Conservation of the biochemical properties of IncA from Chlamydia trachomatis and C. caviae: oligomerization of IncA mediates interaction between facing membranes.

Cédric Delevoye, Michael Nilges$, Alice Dautry-Varsat and Agathe Subtil#

Unité de Biologie des Interactions Cellulaires and $Unité de Bio-Informatique Structurale, Institut Pasteur, CNRS URA 2582 and 2185, 25 rue du Docteur Roux, 75015 Paris, France.

# To whom correspondence should be addressed
Tel: (33) 1 40 61 30 49 Fax: (33) 1 40 61 32 38 Email: asubtil@pasteur.fr

Running title: Oligomerization of IncA anchored on facing membranes

SUMMARY

The developmental cycle of Chlamydiaceae occurs in a membrane compartment called an inclusion. IncA is a member of a family of proteins synthesized and secreted onto the inclusion membrane by the bacteria. IncA proteins from different species of Chlamydiaceae show little sequence similarity. We report that the biochemical properties of C. trachomatis and C. caviae are conserved. Both proteins associate with themselves to form multimers. When artificially expressed by the host cell, they localize to the endoplasmic reticulum. Strikingly, heterologous expression of IncA in the endoplasmic reticulum completely inhibits concomitant inclusion development. Using truncated forms of IncA from C. caviae, we show that expression of the C-terminal cytoplasmic domain of the protein at the surface of the endoplasmic reticulum is sufficient to disrupt the bacterial developmental cycle. On the other hand, development of a C. trachomatis strain that does not express IncA is not inhibited by artificial IncA expression, showing that the disruptive effect observed with the wild type strain requires direct interactions between IncA molecules at the inclusion membrane and on the endoplasmic reticulum. Finally, we modeled IncA tetramers in parallel four helix bundles based on the structure of the SNARE complex, a conserved structure involved in membrane fusion in eukaryotic cells. Both C. trachomatis and C. caviae IncA tetramers were highly stable in this model. In conclusion, we show that the property of IncA proteins to assemble into multimeric structures is conserved between chlamydial species and we propose that these proteins may have co-evolved with the SNARE machinery for a role in membrane fusion.
INTRODUCTION

Chlamydiaceae are obligate intracellular parasites which develop in a host cell within a membrane-bound compartment, termed an inclusion. The membrane of the inclusion is initially formed by the invagination of the plasma membrane, and pinching off of a vesicle containing the infectious form of the bacterium, the elementary body (EB\(^1\)). Thereafter, EBs differentiate into noninfectious but metabolically active reticulate bodies, which proliferate within the expanding inclusion, giving rise to 1000 or more progeny per host cell. The infectious cycle ends after 2 to 3 days depending on the strain, when bacteria that have differentiated back to EBs are released in the extracellular medium.

The composition of the inclusion membrane and the origin of its constituents is not yet fully understood. EB-containing vesicles seem to become unable to fuse with early endosomes soon after entry (1). They also fail to fuse with late endocytic compartments and lysosomes, thus escaping degradation by the host cell (2). The early separation between the chlamydial inclusion and the endocytic trafficking distinguish the inclusion from the parasitophorous vacuoles of several pathogens (reviewed in (3)). Acquisition of lipids necessary for the growth of the inclusion membrane, as well as for incorporation into bacteria (4), must originate from other sources. One origin of the bacterial lipid content is the Golgi apparatus, as sphingomyelin, synthesized in the Golgi apparatus from a fluorescent precursor, is transported to the inclusion and accumulates into the bacteria (5). This process is dependent on bacterial protein synthesis: inhibition of chlamydial early transcription or translation prevents the incorporation of sphingomyelin, and the inclusion eventually fuses with lysosomes (6). The bacteria also appear to acquire host cell cholesterol by the same Golgi-dependent pathway as sphingomyelin (7). Surprisingly, no host cell protein has been found inserted into the inclusion membrane (2). The only host protein known to interact directly with the inclusion is 14-3-3, for which interaction with a bacterial component of the inclusion has been demonstrated (8). Host proteins recruited to the inclusion include dynein, β-catenin and specific Rab GTPases, but the mechanisms of their association with the inclusion are not known (9-11).

In contrast with the seemingly poverty of the inclusion membrane in eukaryotic proteins, a large family of bacterial proteins, termed Inc proteins, are known to be inserted in this compartment (12). These proteins are unique to Chlamydiaceae, and members of the family share little primary sequence identity with each other within one species. Some of the members are somewhat conserved between different Chlamydiaceae species, but in that case the conservation is usually low. They share however one remarkable feature, which allows to predict that a given protein
probably belongs to the family: they possess a very large (50-80 amino acids) bilobed hydrophobic domain. Confirmation that a protein which such a domain is located to the inclusion membrane requires specific antibodies against this protein (13). So far about 10 proteins have been shown to localize to the inclusion membrane, and between 40 to 90 proteins are predicted to belong to the family, from *C. trachomatis* and *C. pneumoniae* genome analysis, respectively (13,14). The topology of the insertion of Inc proteins in the inclusion membrane has not been directly addressed, but microinjection of antibodies against 4 of these proteins demonstrated that at least the carboxy-terminal domain is exposed to the cytosol (15,16). The large hydrophobic domain is probably required for the insertion in the inclusion membrane and would be compatible with a hair-pin insertion, with both extremities of the proteins facing the cytosol, but this needs to be investigated.

The mechanism by which Inc proteins are secreted out of the Chlamydiaceae for insertion in the inclusion membrane has been identified. Chlamydiaceae possess a type III secretion apparatus, which is found in several Gram negative pathogenic bacteria, and which allows for the translocation of bacterial proteins through the bacterial membranes and across a eukaryotic membrane (17). Using heterologous secretion, it was shown that Inc proteins are recognized by type III secretion machineries of other pathogens, strongly suggesting that it is the mechanism used by Chlamydiaceae to secrete these proteins into the inclusion membrane (18,19).

The first member of the Inc family to be identified, IncA, is also the one that attracted most of the attention. First cloned from *C. caviae* (CCA00550), it has homologs in a similar genetic environment in all sequenced genomes (CT119 in *C. trachomatis* serovar D and CPn0186 in *C. pneumoniae* CWL029). The level of sequence identity between homologs is low, and antibodies against IncA do not cross react between different species. Antibodies against IncA from each of these species have been obtained, and have allowed to show an important accumulation of the protein on the membrane of the inclusion of all species (13,20,21), as well as on fibers emanating from the inclusion that are particularly enriched in some species (22). IncA from *C. caviae* was shown to be exposed on the cytoplasmic face of the inclusion, and to be phosphorylated by the host cell (15). Finally, IncA is expressed rather late compared to most Inc proteins, the transcript being detected 12 h post infection with the *C. trachomatis* serovar L2 strain and 16 h post infection with the *C. trachomatis* serovar D strain (23,24).

IncA is the only member of the Inc family for which a function has been proposed, namely a role in the homotypic fusion of inclusions in *C. trachomatis*. Typical *C. trachomatis* isolates occupy inclusions that fuse with each other when the cells are infected at high multiplicities of infection. This fusion is inhibited at low temperature (32°C) and requires bacterial protein synthesis (25). Evidence for the involvement of IncA in this process came from two independent studies.
First, microinjection of anti-IncA antibodies blocked the fusion of inclusions in cells infected at high multiplicities of infection (16). Second, a minority (1.5%) of *C. trachomatis* clinical isolates form multiple non-fusogenic inclusions and do not express IncA (26). Careful analysis of this collection of variants later showed that if most of these variants (24/27) do not express IncA, three non-fusogenic strains do express a normal protein at the inclusion membrane, suggesting that other elements of the fusion machinery are missing in these strains (27). Consistent with the implication of IncA in inclusion fusion is the observation that the majority of inclusion fusions occurs between 10 and 16 h post infection with the serovar L2 (25,28), which correlates with the time when IncA can be detected in this strain (16). Moreover, the inhibition of inclusion fusion at low temperature correlated with an inhibition of IncA export to the inclusion membrane in these conditions (29). However, the temperature block is likely to affect the export of several proteins, which could also account for the inhibition of fusion.

