/94th percentile | 1,2499th percentile | 4.71/98th percentile |
| MolProbity ClashScore/ rankingb | 3.65/5th percentile |                 |                 |

Values in parentheses are for highest-resolution shells

*Rs_mer was calculated using ~5% randomly selected reflections

**Percentile ranking relative to X-ray structures solved at similar resolution

(res. 247–273) does not promote IncA self-association in vitro. The tertiary structure of IncA87–246 consists of four down-up-down-up antiparallel α-helices, named HA, HB, HD. However, IncA87–246 deviates from canonical four-helix bundles in at least three aspects. First, the helix HB is interrupted at position 144 by a glycine that generates two shorter helices, named HB' and HB'' (Fig. 1b, c). We termed this break in helicity as the 'hinge'. Second, the loop connecting helices HB and HC contains a short insertion helix (res. 165–169), which we termed the 'clamp' helix (Hclamp) (Fig. 1b, c). The clamp makes numerous contacts with HA, HB', HC and HD that account for a total of eight hydrogen bonds and 94 non-bonded interactions (Supplementary Fig. 4 and Supplementary Table 2). Third, while helices HA and HC run parallel to each other making an acute angle of ~10° and bonding throughout their entire length (Fig. 1c), the longest helix HD makes a 40° angle from helix HC pointing away from the Hclamp (Fig. 1d) and gives an asymmetric appearance to the helical bundle. Consistent with the unique four-helix-composition of IncA87–246 a search for structural relatives using DALI17 did not identify four-helix bundles with high structural similarities to IncA87–246 despite the abundance of this fold in nature. Instead, DALI found IncA87–246 bears structural similarity (Z-score = 9.0) to the talin-hippi/f12p actin-tethering C-terminal homology (THATCH) domain core of the Huntingtin Interacting Protein 12, (PDB ID 1R0D) (Supplementary Fig. 5), which superimposes to IncA87–246 with a Ca root-mean-square deviation (RMSD) ~ 3.6 Å. This helical bundle, involved in the association between actin and clathrin-coated structures at the plasma membrane and trans-Golgi network18, also contains a clamp helix between HB and HC but, unlike IncA87–246, its C-terminal helix HD is split into two α-helices, HD and HD. Thus, the high-resolution crystal structure of the cytosolic domain of IncA reveals a non-canonical helical bundle.

**Intramolecular contacts maintain IncA as a monomer.** To investigate how the structural determinants in IncA that deviate from a classical four-helix bundle topology affect protein flexibility and the ability to mediate homotypic fusion, we analyzed the anisotropically-refined B-factor of IncA87–246 (Fig. 2a). The hinge and the amino acids preceding Hclamp were found to have significantly higher than average B-factors (~26 Å² vs. ~16 Å²) (Fig. 2a), possibly underscoring intrinsic flexibility. To characterize these regions, we first generated a G144A hinge mutant variant and found that IncA87–246(G144A) (Supplementary Fig. 1c) remains monomeric in solution (Supplementary Fig. 3c, d and Supplementary Table 1) and it has comparable structural stability as IncA87–246 (Supplementary Fig. 6). IncA87–246(G144A) crystallized in a triclinic space group with two IncA87–246(G144A) protomers in the unit cell. Though this crystal form did not diffract as well as the previous one, we were able to collect 88.1% complete data to 1.95 Å resolution (Table 1) and determine an accurate atomic model of IncA87–246.
IncA\textsubscript{87–246}(G144A) using molecular replacement. The structure, solved to a \(R_{\text{work/fre}}\) of 16.9/21.2 at 1.95 Å resolution (Table 1), contains two four-helix bundles (Fig. 2b) where the interrupted B-factor plotted onto the 3D-structure: the diameter of the tube is proportional to the B-factor. The hinge region and the moiety C-terminal of the helix B of IncA plays a global structural role in the architecture of the bundle.

