Location of *C. trachomatis* Inc Proteins during Expression of Their Genes in HeLa Cell Culture

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Plasma constructions including genes encoding *C. trachomatis* inclusion membrane protein as composite proteins with reporter green fluorescent protein were obtained. After transfection of HeLa cell culture with the resultant plasmid constructions the location of inclusion membrane proteins in transfected cell was for the first time detected by confocal microscopy.

Key Words: *Chlamydia; inclusion membrane proteins; green fluorescent protein; composite proteins; gene expression in heterologous systems*

C. trachomatis is an obligate intracellular bacterium causing trachoma, inguinal lymphogranulomatosis, and urogenital infections often leading to serious complications in humans [8]. Chlamydia are characterized by a unique biphasic vital cycle; they are situated in the host cell inside a special membrane vacuole (inclusion). It is believed that chlamydia—host cell interactions are realized through their own unique chlamydial proteins situated in the inclusion membrane, called Inc proteins (inclusion proteins) [5]. Inc proteins were found in virtually all Chlamydia species; no homologues were found in other known live organisms. These proteins appreciably differ from each other by the primary amino acid sequence, but all Inc proteins have a characteristic hydrophobic domain consisting of about 50 amino acids, this presumably determining their location in the inclusion membrane [1]. The functions of Inc proteins are unknown, but the fact that expression of genes encoding some Inc proteins starts during the first half-hour after cell culture infection suggests that they can act as mediators in interactions between Chlamydia and eukaryotic cell. Chlamydial genome contains many open reading frames encoding proteins with similar hydrophobic profile [7]. The absence of systems of genetic transformation the Chlamydia impedes studies of the structure and functions of these proteins. There are virtually no data on the relationship between *C. trachomatis* inclusion membrane proteins and proteins and organelles of the infected cell.

We studied the location of *C. trachomatis* Inc proteins under conditions of expression of genes encoding them in eukaryotic cells.

MATERIALS AND METHODS

Genes encoding *C. trachomatis* Inc proteins were cloned in pEGFP-N1 and pEGFP-C1 plasmid vectors (Clontech). *C. trachomatis* DNA was isolated from McCoy cell culture as described previously [2]. PCR amplification of genes was carried out using primers into which sites for restriction endonucleases were added. The reaction was carried out in a programmed Thermal Cycler Abbott LCX Probe System. The amplification protocol included denaturing (92°C, 30 sec), annealing (55°C, 30 sec), and elongation (72°C, 30 sec). The reaction mixture contained 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 1% Triton X-100, 1 mg/ml BSA, 2 mM $MgSO_4$, dNTP (250 μM each), primers (10 pmol each), and 1.25 U Pfu polymerase (Fermentas). *C. trachomatis* DNA (50 ng) served as the matrix.

Amplification products were purified using Wizard PCR Preps DNA Purification System (Promega)

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and cloned into pGEM-Teasy plasmid (Promega) according to manufacturer's instruction. The resultant plasmid constructions were used for transformation of *E. coli* DH5α cells, plasmid DNA was isolated as described previously [4]. Inc protein genes were subcloned into pEGFP-N1 and pEGFP-C1 plasmid vectors by the respective restriction sites according to standard protocols [5] using Hind III, EcoR I, BamH I restriction endonucleases and T4 DNA ligase (Fermentas). Preparative purification of plasmid constructions was carried out using JETSTAR Plasmid Kit (Genomed) according to manufacturer's instruction.

The correspondence of the cloned fragment to the expected one was verified at each stage by determining the nucleotide sequence of this fragment and the adjacent sites of the vector using Big Dye Terminator v.3.0 Cycle Sequencing thermocycling kit (Applied Biosystems). The reaction mixture (20 μl) contained 300 ng plasmid DNA, 3.2 pmol specific primer, and reaction mixture from the manufacturer (Terminator Ready Reaction Mix).