Altogether, these data argue for a role of IncA in the fusion of inclusions observed with *C. trachomatis* strains. However, several questions remain unsolved. In cells infected at low multiplicity of infection, microinjection of anti-IncA antibodies leads to the septation of the inclusion (16). One explanation is that inclusions, like other eukaryotic organelles, are dynamic entities that can fuse and septate, and that in the presence of antibodies against IncA fusion is slowed down, resulting in multiple inclusions. However, clinical isolates of non-fusogenic phenotypes contain only a single inclusion at low multiplicity of infection, implicating that the absence of IncA does not result in multiple inclusions in this case. Microinjection of whole anti-IncA antibody was shown to induce the aggregation of IncA on the surface of the inclusion, while the protein remained homogenously distributed when Fab fragments of the same antibody were injected (15). Therefore, microinjection of anti-IncA antibody may induce a more general disorganization of the inclusion proteins involved in fusion/septation and some of the consequences of microinjection may be indirect. Even if IncA plays a direct role in *C. trachomatis* inclusion fusion, other roles may be envisioned, especially in other species such as *C. caviae* and *C. pneumoniae*, which appear to be less fusogenic than *C. trachomatis*.

In this report, we investigated the biochemical properties of IncA from *C. caviae* GPIC strain (*Cca*IncA) and from *C. trachomatis* serovar L2 (*Ctr*IncA). We showed that IncA from both species can interact with itself via coiled-coil interactions in its C-terminal cytoplasmic domain. Dynamic modelisation on membrane-proximal domains of *Cca*IncA and *Ctr*IncA showed that tetramers of IncA are compatible with a structure similar to the SNARE complex, which is a conserved complex involved in the fusion of vesicles with their target membrane. We used
heterologous expression of IncA by HeLa cells to further investigate the biochemical properties of IncA.
EXPERIMENTAL PROCEDURES

Cells, bacteria, antibodies and other reagents

The human cervical adenocarcinoma cell line, HeLa 229, was from the American Type Culture Collection (ATCC) and was grown in Dulbecco's modified Eagle's medium with glutamax (Life Technologies) supplemented with 10% fetal calf serum (complete medium). The GPIC strain of *C. caviae* was obtained from Roger Rank (University of Arkansas). *C. trachomatis* serovar L2 strain 434 (VR-902B) and *C. trachomatis* serotype D (27F0734) were from ATCC. *C. trachomatis* serotype D(s)5058 is a clinical isolate which does not express IncA and was kindly given by Drs Dan Rockey and Walter Stamm (27). *Chlamydiaceae* were prepared as described (30). Bovine serum albumin (BSA), saponin, dimethyl sulfoxide (DMSO) and 1,4-diazabicyclo-[2.2.2] octane (DABCO) were purchased from Sigma, paraformaldehyde was from Merck, mowiol was from Calbiochem. Ni-nitrilotriacetic acid (NTA) agarose beads were purchased from Qiagen, dithio-bis-succinimidyl propionate (DSP) was from Interchim (Montluçon, France). The mouse anti-*Chlamydia* antibodies were purchased from Argene, Biosoft (#12-114,Varilhes, France). Rabbit anti-Histidine (sc-803) and rabbit anti-green fluorescent protein (GFP) (sc-8334) antibodies were from Santa Cruz Biotechnology, mouse anti-Histidine from Invitrogen (#46-0693), and rabbit anti-calnexin antibodies were from Stressgen (#SPA-860). Horseradish peroxidase-linked (HRP) anti-rabbit and Cy™-3-conjugated goat anti-mouse antibodies were from Amersham Pharmacia Biotech, alkaline phosphatase-linked secondary antibodies were obtained from Pierce, goat anti-rabbit or mouse Alexa Fluor-488 antibodies were from Molecular Probes and goat anti-rabbit TRITC antibodies were from Immunotech (France). Rabbit anti-*C. caviae* IncA antibodies were prepared as described (30).

Plasmids

Genomic DNA from *C. caviae* strain GPIC and *C. trachomatis* serovar L2 were prepared from bacteria using the RapidPrep Micro Genomic DNA isolation kit (Amersham Pharmacia Biotech). The *incA* genes were amplified by PCR, and cloned in NcoI-KpnI sites of pQE-TriSystem vector for His-tagged proteins (Qiagen) and in EcoRI-KpnI sites in pEGFP-C1 vector for GFP-tagged proteins (Clontech). Sequences of the primers used are listed in Table 1. In some cases, introduction of the NcoI cloning site necessitated a modification of the second amino-acid of the protein. To construct ∆118CcaIncA-NS5B, the last 714 nucleotides of CcaIncA were amplified by PCR and
cloned into pCMVGFPNS5BconC26 digested by NheI and BsrGI a generous gift from Dr. D. Moradpour, Freiburg Hospital University).

Infection

HeLa cells, grown in 10 cm dishes and less than 50% confluent, were washed twice in warmed phosphate buffer saline (PBS). For infection with *C. caviae* strain GPIC and with *C. trachomatis* serovar L2, the cells were incubated in complete medium with bacteria (GPIC) for 90 min at 37 °C, washed twice in PBS, and incubated for the indicated time at 37°C. For infection with *C. trachomatis* serovar D, a centrifugation step of 30 min at 3.000 g was added before incubating the cells at 37°C.

Cross-linking experiments

Cells (6x10⁶ cells per condition) were washed three times in PBS, incubated with 0.5 mM DSP in 1% DMSO or in 1% DMSO alone in cross-linking buffer (150 mM NaCl, 0.2 mM CaCl₂, 0.2 mM MgCl₂, 10 mM Heps, pH 7.3) for 30 min at 4°C and neutralized with 3 washes in 150 mM NaCl, 20 mM Tris, pH 7.3. Cells were lyzed in lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X100, pH 7.5) supplemented with 5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and protease inhibitor cocktail (Sigma) for 30 min at 4°C before scraping and centrifugation at 18.000 g for 20 min at 4°C. In the case of infected cells, the supernatant fractions were boiled in SDS-PAGE sample buffer (62 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue) with or without β-mercaptoethanol and resolved by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE). After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore), and the membrane was used for blotting with anti-CcaIncA or anti-Histidine antibodies followed with HRP- or alkaline phosphatase-conjugated antibodies, and revelation were performed by enhanced chemiluminescence (ECL) or chemifluorescence, respectively, according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

Co-purification of GFP-IncA and IncA-His on Histidine affinity columns

HeLa cells (6x10⁶ per point) were transfected by electroporation with 20 μg of indicated plasmid in 40 mM NaCl solution at 900 μF, 200 V (EasyJet, Eurogentec). Cells were seeded in 10 cm dishes for 24 h, lysed on ice in lysis buffer. Cell lysates were centrifuged at 18.000 g for 20 min at 4°C. Equal volumes of supernatant were incubated for 2 h with 20 μl of Ni-NTA beads at 4°C with
gentle agitation. The beads were washed extensively with lysis buffer at 4°C, boiled in SDS-PAGE sample buffer and proteins bound to the beads were analyzed by SDS-PAGE followed with transfer to a PVDF membrane and western blotting. The same membrane was first probed with anti-Histidine and HRP-conjugated anti-rabbit antibodies, and revealed by ECL. The membrane was then stripped by a 30 min incubation at 50°C in 0.7% β-mercaptoethanol, 2% SDS, 62.5 mM Tris pH 6.8, and probed again, using anti-GFP and HRP-conjugated anti-rabbit antibodies, and revealed by ECL.