To probe the second region of IncA that has a higher-than-average B-factor (Fig. 2a), we generated a mutant, IncA\textsubscript{87–246}(polyA), where eight residues in \(H_D\) that make contact with the helices surrounding the \(H_{\text{clamp}}\) are mutated to alanine, namely L233A, S234A, L237A, T238A, Q240A, I241A, Q244A, and R245A (Fig. 2d and Supplementary Fig. 1d). Although these mutations result in a loss of 12 intramolecular bonds (Supplementary Fig. 8), IncA\textsubscript{87–246}(polyA) had comparable structural stability as IncA\textsubscript{87–246} (Supplementary Fig. 6). Unlike the previous two IncA constructs, IncA\textsubscript{87–246}(polyA) failed to crystallize, limiting our understanding of its structure. In solution, when analyzed by AUC-SV, IncA\textsubscript{87–246}(polyA) migrated as a slightly larger species than IncA\textsubscript{87–246} or IncA\textsubscript{237–246}(G144A), possibly consistent with a trimer or an elongated dimer (Supplementary Fig. 3e, f and Supplementary Table 1). Interestingly, IncA\textsubscript{237–246}, a shorter deletion construct of IncA that lacks many of the same residues on Helix D mutated in IncA\textsubscript{87–246}(polyA) (Supplementary Fig. 1f), is also predominantly dimeric in solution. Thus, when the intramolecular contacts generated by helix \(H_D\) are broken, then a higher oligomeric species spontaneously forms, suggesting that the non-canonical conformation of the \(H_{\text{clamp}}\) may function by locking the helical core of IncA in a monomeric conformation that prevents IncA from self-assembling on the inclusion membrane.

The IncA monomer is highly stable. To determine if the crystallographic structure of IncA\textsubscript{87–246} represents a metastable intermediate, we subjected the 1.12 Å crystallographic model of IncA\textsubscript{87–246} to equilibrium molecular dynamics simulations, along with models of IncA\textsubscript{237–237} and IncA\textsubscript{237–246}(G144A) (Supplementary Fig. 1). These three systems were subjected to 4 μs of equilibrium sampling to explore conformational flexibility and stability of IncA, and to probe the effects of the hinge, \(H_{\text{clamp}}\), and the C-terminus of helix \(H_D\) on dynamics and structure. Our simulations showed that the structure of IncA\textsubscript{87–246} is highly stable and is unlikely to be a metastable intermediate. This conclusion is supported by the rigid, unchanging conformation of IncA\textsubscript{87–246} throughout the equilibrium sampling at physiological conditions (310 K, 150mM NaCl) and the entire time scale of the simulation (Supplementary movie 1). In contrast, IncA\textsubscript{237–237}, which lacks nine additional C-terminal residues, underwent a structure-wide increase in root-mean-square fluctuation (RMSF) compared to both IncA\textsubscript{87–246} and IncA\textsubscript{237–246}(G144A) (Fig. 3a), and experienced an eventual conformational change after ~3.2 μs of sampling (Fig. 3b and Supplementary movie 2). This conformation change involved a repositioning of \(H_{\text{clamp}}\) away from helix \(H_D\) toward helix \(H_A\), where it ultimately formed H-bonds with residues 94 to 100 of \(H_A\) hereinafter referred to as \(H_A(94-100)\). Hydrogen bonds were identified using a contact-analysis with a cutoff at 3.2 Å, between hydrogen, oxygen, and nitrogen atoms of the \(H_{\text{clamp}}\) (res. 165–169) and \(H_A(94-100)\). This hydrogen bond analysis was conducted for all three systems (Fig. 3c). Hydrogen bonds in IncA\textsubscript{87–237} appeared transiently around 2 μs following positional fluctuations of \(H_A(94-100)\), and they persisted once \(H_A(94-100)\) repositioned permanently at 3.2 μs. Beyond the notable structural

![Figure 2](image-url)
instability compared to IncA87–246, this result implies that the C-terminus of helix HD (res. 237–246) plays a significant role in regulating the conformation of IncA87–246. The RMSF analysis shows that the Hclamp is highly flexible in all three structures (Fig. 3a). Moreover, the relatively higher magnitude of RMSF in the Hclamp region of IncA87–237 compared with that of IncA87–246 and IncA87–246(G144A) again makes the structural effect of the C-terminus of helix HD apparent.