HeLa cells were cultured in DMEM (Sigma) with 10% fetal calf serum (HyClone), and 10 μg/ml gentamicin at 37°C and 5% CO_2. For confocal microscopy the cells were cultured on sterile slides for 24 h until formation of 50-70% monolayer. HeLa cell transfection with plasmid DNA was carried out using Lipofectamine 2000 preparation (Invitrogen) according to manufacturer's instruction. Gene expression was analyzed 24 h after transfection. The cells were washed in phosphate buffer (1.5 M NaCl, 17 mM NaH_2PO_4, 170 mM Na_2HPO_4) and fixed in 2% paraformaldehyde for 30 min. Cell nuclei were stained with DAPI stain (Molecular Probes) at a concentration of 0.1 μg/ml for 10 min. The slides were washed in phosphate buffer and examined under Eclipse E800 fluorescent microscope (Nikon) under a ×60 objective with oil immersion. The location of Inc protein components in the cells was studied using C1 confocal modulus (Nikon) with argon laser at λ=488 nm for green fluorescent protein (GFP). The images were obtained at 1-sec interval and recorded using EZ-C1 2.00 Software (Nikon).

RESULTS

In order to locate *C. trachomatis* Inc proteins during expression of their genes in eukaryotic cells, we used pEGFP-N1 and pEGFP-C1 plasmid vectors. These constructions carry *gfp* reporter gene under the control of human cytomegalovirus (CMV) immediate-early promoter. Capacity to visible fluorescence (λ_{em}=508 nm) in UV light (λ_{stim}=395-498 nm for different modifications), requiring no additional substrates and cofactors, except molecular oxygen, is a favorable advantage of the GFP-based reporter systems over other known systems (based on β-galactosidase, α-amylase, luciferase, chloramphenicol acetyltransferase) [9]. The gene cloned in pEGFP-N1 plasmid polylinker, situated between CMV promoter and *gfp* gene, is expressed as a composite protein at the GFP aminoterminal with retained common reading frame (Fig. 1, *a*). The gene cloned in the pEGFP-C1 plasmid polylinker, situated between *gfp* gene and polyadenylation signal, is expressed as composite protein at the GFP carboxyter-