Modelisation of IncA structure

For both CcaIncA and CtrIncA, a stretch of 39 amino acids 23 residues after the end of the second predicted transmembrane helix was modeled. The sequences were aligned by hand to the four helices in the endosomal SNARE complex (31), such that the hydrophobic a and d positions matched. This naturally aligned a central hydrophilic residue (Glutamine for CcaIncA, Threonine for CtrIncA) with the central Glutamine residues in the SNARE complex, in the "d" position. The side chains of CcaIncA and CtrIncA were built onto the coordinates of the central part of the endosomal SNARE complex (31) by a molecular dynamics based strategy essentially as published (32). In this method, missing side-chain atoms are initially placed in random positions and the resulting structure is then minimized in a three-stage protocol: first, simulated annealing with covalent and packing interactions only; second, a short molecular dynamics run with a full molecular dynamics force field in vacuo, followed by conjugate gradient minimization; and third, a molecular dynamics run in explicit solvent. We used the program X-plor (33) for stages 1 and 2, and GROMACS 3.2 (34) for stage 3. We extended the third stage to a molecular dynamics trajectory in explicit solvent of one ns. The structures were embedded in a box of SPC water molecules with minimum distance between the solute and the box boundary of 1 nm. The system consisting of protein, water and Na ions to neutralize the total charge was slowly heated from 50 K to the simulation temperature (300 or 350 K) with positional restraints on the solute during 300 ps. The electrostatic interactions were treated with the Particle-Mesh-Ewald method (35) for interactions beyond 1 nm; weak temperature coupling with a relaxation time of 5 ps, using the Berendsen method, was employed (36).

Immunofluorescence microscopy

HeLa cells grown in 6-well plates were transfected with the indicated plasmids using Fugene reagent (Roche Applied Science). Twenty-four hours later, the cells were washed twice in PBS and
fixed with 4% paraformaldehyde, 120 mM sucrose in PBS for 30 min at room temperature. The cells were washed in PBS, incubated for 10 min in 50 mM NH₄Cl in PBS at room temperature, saturated in 1 mg/ml BSA in PBS and permeabilized in 0.05% saponin, 1 mg/ml BSA in PBS. To observe Histidine-tagged proteins, the cells were first labeled with anti-Histidine antibody before being incubated with anti-rabbit Alexa Fluor-488 antibody. The endoplasmic reticulum and the chlamydial inclusion were labeled with anti-calnexin and anti-\textit{Chlamydia} antibodies, respectively, followed by incubation with Cy\textsuperscript{TM}-3-conjugated goat anti-mouse antibodies. To quantify bacterial entry in cells transfected with \textit{Cca}IncA-His, the cells were fixed 3 h after infection. Extracellular and intracellular bacteria and transfected cells were labeled as described (40), except that transfected cells were identified using anti-His antibodies. Coverslips were mounted in mowiol with 100 mg/ml DABCO and examined under an epifluorescence microscope (Axioskop, Zeiss, Germany) attached to a cooled CDD-camera (Photometrics, Tucson, AZ), using a x63 Apochromat lens. Images were acquired and analyzed using Metamorph software (Universal Imaging Corporation).
RESULTS

*IncA proteins are found in high molecular weight complexes*

IncA proteins share the same general organization: a short amino-terminal domain, a large bilobed hydrophobic domain and a C-terminal cytoplasmic domain (Figure 1). This domain was shown to be exposed on the cytosolic face of the inclusion in the case of *CcaIncA* and *CtrIncA* (15,16). The level of identity between IncA of different species is low (around 20% of identical residues, which are distributed along the whole molecule). Using tools designed to calculate the propensity of a given sequence to form coils, we noticed that all IncA proteins are predicted to engage in coiled-coil structures over the whole length of their C-terminal domain with a high probability. This prediction is strengthened by the presence of one or more leucine-zipper domains in these regions.

The potential of IncA to interact with other molecules via coiled-coil interactions prompted us to look for its partner(s). First, we performed cross-linking experiments on cells infected for 24 h with *C. caviae*. Infected cells were incubated for 30 min at 4°C with the cross-linking reagent DSP before cell lysis and analysis of whole-cell extracts by SDS-PAGE, followed by western blot analysis using anti-*CcaIncA* antibodies (Figure 2A). In control cells, IncA migrated around 37 kD, which corresponds to its expected molecular weight. In cells incubated with the cross-linker, IncA also migrated as a higher molecular weight complex, around 75 kD. This band disappeared when the sample was treated with β-mercaptoethanol, which cleaves the disulfide bridge present in the cross-linker and separates IncA from its partner. A faint upper band (around 150 kD) was also observed in cells treated with DSP, suggesting that IncA may participate in the formation of complexes of molecular weight even higher than 75 kD, that may consist of more than 2 molecules.

Since IncA is found on the cytosolic face of the membrane of the inclusion, which contains several chlamydial proteins, its partners may be of eukaryote or prokaryote origin. To assess if the presence of other chlamydial proteins were necessary for the association of IncA with its partners, we performed cross-linking experiments in cells heterologously expressing IncA. For this purpose, *incA* was cloned in a vector that provided a promoter for expression in eukaryotic cells as well as a carboxy-terminal Histidine tag.

Cells transfected with *CcaIncA*-His were treated with DSP, lysed, and the affinity of the Histidine tag for Ni$^{2+}$ was used to purify the His-tagged protein from cell extracts using Ni-nitrilotriacetic acid (NTA) beads. Ni-NTA associated proteins were analyzed by SDS-PAGE and...
western blot. In cells incubated with DSP, in addition to the band corresponding to *Cca*IncA-His monomers (42 kD), *Cca*IncA-His migrated in an intermediate- (around 90 kD) and high- (around 180 kD) molecular weight complex, which disappeared when the samples were prepared in reducing conditions (Figure 2B). These results are similar to what we observed in cells infected with *C. caviae*, the Histidine tag accounting for the shift in the migration of the different bands. This result shows that the participation of *Cca*IncA to high molecular weight complexes does not require the presence of other chlamydial proteins, and that *Cca*IncA partner is either itself or a protein from the host cell.

To see whether the formation of high molecular weight complexes involving *Cca*IncA was specific to this protein we performed the same cross-linking experiments in cells expressing *Ctr*IncA-His. In cells incubated with DSP, in addition to the band corresponding to *Ctr*IncA-His monomers (35 kD), *Ctr*IncA-His migrated in an intermediate- (around 70 kD) and high- (around 100 kD) molecular weight complexes, which disappeared when the samples were prepared in reducing conditions (Figure 2B). Therefore, the property of IncA to participate in high molecular weight complexes is common to at least two species of Chlamydiaceae.

Altogether, these experiments show that *Cca*IncA-His and *Ctr*IncA-His participate in high molecular weight complexes. Considering the fact that cross-linking is never complete, the relative abundance of intermediate- and even high- molecular weight complexes suggests that most of the IncA molecules are engaged in these complexes.