Structural changes of IncA87–246(G144A) during simulation are minimal compared to simulations of IncA87–246 (Supplementary movies 3 and 4). The latter observation supports the hypothesis that helix HD is a key determinant of structural stability. Furthermore, noting both the lower RMSF of IncA87–246(G144A) (Fig. 3a) and the dissimilarity in the position of HD among IncA87–246(G144A) and IncA87–246 (Fig. 2c), it is evident that the orientation of helix HD with respect to Hclamp is coupled to the overall stability of each construct. Closer proximity between Hclamp and HD in IncA87–246(G144A) prevents the Hclamp from forming hydrogen bonds with HA(94–100) resulting in a more stable conformation (Fig. 3c). The Hclamp of IncA87–246, with a significantly higher RMSF than that of IncA87–246(G144A), forms hydrogen bonds with HA(94–100) after 2 μs of sampling. While less-numerous and denoting no clear structural significance compared with those of IncA87–237, the lack of hydrogen-bonding in IncA87–246(G144A) implies that the position of the C-terminus of HD relative to the Hclamp directly modulates the behavior of the Hclamp. This finding suggests that the overall flexibility of the Hclamp potentially its ability to recognize regulatory proteins, is sensitive to the network of hydrogen bonds it can form with adjacent regions of HD and HA.

Markov State models (MSMs) provide information regarding the thermodynamic stability of a protein and are typically used to characterize long timescale dynamic modes19, such as folding coordinates. For the trajectories in the present study (4.18 μs for IncA87–246 and 5.80 μs for IncA87–237), the high temporal resolution of the input datasets (40,000 states with each
Intramolecular contacts are critical for IncA function. To probe the functional importance of IncA oligomerization, we assessed the fusogenic activities of the multiple IncA constructs (IncA\textsubscript{87–237}, IncA\textsubscript{87–246}, IncA\textsubscript{87–246} (G144A), and IncA\textsubscript{87–246} (polyA)). Supplementary Fig. 1) during Chlamydia infection. We complemented an IncA knock-out (KO) Chlamydia strain\textsuperscript{11} with the different IncA mutants. All IncA mutants expressed the intact N-terminal transmembrane domain (residues 1–86) for efficient secretion to the inclusion membrane, as well as a C-terminal FLAG-tag for rapid identification (see Supplementary Table 3). Expression levels for each IncA\textsubscript{mutant}-FLAG protein were comparable as validated by western blot analysis (Fig. 4a and Supplementary Fig. 10). HeLa cells were infected with these strains at an MOI of 5, and the homotypic fusion of inclusions was quantified 24 h post-infection (hpi). IncA KO Chlamydia complemented with IncA\textsubscript{WT} undergoes comparable levels of homotypic fusion as wild-type Chlamydia (Supplementary Fig. 11) and was used as a positive control, while the IncA KO strain was used as negative control. As shown in Fig. 4b, c, cells infected with Chlamydia expressing IncA\textsubscript{1–240} which lacks 27 C-terminal residues (Supplementary Fig. 1), had 23% fewer cells containing single, fused inclusions compared with IncA\textsubscript{WT}. While this decrease is statistically significant, it demonstrates that the protease-resistant fragment IncA\textsubscript{1–246} remains largely fusion-competent. When the C-terminus was further shortened, the fusogenicity of IncA continued to drop with the deletion of only three additional residues (IncA\textsubscript{1–243}) resulting in a drastic 43% inhibition of fusion. When six additional residues were deleted (IncA\textsubscript{1–237}), we observed ~82% inhibition of fusion (Fig. 4c), demonstrating a nearly complete loss of fusogenicity. Since IncA\textsubscript{1–237} is mostly diolic in solution\textsuperscript{8}, this finding suggests that the self-assembly of IncA could impact its fusogenic activity. Altogether, these data reveal that progressive deletion of the C-terminal residues of helix HD in IncA results in an increased loss of fusogenicity, which correlates with a shift towards self-assembly and a loss of monomeric IncA in solution.

To further address the potential impact of IncA oligomerization on homotypic fusion, we complemented the IncA KO Chlamydia strain with IncA\textsubscript{1–246} (polyA), in which the residue-forming contacting on helix HD have been mutagenized. We observed that IncA\textsubscript{1–246} (polyA) forms oligomers in solution (Supplementary Fig. 3 e, f and Supplementary Table 1). We also observed that IncA\textsubscript{1–246} (polyA) was unable to drive homotypic fusion efficiently, and only ~33% of infected cells contained single inclusions (Fig. 4b, d). This loss of function was specific for these mutations. Cells infected with Chlamydia expressing another mutated IncA, IncA\textsubscript{1–246} (G144A), in which the hinge was genetically eliminated, were fully fusogenic and mostly displayed a single inclusion (Fig. 4b, d). Glycine 144 is not conserved amongst the fusogenic IncA proteins, further supporting our functional data, which indicate this particular amino-acid does not play a critical role in fusion.

