BIOPHYSICS AND BIOCHEMISTRY

Hydrophobic Domains Determine Localization of IncC and IncG Full-Length Proteins of *C. trachomatis* during Their Expression in Cultured HeLa Cells

Yu. I. Basovskiy, M. M. Shkarupeta, S. A. Levitskiy, E. S. Kostryukova, V. N. Lazarev, and V. M. Govorun

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 145, No. 4, pp. 402-407, April, 2008
Original article submitted November 13, 2007.

Plasmid vectors encoding hydrophilic (IncB, IncC, IncE, IncG) and hydrophobic (IncC, IncG) domains of *C. trachomatis* incorporation membrane proteins and reporter green fluorescing proteins were constructed. After transfection of HeLa cells with these plasmid constructs, localization of the complex proteins was determined by laser confocal microscopy. Tropism of hydrophobic domains to compartments constituting the exocytotic pathway in the cell was demonstrated. Location of signal/sorting sequences responsible for specific localization was determined.

Key Words: *chlamydia; inclusion membrane proteins; signal sequence; green fluorescing protein; exocytotic pathway*

Chlamydia trachomatis is an obligate intracellular parasite causing serious diseases such as trachoma (damage to eye mucosa leading to blindness), inguinal lymphogranulomatosis, and urogenital chlamydiosis, one of the most prevalent sexually-transmitted infections [5]. Intracellular cycle of Chlamydia takes place in a special membrane vacuole (inclusion) modified by unique proteins (Inc proteins) not homologous to known proteins of other organisms [8]. A specific feature of Inc proteins is the presence of a bilobed hydrophobic domain consisting of 60-80 amino acid residues, which probably determines localization of these proteins in the membrane [2].

Genes encoding the majority of Inc proteins are expressed at the early stages of infection (1-2 h), which confirms their important role in Chlamydia life cycle and interaction with the host cell [3,8].

Functions of the majority of inclusion membrane proteins are unknown. This can be explained by the absence of systems for genetic transformation of Chlamydia and uniqueness of Inc proteins. That is why investigators have to search alternative ways for evaluation of the role of these proteins in Chlamydia life cycle. A possible way to evaluation of the role of some proteins is the study of the expression of the corresponding genes, intracellular trafficking, and localization these proteiuns in cultured mammalian cells [1,7,9].

In eukaryotic cells, most synthesized proteins enter the exocytotic pathway and their further sorting and localization depend on the presence of specific "topogenic signals". Common conditions for sorting of exocytotic pathway proteins are the presence of targeting signals in protein molecules and bivalent receptors interacting with both exo-

Research Institute of Physicochemical Medicine, Federal Agency for Health Care and Social Development, Russia. ***Address for correspondence:*** yur84@mail.ru. Yu. I. Basovskiy.

0007-4888/08/1454-0425

cytosed protein and components of the transporting apparatus responsible for the delivery of the protein to this or that subcellular compartment [6,10].

We previously determined specific location of full-length GFP-fused Inc proteins in cultured HeLa cells. Of particular interest were the data obtained for recombinant full-length IncC and IncG proteins [1]. Specific location of these proteins in the cell can be determined by the presence of signal/sorting sequences.

Here we determined sites of Inc proteins determining localization of IncC and IncG proteins in cultured HeLa cells by the method of confocal laser scanning microscopy (CLSM).

MATERIALS AND METHODS

We used *Escherichia coli* strain *DH5α* (Life Technologies) and HeLa229 cells. Plasmid vectors pEGFP-N1 and pEGFP-C1 (Clontech) and constructs containing full-length genes *IncB*, *IncE*, and *IncG*, (pEGFP-C1/IncB,pEGFP-C1/IncC,pEGFP-C1/IncG) previously obtained in Laboratory for Gene Engineering (Institute of Physicochemical Medicine) [1] were used for cloning.