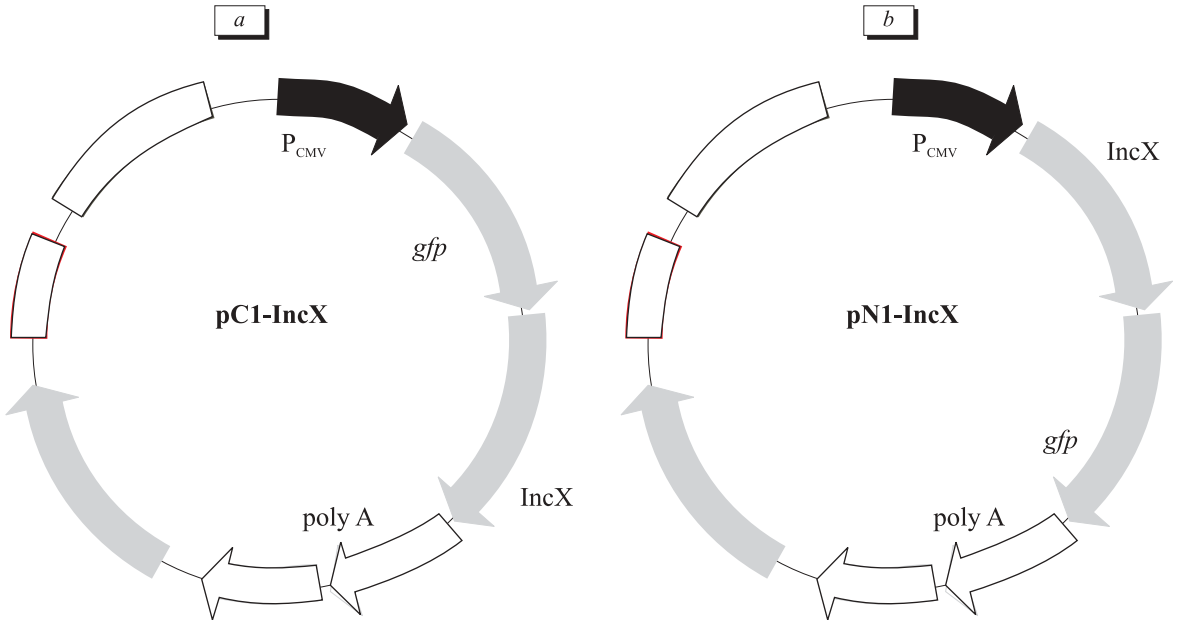
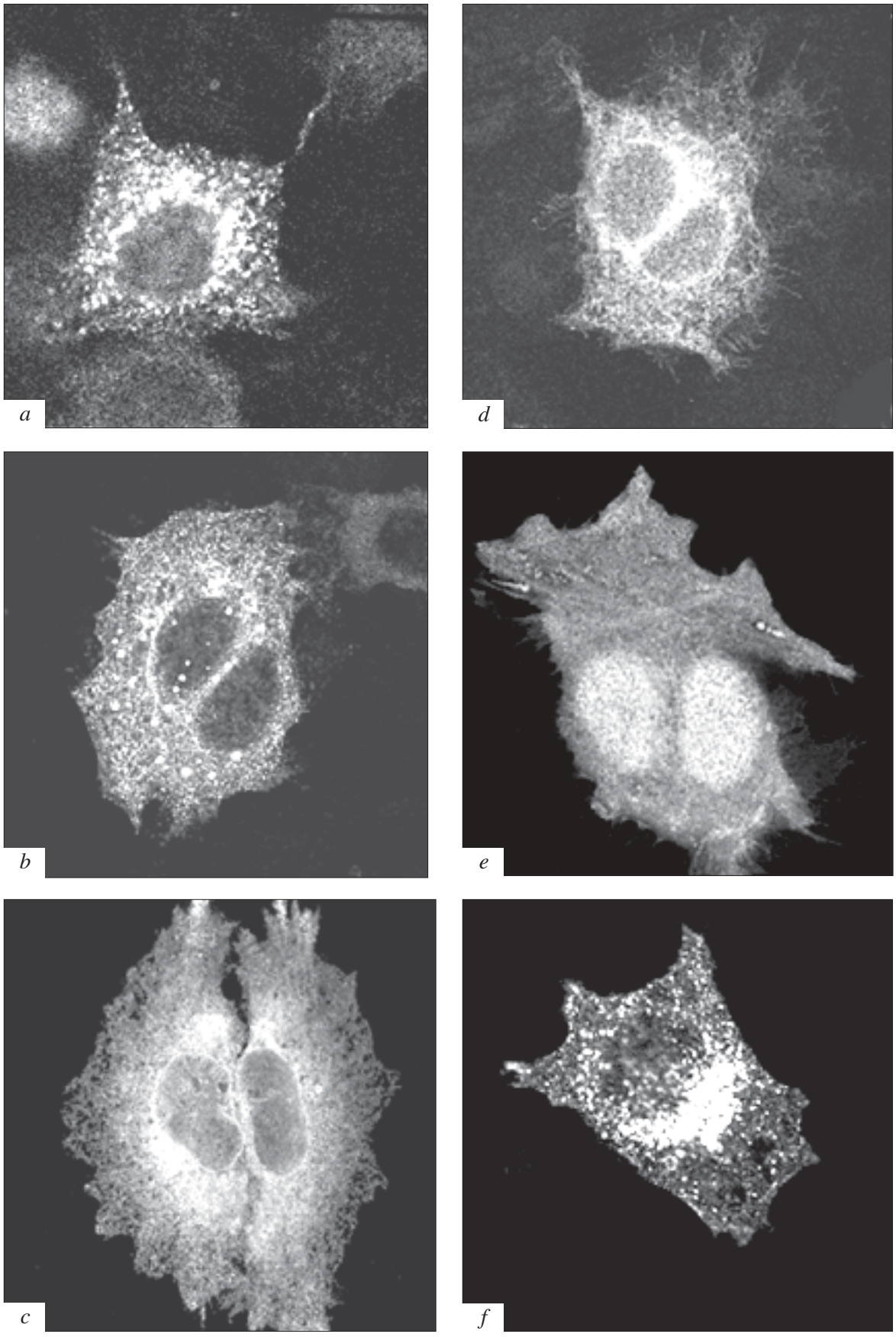