Altogether, these data suggest that the intramolecular contacts generated by helix HD likely maintain IncA in a monomeric fusion-competent state. We observed that the IncA mutants that form oligomers in solution are all non-fusogenic when expressed on the inclusion membrane. Although the oligomerization state of IncA on the membrane is unknown, these results suggest that IncA self-association may lead to its inactivation.

Wild-type IncA rescues fusion of non-fusogenic IncA. To further probe the mechanism controlling IncA function and assess the importance of trans-interactions between IncA present on opposing membranes, we conducted a series of co-infection experiments in which cells were infected with both non-fusogenic IncA\textsubscript{mutant} complemented-IncA KO C. trachomatis expressing GFP (MOI 5) and wild-type C. trachomatis expressing mCherry (L2\textsubscript{mCherry}, MOI 2) (Fig. 5). Wild-type C. trachomatis transformed with mCherry (L2\textsubscript{mCherry}) undergoes the same level of fusion as wild-type Chlamydia (Supplementary Fig. 11). We used the IncA KO Chlamydia strain complemented with IncA\textsubscript{1–246} and the IncA KO (non-induced) strain as positive and negative controls, respectively. As expected, IncA\textsubscript{1–246} was able to promote fusion with IncA\textsubscript{WT}, resulting in a mixed inclusion containing both GFP and mCherry Chlamydia (Fig. 5a). Strikingly, the...
mutants for which homotypic fusion was significantly impaired (IncA_{1–237}, IncA_{1–243}, and IncA_{1–246(polyA)}), were all able to promote “heterotypic” fusion when IncAWT was present on the opposing membrane, thus resulting in complete rescue with almost all of the cells (97%, 96%, 94%, respectively) displaying single red wild-type inclusions that fused with green mutant inclusions (Fig. 5), compared with far fewer of the cells (~18%, 55%, and 35%, respectively) having homotypically fused inclusions (Fig. 4). These results suggest that IncAWT was able to form trans-interactions, likely by disassembling cis-oligomers (see Discussion). Inclusions generated by the IncA KO Chlamydia strain were unable to undergo fusion even with WT inclusions (Fig. 5a, b, non-induced). This observation confirms that IncA needs to be present on both inclusion membranes to promote fusion. Altogether, these data suggest a model whereby IncAWT is able to disassemble cis IncAmutant oligomers to promote IncAWT:IncAmutant fusion.

Discussion

Fusogenic viral proteins\(^\text{21}\) and eukaryotic SNAREs\(^\text{22-24}\) have been extensively studied, and much is known about the key structural determinants and, in some cases, mechanisms of membrane fusion. A common denominator of fusogenic proteins is their intrinsic structural plasticity and ability to undergo dramatic conformational changes upon fusion, typically adopting a metastable conformation in the pre-fusogenic state and a thermodynamically stable structure post-fusion\(^\text{25,26}\). In contrast, the key players and mechanisms that mediate fusion of chlamydial inclusions remain largely unknown. Chlamydial inclusions are extraordinarily challenging to study due to the fragility of the lipid membrane, the poor conservation of Inc proteins, and the lack of an in vitro fusion assay. In addition, the homotypic fusion of chlamydial inclusions presents a formidable topological challenge because the proteins responsible for this event are identical on both membranes\(^\text{27}\). In this paper, we describe the molecular...
architecture of the prototypical bacterial SNARE-like protein IncA, which mediates the homotypic fusion of chlamydial inclusions\textsuperscript{10,11}.

Our work sheds light on three aspects that are important for deciphering the mechanisms of chlamydial inclusion membrane fusion. First, the cytosolic domain of IncA folds into a non-canonical four-helix bundle characterized by a segmented helix H\textsubscript{B}, a clamp, and a long C-terminal helix H\textsubscript{D}. This structure differs profoundly from the four-helix bundle structure of eukaryotic SNAREs previously used to describe IncA topology (Supplementary Fig. 12) but shares similarity with the THATCH domain core of the Huntingtin Interacting Protein 12 that mediates associations between actin and clathrin-coated structures.