*IncA interacts with itself*

Since the size of the intermediate-molecular weight complexes were compatible with the formation of dimers of IncA, we investigated further whether this protein was able to form multimers. There is no useful gene manipulation technique to manipulate the genome of Chlamydiaceae, and to address reporter molecules to the membrane of the inclusion. Therefore, to determine whether *Cca*IncA was able to interact with itself, we expressed *Cca*IncA in HeLa cells with two different tags: a C-terminal Histidine tag (*Cca*IncA-His), and a N-terminal Green Fluorescent Protein tag (GFP-*Cca*IncA). The affinity of the Histidine tag for Ni$^{2+}$ was used to purify the His-tagged protein from cell extracts using Ni-NTA beads. The beads were washed and Ni-NTA associated proteins were analyzed by SDS-PAGE and western blot. Probing of the membrane with anti-Histidine antibody showed that, as expected, *Cca*IncA-His was retained on the beads (Figure 2C, left, lane 1). The membrane was then stripped and re-used for probing with anti-GFP antibody,
which revealed the presence of GFP-CcaIncA in the Ni-NTA associated fraction (Figure 2C, right, lane 1). In control experiments, when the cells had been transfected with GFP and CcaIncA-His, or with GFP-CcaIncA alone, no signal was seen after the anti-GFP blotting, showing that neither GFP nor CcaIncA interacts directly with the NiNTA beads (data not shown).

An identical experiment was performed using cells co-expressing CtrIncA-His and GFP-CtrIncA. Probing with anti-GFP antibody showed that GFP-CtrIncA was retained with CtrIncA-His on the Ni-NTA beads, demonstrating that CtrIncA is also able to form dimers (Figure 2C, left and right, lanes 2).

These experiments show that both CcaIncA and CtrIncA were able to form dimers, which resisted to the detergent (0.5 % Triton) present in the cell extracts. It is therefore very likely that the intermediate-molecular weight complexes revealed by cross-linking in the previous experiments correspond to IncA dimers.

Modelisation of IncA tetrameric interactions is compatible with a four helix bundle structure similar to that of the SNARE complex

Upon closer examination of IncA sequences, we noticed that CcaIncA showed sequence similarity with a well characterized domain of the eukaryotic fusion machinery, the SNARE domain. In eukaryotic cells, fusion of compartments is preceded by assembly of the fusion machinery, which involves mainly proteins of the SNARE superfamily. The hallmark of all SNARE proteins is that they contain conserved heptad repeat sequences that form coiled-coil structures called the SNARE complex, with a highly conserved Glutamine or Arginine residue at the center of the complex (37). Importantly, the SNARE domain is close to the transmembrane domain of the SNARE proteins (or brought in proximity of the membrane during SNARE complex formation). This position brings opposite membranes together during SNARE complex formation and is essential for membrane fusion. Strikingly, CcaIncA first leucine zipper (amino acids 140 to 178) consists of 6 heptad repeats with leucine residues in positions $a$ and other hydrophobic residues in positions $d$, except for the Glutamine residue present in $d$ in the center of the domain (Figure 3A). This similarity with SNARE domains, together with the fact that we showed that IncA dimerizes and probably tetramerizes, suggested to us a parallel organization of four CcaIncA helices similar to that found in the SNARE complex. We modeled a 39 residue stretch of the CcaIncA and CtrIncA sequences, 23 residues after the predicted transmembrane domain, as a parallel tetrameric coiled coil. These sequences are shown in Figure 3A, aligned with the four helices of the SNARE domain of the endosomal SNARE complex (31). Figure 3B shows helical wheel representation of the
CcaIncA tetramer, with some key interactions indicated by lines. Fig. 3C and D show the tridimensional structures of CcaIncA and CtrIncA models, respectively. In these models, the helices are connected by layers of hydrophobic amino acids with the exception of the hydrophilic central layer, like in the SNARE complex. The association of neighbouring helices is favored by numerous polar interactions. We next analyzed the stability of the modeled tetramer over time in aqueous solution. The simulations were performed at two temperatures, 27°C and 77°C. Both simulations showed that the modeled tetramers were very stable, presenting only small fluctuations around the average structure. Indeed, during the whole simulation, we observed neither separation of the monomers nor modification of the secondary structure. For comparison, we performed the same simulation on the SNARE complex. We observed that the molecular dynamics trajectory of the IncA models were very similar to those of the SNARE complex itself. The only difference was that the four central polar residues of the IncA tetramers exchanged conformations several times during the simulations while those of the SNARE complex did not. It is noteworthy that such exchanges of conformations were observed experimentally in the case of a dimeric leucine zipper (38). Movies of the simulations are available as supplementary material (Fig. 3Csup, 3Dsup and 3Esup). Altogether, our modelisation shows that CcaIncA and CtrIncA sequences are compatible with the formation of very stable four parallel helix bundles, resulting in a structure similar to the SNARE complex.

**IncA-His expressed by HeLa cells localises to the endoplasmic reticulum**

Using biochemical approaches, we showed that IncA molecules interact similarly when they localize in the inclusion, during normal infection, or when they are artificially expressed by a cell in the absence of inclusion. To determine the localisation of IncA in the latter case, we labelled cells transfected with IncA-His with anti-His antibody. Both CcaIncA-His and CtrIncA-His were found in a reticulate compartment extending throughout the cell. This compartment was identified as the endoplasmic reticulum (ER) using antibodies to calnexin, a well characterized ER marker (39) (Figure 4A). This distribution was not due to the His tag as it was also observed in cells transfected with GFP-tagged IncA constructs (see Figure 7A).

To determine the domains of IncA that are responsible for the cellular localisation of the protein, we constructed several truncated forms of CcaIncA-His (Figure 4B). Deletion of the amino-terminal domain (Δ53CcaIncA-His) had no effect on the distribution of the protein. Further deletion of the hydrophobic domain (Δ118CcaIncA-His) resulted in a cytosolic distribution, showing that this domain is necessary for membrane anchoring of the protein. Finally, truncation of
the last 135 amino acids of CcaIncA (CcaIncAΔ135His) resulted in redistribution of the protein, which was enriched at the plasma membrane, suggesting that this mutant was able to exit from the ER. Altogether, these experiments show that the large hydrophobic domain of CcaIncA is necessary to anchor the protein in a membrane, but is not sufficient for ER retention, for which part of the carboxy-terminal domain is necessary.

*Heterologous expression of IncA disrupts the infectious cycle*

We have shown that IncA molecules interact with each other when they localize in a cellular membrane, either the inclusion, during normal infection, or the ER, when experimentally expressed in eukaryotic cells. In both cases, IncA can form dimers and higher molecular complexes. We also showed that IncA could form a tetrameric structure similar to that of the four helix bundle of the SNARE complex. In the latter case, the complex involves SNARE proteins localized on two different membrane compartments. To test the relevance of our structural model in vivo, we asked whether IncA molecules expressed on two different compartments were able to interact with each other. To that end, cells were first transfected with CcaIncA-His for 24 h, or with GFP as a control, then infected with *C. caviae* GPIC for another 24 h before fixation and labeling of the inclusion and of CcaIncA-His by immunofluorescence. Unexpectedly, we observed that none of the transfected cells were infected, although in GFP-transfected cells infection had occurred normally (Figure 5A). Similarly, *C. trachomatis* serovar L2 or D were not able to develop in cells transfected with CtrIncA-His. The same result was observed when GFP-IncA was used instead of IncA-His (see below). This suggested that some step(s) in the infectious cycle was inhibited in cells expressing IncA. Inhibition on chlamydial development was complete only when IncA corresponding to the strain used for the infection was expressed. However, in cells transfected with GFP-CcaIncA, *C. trachomatis* serovar L2 development was inhibited by 50% and in cells transfected with GFP-CtrIncA, *C. caviae* development was inhibited by 20% (*data not shown*). Using an assay that allows to measure bacteria entry (40), we quantified *C. caviae* GPIC entry 3 h after infection in cells transfected with CcaIncA-His and in nontransfected cells. We observed no difference in the efficiency of bacteria entry between the two cell types, indicating that the expression of IncA-His did not affect bacteria attachment nor entry (Fig. 5B). To get further insight into the mechanism by which IncA inhibits chlamydial development, we performed the experiment in the reverse order: cells were first infected for 24 h, then transfected with GFP-IncA, and fixed 8 h after transfection, when expression of GFP-IncA becomes detectable by immunofluorescence. In both infected and...
non infected cells, GFP-IncA distributed in a reticulate compartment. However, in infected cells, this compartment was distorted (Figure 5C). The inclusion itself showed large changes in morphology, as the bacteria scattered in the cell and it was not clear whether the integrity of the inclusion membrane was intact. When cells were observed later after transfection (16 h), very few transfected cells that contained bacteria were visible and many dead cells were present in the culture dishes, suggesting that heterologous IncA expression eventually resulted in the death of infected cell. Altogether, these experiments show that experimental IncA expression in the host cell ER inhibits inclusion development.