Cloning of genes encoding hydrophilic and hydrophobic domains of Inc proteins (IncC, IncG) was performed according to the following scheme. PCR amplification of genes was carried out using specific primers containing sites recognized by restriction endonucleases. The reaction was performed in Thermal Cycler Abbott LCX Probe System programmed thermostat, amplification program included denaturation (94°C, 20 sec), annealing (60°C, 20 sec), and elongation (72°C, 20 sec) stages. The reaction mixture contained 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KC1, 1% Triton X-100, 1 mg/ml BSA, 2 mM $MgSO_4$, dNTP (250 μM each), 7 pM each primer, 1.25 U Taq-polymerase. Plasmid DNA (10 ng) containing full-length genes of Inc proteins was used as the template.

Purification of amplification products was performed using Agarose Gel DNA Extraction Kit (Roche Diagnostics). The genes containing hydrophilic and hydrophobic domains of Inc proteins were cloned in plasmid vectors pEGFP-N1 and pEGFP-C1 using restriction endonucleases Hind III, PstI, and T4 DNA-ligase (MBI Fermentas). *E. coli DH5α* cells were transformed with these plasmid constructs, plasmid DNA was isolated as described previously [4]. Preparative purification of plasmid constructs was performed using JETSTAR Plasmid Kit (Genomed) according to manufacturer's instructions.

The correspondence of the cloned fragment to the target one was verified by sequencing of this fragment with flanking vector regions using Big Dye Terminator v.3.0 Cycle Sequencing kit (Applied Biosystem) according to manufacturer's instructions. The reaction products were analyzed using ABI Prism Genetic Analyzer 3100 automated sequenator (Applied Biosystem, Hitachi).

Plasmid vectors encoding hydrophilic domains IncB, IncE, and IncG fused with hydrophobic domain IncC were obtained by subcloning in plasmid vector pEGFP-C1/fobC prepared according to the above method at the sites specific for HindIII and XhoI restriction endonucleases.

HeLa 229 cells were routinely cultured in Eagle's medium (PanEco) supplemented with 10% FCS. Transfection of HeLa cells with plasmid DNA was carried out using Lipofectamine^{tm} (Invitrogen) according to manufacturer's instructions. Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde for 5 min at room temperature. After washout and drying, the cells were analyzed by CLSM using Confocal microscope system ECLIPSE E800 with VEM Epi-Fluorescence and argon laser (488 nm, Nikon corporation). Images of sections (prepared with an interval of 0.25 μ) were acquired using EZ-C1 2.00 Software (Nikon corporation). Z-projection was made with ImageJ software (Media Cybernetics).

RESULTS

At the first stage of the experiment, sequences encoding hydrophilic (IncC 1-291 b.p., IncG 271-504 b.p.) and hydrophobic (IncC 283-537 b.p., IncG 1-276 b.p.) domains of IncC and IncG proteins were

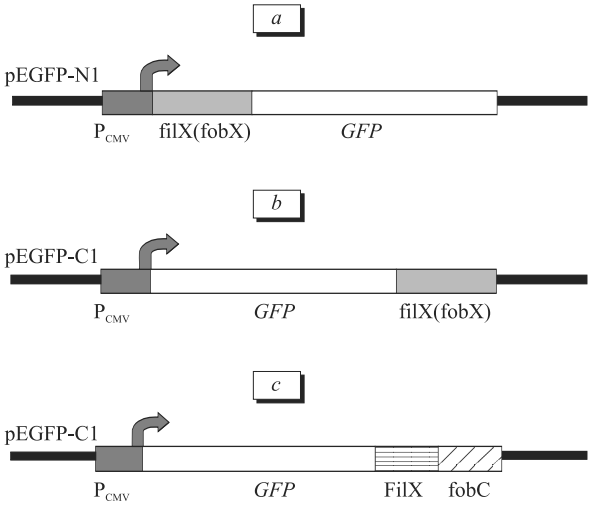

Fig. 1. Scheme of plasmid vectors IncXfil(fob)-N1 (*a*), IncXfil(fob)-C1 (*b*), fil(IncX)-fob(IncC) (*c*) expressing genes encoding hydrophilic and hydrophobic domains of inclusion membrane proteins of *C. trachomatis* with GFP.