Combining biochemical, biophysical, and functional methodologies, we provide evidence that the C-terminal helix H\textsubscript{D} of IncA makes key intramolecular contacts with H\textsubscript{clamp} and helix H\textsubscript{B}, thus locking the IncA bundle into a stable monomeric pre-fusion conformation. These contacts, in particular, S234, Q240, I241, and Q245, are conserved in all known fusogenic IncA proteins (i.e., CtrIncA expressed by \textit{C. trachomatis}, CmuIncA expressed by \textit{C. muridarum}, and G3IncA expressed by \textit{C. suis}), supporting the idea that this region is important for IncA function. Second, using molecular dynamics simulations, we establish that the crystallographic structure of the IncA cytosolic core is thermodynamically stable and unlikely to undergo major tertiary structural conformational changes spontaneously. Local conformational changes in IncA occur during membrane fusion, but they likely remain confined to regions of the bundle characterized by higher RMSD, such as the H\textsubscript{clamp} and the C-terminal helix H\textsubscript{D}. Third, by assessing the fusion of the inclusions in HeLa cells infected with genetically manipulated \textit{Chlamydia} strains, we establish a direct correlation between the monomeric state of IncA in solution and its fusogenic activity. Deletion of just nine amino acids of the C-terminal helix H\textsubscript{D} results in the formation of stable IncA dimers in vitro and the drastic loss of fusogenic activity during infection. This inhibition of fusion was nonetheless completely rescued when cells infected by \textit{Chlamydia}-expressing IncA\textsubscript{mutant} were co-infected with \textit{Chlamydia}-expressing IncA\textsubscript{WT}, suggesting that the full-length bundle exerts a dominant function in \textit{trans} to restore the fusogenic activity of IncA\textsubscript{mutant}.

Using these data, we postulate a model in which IncA\textsubscript{WT} promotes the fusion of inclusion membranes by engaging in homotypic interactions in \textit{trans} (i.e., \textit{trans} homodimers in Fig. 6) mediated by the C-terminal helix H\textsubscript{D}. Such an interaction is unlikely to be a simple swap of the C-terminal helix because molecular dynamic simulations argue against a global unfolding of the IncA four-helix bundle. Instead, this interaction appears limited to a molecular contact between two IncA bundles. The ability of IncA\textsubscript{WT} to rescue IncA\textsubscript{mutant} in \textit{trans} suggests that the association between IncAs is not restricted to the C-terminal helix and may use determinants in the H\textsubscript{clamp}.

The non-canonical helical structure of IncA is key to solving the topological conundrum of homotypic fusion. In this context, the intramolecular contacts within IncA C-terminal helix H\textsubscript{D} and H\textsubscript{clamp} are essential for its fusogenic activity as it allows for homotypic interactions to occur only in \textit{trans}. By removing the intramolecular interactions through point mutations (IncA\textsubscript{1-246(polyA)}) or truncation (IncA\textsubscript{1-237}), we show that IncA\textsubscript{mutant} self-assemble in solution, likely leading to the formation of cis homodimers on the inclusion membrane (Supplementary Fig. 13). Consequently, the \textit{cis} homodimers cannot interact with IncA in \textit{trans} and thus are unable to promote homotypic fusion.

This model is supported by a previous analysis of the oligomeric state of IncA during infection, which found that IncA can naturally switch between monomeric and oligomeric states\textsuperscript{5,8,10} but cannot rule out the existence of an additional host or \textit{Chlamydia} factor that assists in the fusogenic bundling of IncA. One possibility is that a soluble or membrane-bound regulatory protein could catalyze the formation of the IncA \textit{trans}-complex, leading to membrane fusion. It is also possible that the unstructured moiety at the C-terminus of IncA helix H\textsubscript{D} or perhaps an intramolecular interaction between the helical bundle and the N-terminal membrane-embedded hairpin stabilizes the auto-inhibitory state that keeps IncA monomeric and competent for fusion.

In summary, the structure of the cytoplasmic domain of IncA described in this study sheds light on how this unique bacterial protein engages in functional complexes to control membrane fusion. Our studies also provide an invaluable template for homology modeling of Inc-orthologs from other fusogenic and non-fusogenic \textit{Chlamydia} strains\textsuperscript{10,28}, and will help guide us towards a more mechanistic understanding of how mutations and sequence insertions in IncA affect its fusogenic activity.
Methods