Expression of IncA C-terminal cytoplasmic domain on the ER is sufficient to inhibit the inclusion development.

We tested the CcaIncA-His mutants for their ability to disrupt C. caviae infection, in order to determine the regions of IncA which are responsible for this effect. Cells expressing ∆53CcaIncA-His could not be infected by C. caviae, indicating that even in the absence of IncA N-terminal domain the disruptive effect was total (Figure 6A). To know whether CcaIncA hydrophobic domain played a role in this effect, we created a chimera between hepatitis C virus nonstructural protein NS5B and CcaIncA C-terminal domain. The hydrophobic carboxy-terminal domain of NS5B forms a transmembrane α-helix that contains a signal for retention in the ER. It has been shown that this domain was sufficient to target a protein on the cytosolic side of the ER (41). Indeed, fusion of 26 amino acids from NS5B transmembrane domain with CcaIncA C-terminal cytoplasmic domain resulted in a chimera that had a distribution characteristic of the ER (Figure 6B). HeLa cells expressing this chimera presented the same defect in the development of bacteria as HeLa cells expressing CcaIncaA-His, showing that IncA hydrophobic domain is not required for the inhibition of chlamydial development (Figure 6A). Finally, transfection of CcaIncA C-terminal cytoplasmic domain (∆118CcaIncA-His), which has a cytosolic distribution, had no effect on subsequent infection by C. caviae, which developed into similar inclusions in transfected as in non-transfected cells. Altogether, these data show that, when anchored to the ER, CcaIncA C-terminal cytoplasmic domain is sufficient to disrupt the infection.
IncA-His interacts with endogenous IncA at the membrane of the inclusion and disrupts bacterial development.

The effect of ER-located IncA on the development of the inclusion might be due to interactions with endogenous IncA on the membrane of the inclusion, or with any other inclusion-associated protein. To test these hypotheses, we used a strain of *C. trachomatis* that does not express IncA, D(s)5058 (27). This strain develops forming multiple inclusions that are non-fusogenic. When HeLa cells transfected with GFP-CtrIncA for 24 h were infected with D(s)5058, inclusions developed in transfected cells as efficiently as in non-transfected cells, and the ER had a normal appearance (Figure 7). This experiment demonstrates that in the absence of IncA on the membrane of the inclusion there was no disruptive effect of heterologous IncA expression on inclusion development. This result, together with the biochemical experiments demonstrating that IncA interacts with itself, shows that the disruption of the inclusion development is mediated by interactions between IncA proteins on the inclusion and on the ER. Therefore, we concluded that IncA molecules anchored in facing membranes associate to form multimeric structures.
DISCUSSION

In this paper we show that IncA from two species, *C. trachomatis* and *C. caviae*, associate with themselves to form multimers. First, in infected cells, we showed using a cross-linker that CcaIncA participated in high molecular weight complexes whose sizes were compatible with the cross-linking of two or more CcaIncA molecules. High molecular weight complexes were also present in cells transfected with CcaIncA and CtrIncA treated with a cross-linker, showing that no other bacterial protein than IncA was necessary for their formation. Finally, we showed, using cells transfected with two IncA constructs linked to different tags, that IncA molecules interacted with each other in cell extracts. Our results are in partial agreement with a previous report in which a yeast two-hybrid system was used to show interactions between CtrIncA molecules (16). However, interactions between CcaIncA molecules were hardly detected with this technique, supporting the authors’ conclusion that the function of IncA proteins may differ between species. Here we show that the ability of IncA to form multimers is common to the two species, and we propose that this property underlies similar function(s) during infection.

When artificially expressed in HeLa cells, IncA was localized in the ER, as shown by colocalization with an ER marker, calnexin. The same distribution was observed for other Inc proteins heterologously expressed by the host cell, although some were detected on the plasma membrane (data not shown). Inc proteins are normally synthesized by the bacteria and are transported through the bacterial and inclusion membranes via a type III mechanism. Their insertion in the inclusion membrane may be coupled to the translocation process. In the case of heterologous expression, Inc proteins may be recognized by the cellular machinery as a substrate for ER-associated ribosomes and may be inserted into the ER membrane co-translationally. Alternatively, the association might be post-translational, due to a better affinity for the ER membrane lipid composition than for other cellular membranes, or via interactions with ER membrane proteins. The large bilobed hydrophobic domain which is common to all Inc proteins is probably essential for their interaction with membranes, and deletion of this domain in CcaIncA (Δ118CcaIncA-His) resulted in a cytosolic distribution. However the hydrophobic domain is not sufficient to ensure ER location as deletion of part of CcaIncA C-terminal cytoplasmic domain (CcaIncAΔ135His), preserving the entire hydrophobic domain, resulted in preferential localization to the plasma membrane. We did not directly address the topology of IncA insertion into the ER membrane. However, we observed, as described previously, that heterologously expressed CcaIncA-His showed the same migration profile in SDS-PAGE as endogenous CcaIncA (Figure 2B), with three
bands corresponding to three phosphorylation states of the protein (15). This finding strongly supports the hypothesis that heterologously expressed *Cca*IncA-His is inserted in the ER membrane with the same topology as in the inclusion membrane, with at least the carboxy-terminal domain exposed to the cytosol (16).

Expression of GFP-IncA in infected cells had a very striking effect. Soon after the beginning of appearance of IncA, the morphology of the inclusion was modified, with a scattering of the bacteria and simultaneous distortion of the ER. Later after transfection, all infected cells had disappeared from the transfected population and many dead cells had detached from the culture plate, strongly suggesting that heterologous expression of IncA in the ER of infected cells resulted in cell death. When the experiment was performed in the reverse order, by infecting cells which had been transfected prior to infection, no inclusion developed in the transfected population. This was not due to an inhibition of bacteria entry, as we measured that bacteria entry was not affected by IncA expression. In fact, infection with *C. caviae* seems to proceed normally during the first 10 hours. Fifteen hours after infection, the inclusions were disrupted. This correlates with the kinetics of *incA* expression, as its transcript was first detected by RT-PCR at 10 hours post infection in *C. caviae* infected cells (not shown).