Fig. 1. Scheme of plasmid vectors pN1-IncX (*a*) and pC1-IncX (*b*) expressing the genes of *C. trachomatis* inclusion membrane composite proteins with GFP. P_{CMV}: CMV promoter; *gfp*: reporter gene; poly A: polyadenylation signal.


a
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c
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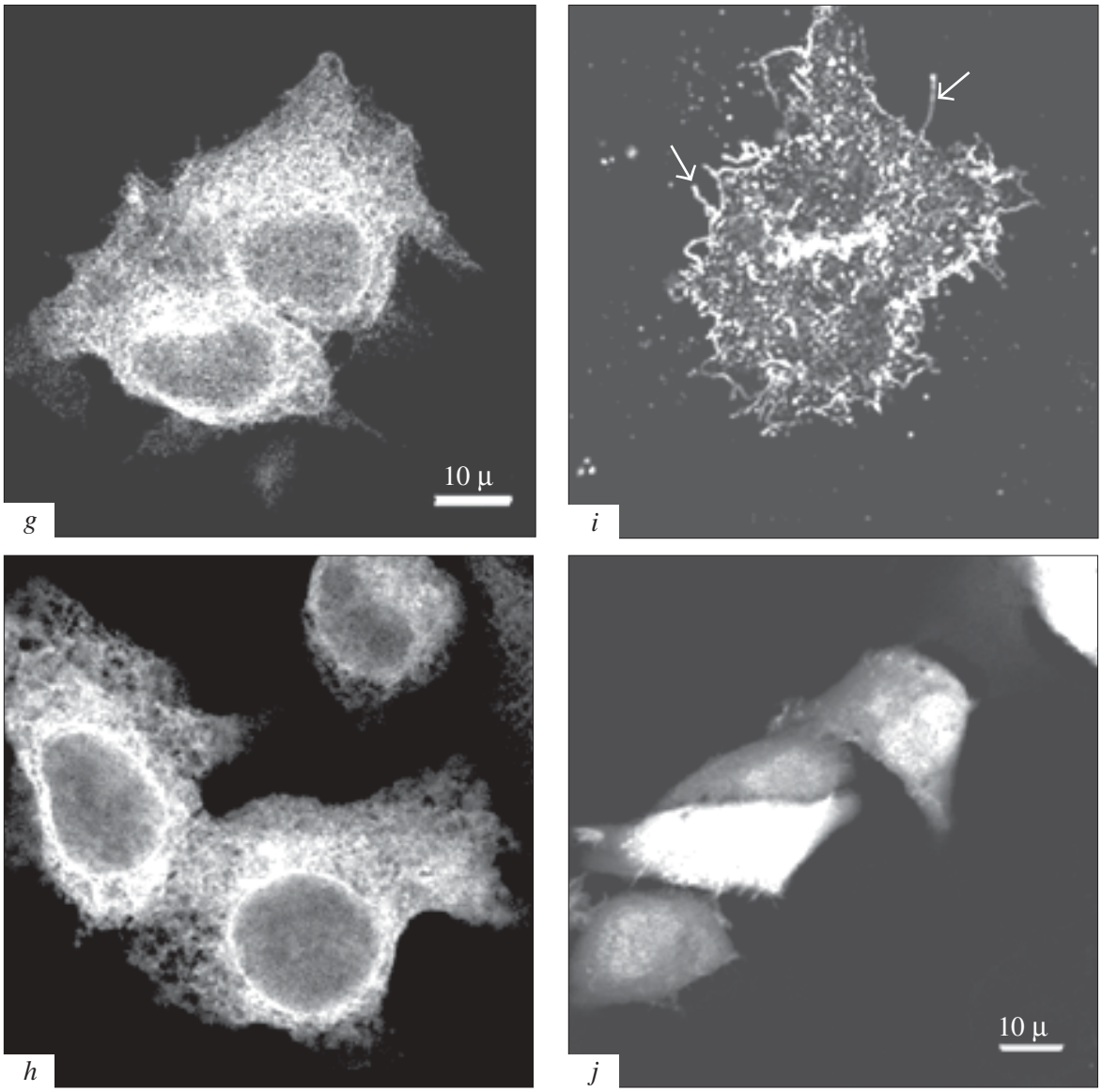