Gene cloning and mutagenesis. All primers and plasmids used in this study are detailed in Supplementary Tables 4 and 5, respectively. IncA\textsubscript{246-246-His\textsubscript{6}} (FD578) was constructed by PCR amplification of the region Thr87 to Lys246 using primers FO515/FO516 and full-length IncA as a template. The PCR product was digested with NcoI and XhoI and ligated into pET28a. IncA87\textsubscript{246-246-His\textsubscript{6}} (FD930) was constructed by PCR amplification of the region spanning Met1 to Lys246 using primers FO1023/FO1024 and full-length IncA as the template. The PCR product was digested with NcoI and XhoI and ligated into pET28a. IncA87\textsubscript{246-246-FLAG} (FD930) was constructed by PCR amplification of the region spanning Met1 to Lys246 using primers FO1023/FO1143 and full-length IncA as the template. The PCR product was digested with NcoI and SalI and ligated into pBOMB3-Tet. pBOMB3-Tet plasmid (FD9299) was constructed by removing the tetracycline promoter from pBOMB4-Tet with XhoI and NolI followed by ligation into pBOMB3 (FD2329). IncA\textsubscript{246-246-His\textsubscript{6}} (FD9944) was constructed by PCR amplification of the region spanning Met1 to Lys246 using primers FO1023/FO1143 and full-length IncA as the template. The PCR product was digested with NolI and SalI and ligated into pBOMB3-Tet. IncA\textsubscript{1-246 (G144A)-FLAG} (FD9495) was constructed by PCR amplification of the region spanning Met1 to Lys246 using primers FO515/FO516 and full-length IncA as a template. The PCR product was digested with NolI and SalI and ligated into pBOMB3-Tet. IncA\textsubscript{1-246 (G144A)-His\textsubscript{6}} (FD9395) was transformed into BL21(DE3) E. coli (Invitrogen, cat #44-0049) and grown in Luria-Bertani media to an optical density (at 600 nm) of 0.8. Protein expression was induced with 0.2 mM isopropyl \(\beta\)-D-thiogalactopyranoside (IPTG) for 24 h. After centrifugation, bacteria pellets containing IncA\textsubscript{246-246-His\textsubscript{6}} were resuspended in 25 mM Hepes pH 7.4, 75 mM NaCl, 10% (v/v) glycerol, 0.5 mM Tris\textsubscript{(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), 1 mM phe- nylmethylsulfonyl fluoride (PMSF), 10 mM Leupeptin, and 1.5 mM Pepstatin A; IncA\textsubscript{246-246-His\textsubscript{6}} was resuspended in 8.45 mM Potassium phosphate mono-basic/ 40.5 mM Sodium phosphate di-basic pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP-HCl, 1 mM PMSF, 10 mM Leupeptin, and 1.5 mM Pepstatin A; IncA\textsubscript{246-246 (G144A)-His\textsubscript{6}} was resuspended in 25 mM Hepes pH 7.4, 75 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP-HCl, 1 mM PMSF, 10 mM Leupeptin, and 1.5 mM Pepstatin A; and IncA\textsubscript{246-246 (G144A)-His\textsubscript{6}} was resuspended in 8.45 mM Potassium phosphate mono-basic/ 40.5 mM Sodium phosphate di-basic pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP-HCl, 1 mM PMSF, 10 mM Leupeptin, and 1.5 mM Pepstatin A. Resuspended proteins were resolved on a 4-12% gradient SDS-PAGE gel, and protein bands were visualized by staining with Coomassie Blue R-250. Protein concentrations were determined by measuring Abs at 280 nm.

Western blot. Infected cells were lysed 24 hpi by scraping into NuPage LDS sample buffer (Invitrogen) containing 2 mM 

Homotypic fusion assay. In all experiments, 6 x 10\(^4\) HeLa cells (ATCC) were seeded on glass coverslips 24 h prior to being infected with wild-type C. trachomatis or various C. trachomatis mutants at a moi of 5 in DME supplemented with 10% CAF, acid-rich serum, 2 mM t-glutamine, 1 mM sodium pyruvate, non-essential amino acids, and 1 µg/ml gentamicin. The infection was synchronized by centrifugation for 1 h at 10,000 x g, for 1 h at 4 °C. The infected cells were then transitioned to 37 °C and 5% CO\textsubscript{2} for the duration of the experiment. IncA\textsubscript{246-246-His\textsubscript{6}} was resuspended in 8.45 mM Potassium phosphate mono-basic/ 40.5 mM Sodium phosphate di-basic pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP-HCl, 1 mM PMSF, 10 mM Leupeptin, and 1.5 mM Pepstatin A. Resuspended proteins were resolved on a 4-12% gradient SDS-PAGE gel, and protein bands were visualized by staining with Coomassie Blue R-250. Protein concentrations were determined by measuring Abs at 280 nm.