To determine which domain of IncA was involved in the inhibition of chlamydial development, when expressed by transfection, we measured infection in cells transfected with truncated forms of IncA. Deletion of the amino-terminal domain did not suppress the deleterious effect of IncA expression showing that IncA amino-terminal domain was not required. In fact, the C-terminal cytoplasmic domain alone, targeted to the ER using a transmembrane ER targeting signal from hepatitis C virus non-structural protein NS5B, was sufficient to produce the inhibitory effect observed with full length IncA. Finally, expression of *Cca*IncA C-terminal cytoplasmic domain (Δ118*Cca*IncA-His), which has a cytosolic distribution, had no effect on the infection. These data suggest that to disrupt the infection, *Cca*IncA C-terminal cytoplasmic domain needs to be anchored to a cellular compartment. We do not know at this stage whether this compartment needs to be the ER, which is naturally distributed in the whole cytosol and therefore in proximity to the inclusion. It may be that expression of IncA at another intracellular compartment, such as the Golgi apparatus, also found in close proximity to the inclusion, would similarly disturb inclusion development. However this hypothesis would be difficult to test, as the only known signals for targeting proteins to the Golgi apparatus are in the cytosolic portions of these proteins, which would need to be replaced with IncA carboxy-terminal domain.
The deleterious effect on chlamydial development was observed only with IncA expression, not with *C. caviae* IncB, which is also expressed on the ER by transfection, showing that the effect was specific of this Inc protein (data not shown). Using a strain that does not express IncA on the surface of its inclusions, we were able to demonstrate that the deleterious effect of IncA expression on chlamydial development required the presence of IncA on the membrane of the inclusion. This result, together with our demonstration that IncA molecules interact with each other, show that the disruption of the inclusion development is mediated by direct interactions between IncA molecules from the two compartments in which they localize. Inhibition on chlamydial development was complete only when IncA corresponding to the strain used for the infection was expressed, showing that the effect is very specific. However partial interspecies inhibition was also observed, suggesting that, although very different in terms of sequence, *C. caviae* and *C. trachomatis* IncA biochemical properties are sufficiently close so that they can interact with each other to some extent. It supports the idea that IncA may fulfill similar roles during infection by different species.

In infected cells, expression of IncA in the ER leads to the disruption of the inclusion, and to changes in the morphology of the ER. Our microscopy observations are consistent with the hypothesis that these interactions lead to fusion events between the inclusion and the ER. Observation at a better resolution would be necessary to support this hypothesis. Unfortunately, the process is not easy to observe because infected cells are transfected with a low efficiency (independently of the transfected gene), and the few infected and transfected cells die rapidly after IncA expression. Our data therefore support previous reports indicating that IncA may play a role in the fusion of inclusions. The aim of the modeling and molecular dynamics studies was to get insights into possible mechanisms of IncA-induced in vesicle fusion. Amphipatic helices alone can be sufficient to induce membrane fusion in vitro (42). However, a fusion mechanism similar to the one involving the formation of the SNARE complex is attractive because of the similarity of the sequences and their properties: there is strong coiled-coil or leucine zipper propensity; the coiled-coil sequence starts shortly after a putative transmembrane region; there is a conserved hydrophilic residue in the interface that may serve to organize the helices, infer specificity, or to bias the equilibrium between multiple possible oligomeric states; finally, the tetramer proved very stable in molecular dynamics calculations at 300 and 350 K.

The model is based on the capability of the IncA sequences to form stable oligomers. Whereas the stability in molecular dynamics calculations is an indication that the model is
energetically favorable, we cannot directly compare the stability to that of a different oligomeric state (such as a dimer). For mutants of GCN4, Harbury et al. investigated which properties of coiled coil sequences favor tetramerization (43). These properties are met in our structural modelisation: (i) beta-branched residues at "a" positions, that could disfavor tetramers due to rotamer preferences of these residues, are absent in both modeled sequences. (ii) a polar residue in the interface, common in coiled coils and which may serve to organize the four helices such that the four hydrophilic residues form one layer, is found in both modeled sequences. (iii) residues on the surface of the tetramers form interactions; as an example, the polar interhelical interactions involving "b", "c", "g" and "e" positions are shown in Figure 3B. (iv) interactions involving "b" and "c" positions are more likely in tetrameric than in dimeric coiled coils because of the different relative orientations of the helices in tetramers. (v) there are no repulsions between residues of like charge in neighboring helices. For CcaIncA, an antiparallel orientation of the helices, on the other hand, would bring the residues on the "g" position on one helix into contact with those on the neighboring helix, thus leading to six Arginine residues into close proximity. We conclude that a parallel orientation of the helices is more likely.

It is generally agreed that these membrane-bridging SNARE complexes mediate membrane fusion directly (44,45). Our IncA model proposes a parallel tetramer formed from helices from two different vesicles. The transmembrane regions of the four monomers are all on the same side of this rod-like structure, similar to proteins involved in viral fusion and in membrane fusion involving the SNARE complex. The formation of the tetramer would therefore induce close approach of the membranes, and possibly destabilize the membranes locally close to the transmembrane domains (46). In that case, one could expect expression of IncA to disturb the ER structure, although this compartment has homotypic fusion abilities of its own. Indeed, we noted that expression of CtrIncA slightly modified the ER, whose network had a somewhat rougher appearance compared to that in non transfected cells (see Fig. 4A). Whether formation of IncA tetramers by itself is sufficient to promote fusion remains to be examined. The difference in the fusogenicity of C. trachomatis and C. caviae inclusions may indicate that IncA by itself is not sufficient to trigger fusion between inclusions, and that accessory molecules, absent from C. caviae, participate in the fusion of C. trachomatis inclusions. This hypothesis is supported by the observation that some of the non-fusogenic isolates do express IncA, suggesting that other factors necessary for the fusion to occur are missing from these strains (27).

Interestingly, we found, using BLAST analyses, that the genome of the chlamydia-related symbiont of free-living amoebae (UWE25) encodes an ORF annotated pc0399 which presents some sequence similarity with IncA (48). Moreover, it shares the characteristic organization of IncA
proteins, with a short amino-terminal domain, a large bilobed hydrophobic domain and a carboxy-terminal domain that is predicted to engage in coiled-coils interactions. If this molecule is present on the surface of the inclusion, it suggests that IncA has coevolved with its host cells since the divergence of the pathogenic and symbiotic chlamydiae, more than 700 million years ago.

Finally, we would like to speculate that, in addition to its role in the fusion of inclusions, IncA may participate in the fusion of cellular vesicles with the inclusion membrane. The fact that IncA structure is compatible with the participation to a SNARE-like complex suggests that this molecule may have evolved to interact not only with itself but also with cellular SNAREs to form fusion-competent SNARE complexes. One possibility is that IncA mimicry with host SNARE proteins could enable the bacteria to hijack part of the cellular traffic by allowing fusion of vesicles with the inclusion.

**FOOTNOTES**

1Abbreviations: BSA, bovine serum albumine; DMSO, dimethyl sulfoxide; DSP, dithio-bis-succinimidyl propionate; EB, elementary body; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; GFP, green fluorescent protein; HRP, horseradish peroxidase; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; SDS, sodium dodecyl sulfate.

**Acknowledgements:** We thank Stéphanie Perrinet for bacteria preparation. We thank Drs Dan Rockey and Walter Stamm for the kind gift of the D(s)5058 strain from the University of Washington *Chlamydia* Repository, and Dr Dan Rockey for critical reading of the manuscript. C. D. was supported by an Allocation de Recherche du Ministère de l’Enseignement Supérieur et de la Recherche.
FIGURE LEGENDS

Table 1. Primers used for plasmid construction by PCR.

Fig. 1. A schematic representation of IncA from different species. The scale refers to amino acid numbering.