Fig. 2. Fluorescent microscopy of HeLa cells transfected by resultant plasmid constructions 24 h after transfection. *a*) location of IncA-N1 protein; *b*) IncA-C1; *c*) IncB-N1; *d*) IncB-C1; *e*) IncE-N1; *f*) IncE-C1; *g*) IncF-N1; *h*) IncF-C1; *i*) IncC-C1; *j*) pEGFP-N1; ×60.

minal end with retained common reading frame (Fig. 1, *b*). We obtained paired plasmid constructions ensuring the expressions of genes encoding *C. trachomatis* Inc proteins as composite proteins with GFP at the aminoterminal and carboxyterminal ends; the proteins can be located in cells without using specific antibodies.

According to confocal microscopy data, IncA-N1 protein was located in cells as 0.5-3.0-μ granules evenly distributed in the cytoplasm (Fig. 2, *a*). At later terms of expression the number of small granules decreased and the number of large complexes increased. The expression of IncA-C1 construction was also detected as small and large granules situated in the cytoplasm (Fig. 2, *b*). Protein IncA formed dimers, thus realizing the function of fusion of several inclusions with elementary corpuscles into one vacuole [3]. Presumably, IncA does not lose the capacity to oligomerization in eukaryotic cells during expression as a composite protein and forms accumulations of different size.

Expression of IncB-N1 and IncB-C1 proteins was detected in membrane structures resembling vesicles (Fig. 2, *c*, *d*). This network of membrane structures filled the entire cell, except the nucleus. IncB was located in the nuclear membrane in the majority of cases, while the plasma membrane remained free from protein.

A similar morphological picture was observed for IncF-N1 and IncF-C1 constructions. These Inc proteins were located in the nuclear membrane and as a

dense network of membrane vesicles in the entire cell (Fig. 2, *f*, *g*). The relationship of the composite protein to the plasma membrane was in some cases detected for protein IncF-N1. We failed to detect differences between the location of IncB and IncF proteins carrying GFP reporter gene on the carboxyterminal and aminoterminal ends of the molecule.

All Inc proteins had a very long hydrophobic domain, a discriminating sign of these proteins determining their location in the inclusion membrane. In IncB and IncF proteins these domains were situated closer to the carboxyl terminal of the molecule, and presumably the tertiary structure of these proteins connected to GFP leaves the domains functional, permitting insertion in cell membrane structures. Different cellular location of composite proteins was observed for IncE-N1 and IncE-C1 constructions.

IncE-N1 protein was homogeneously distributed in the cell including the nucleus, and was not co-located with certain cell structures (Fig. 2, *e*). This location virtually completely corresponded to the expression of pEGFP-N1 and pEGFP-C1 constructions in HeLa cells (Fig. 2, *i*). On the other hand, IncE-C1 composite protein formed accumulations presenting as granules mainly in the polar perinuclear region (Fig. 2, *f*). This location of protein complexes presumably reflects their interactions with cell organelles in this area, specifically, with Golgi complex, but immunocytochemical co-location of these structures with IncE should be carried out in order to clear out this relationship.

The morphology of cells transfected by IncC-C1 protein changed appreciably (Fig. 2, *h*). The entire bulk of composite protein was concentrated in the plasma membrane; numerous fine processes formed on the cell surface (Fig. 2, *h*, arrows). Some processes were true phylopodias containing actin filaments, others were so thin that they were torn from the cell surface. The same impaired morphology of HeLa cells was observed during expression of native IncC without GFP (data not presented).

Hence, we first studied the location of the main proteins of *C. trachomatis* inclusion membrane during the expression of their genes as composite proteins with GFP in HeLa cells. The model system of Inc protein expression in eukaryotic cells, developed in our study, can be further used for investigating the functions of Inc proteins, which remain unclear, and of the main aspects of Chlamydial interactions with the host cell.

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