Complementation of IncA KO C. trachomatis with IncA mutants. IncA KO C. trachomatis 12 and the IncA KO complemented with IncA\textsubscript{246-246-FLAG} and IncA\textsubscript{1-246 (G144A)-FLAG} were previously generated\textsuperscript{11}. To complement IncA KO Chlamydia with IncA-FLAG mutants, 5 x 10\(^4\) IncA KO elementary bodies and 10 µg of unmethylated DNA were mixed with 100 µl of 2x transformation buffer (20 mM Tris, pH 7.5, 100 mM CaCl\textsubscript{2}) and brought up to 200 µl with sterile water. Transformed cells were incubated at 35°C for 18 h before being plated with 13 ml of cell culture medium (Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% calf serum, 2 mM L-glutamine, non-essential amino acids, 1 mM sodium pyruvate). Two milliliters of transformation was added to each well of a 6-well plate containing confluent Vero cells. Antibiotic selection was initiated at 18 hpi with 100 ng/ml chloramphenicol. At 46 hpi, the cells were lysed in sterile water and the lysate was used to infect four T75 flasks containing confluent Vero cells. The chloramphenicol concentration was increased to 200 ng/ml and 1 µg/ml cycloheximide was added. At 46 hpi, the cells were lysed in sterile water and the lysate was used to infect two T75 flasks containing confluent Vero cells. The chloramphenicol concentration was increased to 400 ng/ml and 1 µg/ml cycloheximide was added. At 46 hpi, the cells were lysed in sterile water and the lysate was used to infect one T75 flask containing confluent Vero cells. The chloramphenicol concentration was increased to 400 ng/ml and 1 µg/ml cycloheximide was added. At 46 hpi, the cells were lysed in sterile water and the lysate was used to infect one T75 flask containing confluent Vero cells. The chloramphenicol concentration was increased to 400 ng/ml and 1 µg/ml cycloheximide was added. At 46 hpi, the cells were lysed in sterile water and the lysate was used to infect one T75 flask containing confluent Vero cells. The chloramphenicol concentration was increased to 400 ng/ml and 1 µg/ml cycloheximide was added.
Chlamydia Heterotypic fusion (co-infection) assay were measured using the Pisa server\textsuperscript{43} and PDBsum\textsuperscript{44}, and secondary structure using all re...dissolved at 75 M (corresponding to 1.5 and 3 mg ml\textsuperscript{-1}) to a continuous sedimentation coefficient. The triclinic structure of IncA\textsubscript{246}(G144A) was solved by molecular replacement using the wild-type IncA\textsubscript{246} structure as the search model, as implemented in PHASER\textsuperscript{87}. The best solution was subjected to positional and B-factor refinement in phenix.refine\textsuperscript{88} using all reflections between 15–1.95 Å resolution. The final models were refined to a \(R_{work}/R_{free}\) of 14.1/16.8% (IncA\textsubscript{246}) and 16.9/21.2% (IncA\textsubscript{246}(G144A)) using all diffraction data between 15–1.2 Å and 15–1.95 Å, respectively. Final model validation was done using MolProbity\textsuperscript{89}. Crystallographic data collection and refinement statistics are shown in Table I. Ribbon diagrams and surface representations were prepared using the program PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC). Intramolecular contacts were measured using the Pisa server\textsuperscript{43} and PDBeSum\textsuperscript{44}, and secondary structure superimpositions were carried out in Coot\textsuperscript{85}.

**Analytical ultra centrifugation sedimentation velocity assay.** AUC-SV analysis was carried out in a Beckman XL-A Analytical Ultracentrifuge. IncA samples were dissolved at 75 M and 150 M (corresponding to 1.5 and 3 mg ml\textsuperscript{-1}) in 20 mM HEPES pH 7.5, 130 mM NaCl, 1 mM DTT and were spun at 45,000 rpm at 4°C. Absorption was also analyzed at 250 285 nm to fit to a continuous sedimentation coefficient (c(s)) distribution model in SEDFET\textsuperscript{45}. Data were visualized and presented using GUSSI (University of Texas Southwestern Medical Center).
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Author contributions

Conceived and designed experiments: G.C., J.W., J.R.P., F.P.; Performed the experiments: G.C., J.W., J.R.P., F.P.; Contributed reagents/materials/analysis tools: G.C., J.R.P., F.P.; Wrote the paper: G.C., A.B., J.W., J.R.P., F.P.;}
Additional information

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