Fig. 2. IncA participates in high molecular weight complexes by self-interaction. A- HeLa cells were infected with *C. caviae* for 24 h before cross-linking, cell lysis and centrifugation. The supernatant fractions of control cells or cells treated with 0.5 mM DSP were treated with or without 5% β-mercaptoethanol (β-me) before SDS-PAGE and western blot analysis using anti-*Cca*IncA antibody. The three forms of IncA around 40 kD correspond to different phosphorylation states (P₀, P₁, P₂) of IncA (15). B- HeLa cells untransfected (left) or transiently expressing *Cca*IncA-His (middle) or *Ctr*IncA-His (right) were treated with DSP, lysed and centrifuged. The supernatant fractions were incubated with Ni-NTA agarose beads and the bound fractions were treated with or without 5% β-mercaptoethanol, before analysis by SDS-PAGE and western blot using anti-His antibody. In addition to the specific signal, the anti-His antibody gives a non specific signal (ns) around 70 kD on cell extracts. C- HeLa cells were co-transfected with plasmids coding for *Cca*IncA-His and GFP-*Cca*IncA (lane 1, left and right), or for *Ctr*IncA-His and GFP-*Ctr*IncA (lane 2, left and right). The following day, the cells were lysed, centrifuged and the supernatant fractions were incubated with Ni-NTA beads. Ni-NTA-bound molecules were analyzed by SDS-PAGE and western blot. His-tagged proteins were first detected by blotting with anti-His antibody (left), then the membrane was stripped and re-used for blotting with anti-GFP antibody (right).

Fig. 3. Modelisation of IncA structure A- Alignment of the modeled regions of the sequences of *Ctr*IncA and *Cca*IncA, with the sequences forming the central part of the endosomal SNARE complex (31). The modeled region starts 23 residues after the end of the second predicted transmembrane helix. The alignment with the SNARE complex was done manually based on predicted hydrophobic a and d positions. The central polar residue is Threonine in the case of *Ctr*IncA and Glutamine in the case of *Cca*IncA. Hydrophobic residues on a and d positions are colored in blue, mismatches in yellow; the central polar residue is marked in green, or in red (for the Arginine in the SNARE complex). B- Helical wheel representation of *Cca*IncA as a parallel tetramer. The polar interactions observed in the model are indicated. C, D- Side view of the
CcaIncA (C) and CtrIncA (D) tetramers, showing one monomer (left), one monomer with the backbone of the other 3 helixes (middle) and the tetramer (right). The backbone is shown as a yellow ribbon. Lysine, Arginine and Histidine residues are shown in blue, Aspartate and Glutamate in red, other polar residues in green, hydrophobic residues in white. The figure was generated with VMD (47).

**Fig. 3 supplementary**: Molecular dynamics trajectories of the modeled structures of CcaIncA (C), CtrIncA (D) and the SNARE domain (E) at 27°C during 1 ns.

**Fig. 4. Distribution of IncA and of CcaIncA mutants.** A- HeLa cells expressing CcaIncA-His or CtrIncA-His were fixed, permeabilized and probed with mouse anti-His and rabbit anti-calnexin antibodies, followed with Alexa Fluor-488-coupled anti-mouse (left) and TRITC-coupled anti-rabbit (center) antibodies. The superimposition of the two images is shown on the right. B- Cells transiently expressing the indicated construct were fixed, permeabilized and stained with anti-His antibody. Numbers on the scheme refer to the first and last amino acids from CcaIncA incorporated in the construct.

**Fig. 5. Expression of IncA in the ER disrupts inclusion development.** A- Cells transfected with the indicated constructs were infected with *C. caviae* or *C. trachomatis* serovar L2 or D 24 h after transfection, and fixed one day later. Infected cells were labeled using anti-*Chlamydia* antibodies and cells transfected with IncA constructs were identified using anti-His antibodies. The percentage of infected cells was quantified in the GFP-transfected (open bars) and IncA-transfected (black bars) populations. The experiment is representative of three experiments. GFP-transfected cells were infected as efficiently as non-transfected cells. B- HeLa cells were transfected with CcaIncA-His and infected with *C. caviae* the following day. Three hours later, extracellular and intracellular bacteria, as well as transfected cells, were labeled as described in the Methods section. The number of intracellular bacteria was quantified in the transfected and non-transfected populations (n>30 cells). The average of two experiments is represented. C- HeLa cells were infected with *C. caviae* (2 top panels) or with *C. trachomatis* serovar L2 (2 bottom panels) for 24 h before transfection with the indicated constructs. Eight hours later, the cells were fixed and IncA-transfected cells were stained with rabbit anti-His antibody followed by Alexa Fluor-488-coupled anti-rabbit antibodies (left) while bacteria were labeled with mouse anti-*Chlamydia* antibodies and CyTM-3 coupled anti-
mouse antibodies (middle). On the superimposition of the images, IncA and GFP appear in green and the bacteria in red (right).

**Fig. 6. Expression of IncA C-terminal cytoplasmic domain on the ER is sufficient to disrupt the development of the inclusions.** A- Cells transfected with the indicated constructs were infected with *C. caviae* 24 h after transfection, and fixed one day later. Infected and transfected cells were identified using anti-*Chlamydia* and anti-His antibodies, respectively. The percentage of infected cells was quantified in the non-transfected (open bars) and transfected (black bars) populations. The experiment is representative of two. B- A schematic representation of ∆118*Cca*IncA-NS5B in which the yellow box represents the last 26 amino acids of NS5B. Cells transfected with ∆118*Cca*IncA-NS5B were fixed, permeabilized and stained with anti IncA antibodies. Note that in addition to the reticulate distribution characteristic of the ER, ∆118*Cca*IncA-NS5B shows some association with a perinuclear compartment that may be the Golgi apparatus.

**Fig. 7. Disruption of the infectious cycle is mediated by direct interactions between IncA molecules on the inclusion and on the ER membranes.** A- Cells transfected with GFP-*Ctr*IncA were infected with the IncA negative *C. trachomatis* serovar D(s)5058 strain 24 h after transfection, and fixed one day later. Bacteria were labeled using anti-*Chlamydia* (red). Note that this IncA negative strain is non fusogenic and forms multiple inclusions. B- The percentage of infected cells was quantified in the non-transfected (open bars) and transfected (black bars) populations. The experiment is representative of three.
REFERENCES

1. Scidmore, M. A., Fischer, E. R., and Hackstadt, T. (2003) *Infect. Immun.* 71 (2), 973-984
2. Fields, K. A., and Hackstadt, T. (2002) *Ann. Rev. Cell Dev. Biol.* 18, 221-245
3. Meresse, S., Steele-Mortimer, O., Moreno, E., Desjardins, M., Finlay, B., and Gorvel, J. P. (1999) *Nature Cell Biology* 1 (7), E183-E188
4. Hatch, G. M., and McClarty, G. (1998) *Infect. Immun.* 66, 3727-3735
5. Hackstadt, T., Scidmore, M. A., and Rockey, D. D. (1995) *Proc. Natl. Acad. Sci. USA* 92, 4877-4881
6. Scidmore, M. A., Rockey, D. D., Fischer, E. R., Heinzen, R. A., and Hackstadt, T. (1996) *Infect. Immun.* 64, 5366-5372
7. Carabeo, R. A., Mead, D. J., and Hackstadt, T. (2003) *Proc. Natl. Acad. Sci. USA* 100 (11), 6771-6776
8. Scidmore, M. A., and Hackstadt, T. (2001) *Mol. Microbiol.* 39 (6), 1638-1650
9. Grieshaber, S. S., Grieshaber, N. A., and Hackstadt, T. (2003) *J. Cell Sci.* 116 (18), 3793-3802
10. Prozialeck, W. C., Fay, M. J., Lamar, P. C., Pearson, C. A., Sigar, I., and Ramsey, K. H. (2002) *Infect. Immun.* 70 (5), 2605-2613
11. Rzomp, K. A., Scholtes, L. D., Briggs, B. J., Whittaker, G. R., and Scidmore, M. A. (2003) *Infect. Immun.* 71 (10), 5855-5870
12. Rockey, D. D., Scidmore, M. A., Bannantine, J. P., and Brown, W. J. (2002) *Microbes and Infection* 4 (3), 333-340
13. Bannantine, J. P., Griffiths, R. S., Viratyosin, W., Brown, W. J., and Rockey, D. D. (2000) *Cell. Microbiol.* 2, 35-47
14. Toh, H., Miura, K., Shirai, M., and Hattori, M. (2003) *DNA Res.* 10 (1), 9-17
15. Rockey, D. D., Grosenbach, D., Hruby, D. E., Peacock, M. G., Heinzen, R. A., and Hackstadt, T. (1997) *Mol. Microbiol.* 24, 217-228
16. Hackstadt, T., Scidmore-Carlson, M. A., Shaw, E. I., and Fischer, E. R. (1999) *Cell. Microbiol.* 1, 119-130
17. Hueck, C. (1998) *Microbiol. Mol. Biol. Rev.* 62, 379-433
18. Fields, K. A., Mead, D. J., Dooley, C. A., and Hackstadt, T. (2003) *Mol. Microbiol.* 48 (3), 671-683
19. Subtil, A., Parsot, C., and Dautry-Varsat, A. (2001) *Mol. Microbiol.* 39 (3), 792-800
20. Bannantine, J., Stamm, W., Suchland, R., and Rockey, D. (1998) *Infect. Immun.* 66, 6017-6021
21. Rockey, D. D., Heinzen, R. A., and Hackstadt, T. (1995) *Mol. Microbiol.* **15**, 617-626
22. Brown, W. J., Skeiky, Y. A. W., Probst, P., and Rockey, D. D. (2002) *Infect. Immun.* **70** (10), 5860-5864
23. Shaw, E. I., Dooley, C. A., Fischer, E. R., Scidmore, M. A., Fields, K. A., and Hackstadt, T. (2000) *Molecular Microbiology.* [print] **37** (4), 913-925
24. Belland, R. J., Zhong, G. M., Crane, D. D., Hogan, D., Sturdevant, D., Sharma, J., Beatty, W. L., and Caldwell, H. D. (2003) *Proc. Natl. Acad. Sci. USA* **100** (14), 8478-8483
25. Van Ooij, C., Homola, E., Kincaid, E., and Engel, J. (1998) *Infect. Immun.* **66**, 5364-5371
26. Suchland, R. J., Rockey, D. D., Bannantine, J. P., and Stamm, W. E. (2000) *Infect. Immun.* **68**, 360-367
27. Rockey, D. D., Viratyosin, W., Bannantine, J. P., Suchland, R. J., and Stamm, W. E. (2002) *Microbiology-Sgm* **148**, 2497-2505
28. Matsumoto, A., Bessho, H., Uehira, K., and Suda, T. (1991) *J. Electron. Microsc.* **40**, 356-363
29. Fields, K. A., Fischer, E., and Hackstadt, T. (2002) *Infection & Immunity* **70** (7), 3816-3823
30. Boleti, H., Benmerah, A., Ojcius, D., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999) *J. Cell Sci.* **112**, 1487-1496
31. Antonin, W., Fasshauer, D., Becker, S., Jahn, R., and Schneider, T. R. (2002) *Nat. Struct. Biol.* **9** (2), 107-111
32. Nilges, M., and Brunger, A. T. (1991) *Protein Eng.* **4** (6), 649-659
33. Brunger, A. T. (1992) *X-plor*, Yale University Press
34. Lindahl, E., Hess, B., and van der Spoel, D. (2001) *Journal of Molecular Modeling* **7** (8), 306-317
35. Darden, T., York, D., and Pedersen, L. (1993) *J. Chem. Phys.* **98** (12), 10089-10092
36. Berendsen, H. J. C., Postma, J. P. M., Van Gunsteren, W. F., Dinola, A., and Haak, J. R. (1984) *J. Chem. Phys.* **81** (8), 3684-3690
37. Fasshauer, D., Sutton, R. B., Brunger, A. T., and Jahn, R. (1998) *Proc. Natl. Acad. Sci. USA* **95** (26), 15781-15786
38. Junius, F. K., Odonoghue, S. I., Nilges, M., Weiss, A. S., and King, G. F. (1996) *J. Biol. Chem.* **271** (23), 13663-13667
39. David, V., Hochstenbach, F., Rajagopalan, S., and Brenner, M. B. (1993) *J. Biol. Chem.* **268** (13), 9585-9592
40. Subtil, A., Wyplosz, B., Balañá, M. E., and Dautry-Varsat, A. (2004) *J. Cell Sci.* **117**, 3923-3933
41. Schmidt-Mende, J., Bieck, E., Hugle, T., Penin, F., Rice, C. M., Blum, H. E., and Moradpour, D. (2001) *J. Biol. Chem.* **276** (47), 44052-44063
42. Jahn, R., and Grubmuller, H. (2002) *Curr. Opin. Cell Biol.* **14** (4), 488-495
43. Harbury, P. B., Zhang, T., Kim, P. S., and Alber, T. (1993) *Science* **262** (5138), 1401-1407
44. Ungar, D., and Hughson, F. M. (2003) *Ann. Rev. Cell Dev. Biol.* **19**, 493-517
45. Hu, C., Ahmed, M., Melia, T. J., Sollner, T. H., Mayer, T., and Rothman, J. E. (2003) *Science* **300** (5626), 1745-1749
46. Skehel, J. J., and Wiley, D. C. (1998) *Cell* **95** (7), 871-874
47. Humphrey, W., Dalke, A., and Schulten, K. (1996) *J. Mol. Graph.* **14** (1), 33-8, 27-8&
48. Horn, M., Collingro, A., Schmitz-Esser, S., Beier, C. L., Purkhold, U., Fartmann, B., Brandt, P., Nyakatura, G. J., Droegge, M., Frishman, D., Rattei, T., Mewes, H. W., and Wagner, M. (2004) *Science* **304** (5671), 728-730
| Constructions       | 3’primer                                           | 5’primer                                           |
|---------------------|----------------------------------------------------|----------------------------------------------------|
| CcaIncA-His         | agaattgccccgctatccacagacaacacagt                 | aagtcgtaaggatctccacagccactacagattg                |
| ∆53 CcaIncA-His     | atcgtaaccatggagctgaaggaagaagc                    | aagtcgtaaggatctccacagccactacagattg                |
| ∆118 CcaIncA-His    | atcgtaaccatggatgcacaccgtctcaa                    | aagtcgtaaggatctccacagccactacagattg                |
| CcaIncA ∆233-His    | agaattgccccgctatccacagacaacacagt                 | aagtcgtaaccgggtggtgcacactctgaa                    |
| CcaIncA ∆135-His    | agaattgccccgctatccacagacaacacagt                 | aagtcgtaaccgggtggtgcacactctgaa                    |
| CtrIncA-His         | atcgtaaccatggccgcctctctatgtgaa                   | aagtcgtaaccgggtggtgcacactctgaa                    |
| GFP-CcaIncA         | cccggaattgtgacagtatccacagacaacacg              | ctgctctgacattcacttttagtagctgaccc                 |
| GFP-CtrIncA         | agtgaattctgatcagactgtctctactttagc              | agtgaattctgatcagactgtctctactttagc              |
| ∆118CcaIncA-NS5B    | atcgtagctagctgtgagccgacctctctctct               | tctgtgtatctgtgtacctgtctccacagctgatg             |
Fig. 1
**Fig. 4A**

**Fig. 4B**
FIG. 6A

FIG. 6B

Δ118CcaIncA-NS5B

119 352
Conservation of the biochemical properties of IncA from chlamydia trachomatis and C. caviae: oligomerization of IncA mediates interaction between facing membranes

Cédric Delevoye, Michael Nilges, Alice Dautry-Varsat and Agathe Subtil

J. Biol. Chem. published online August 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407227200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/09/09/M407227200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2004/08/16/jbc.M407227200.citation.full.html#ref-list-1