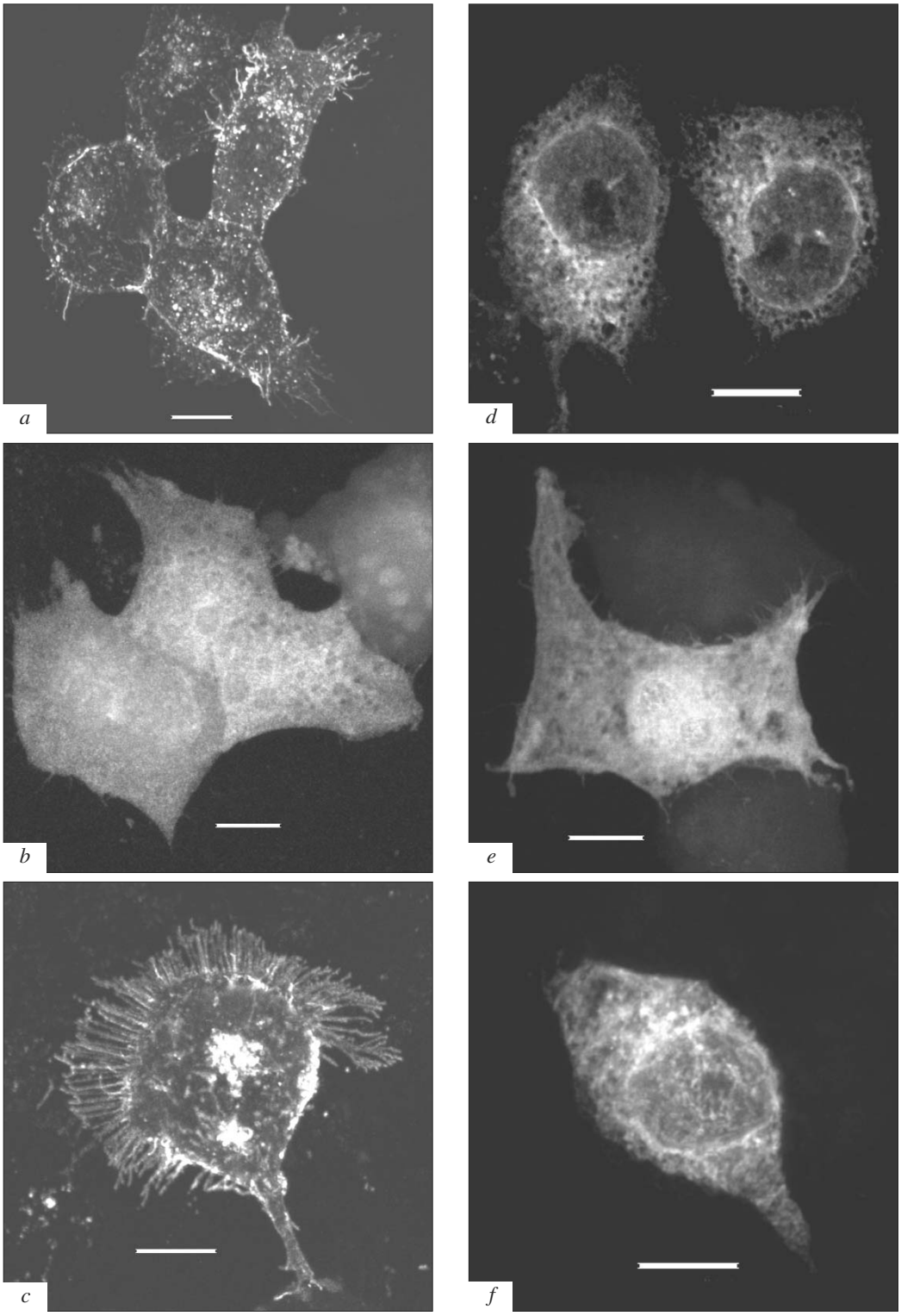

Fig. 2. Confocal microscopy of HeLa cells transfected with obtained plasmid constructs 24 h after transfection (×60): Z-projection of protein localization. *a*) IncC-C1; *b*) IncCfil-C1; *c*) IncCfob-C1; *d*) IncG-C1; *e*) IncGfil-C1; *f*) IncGfob-C1.

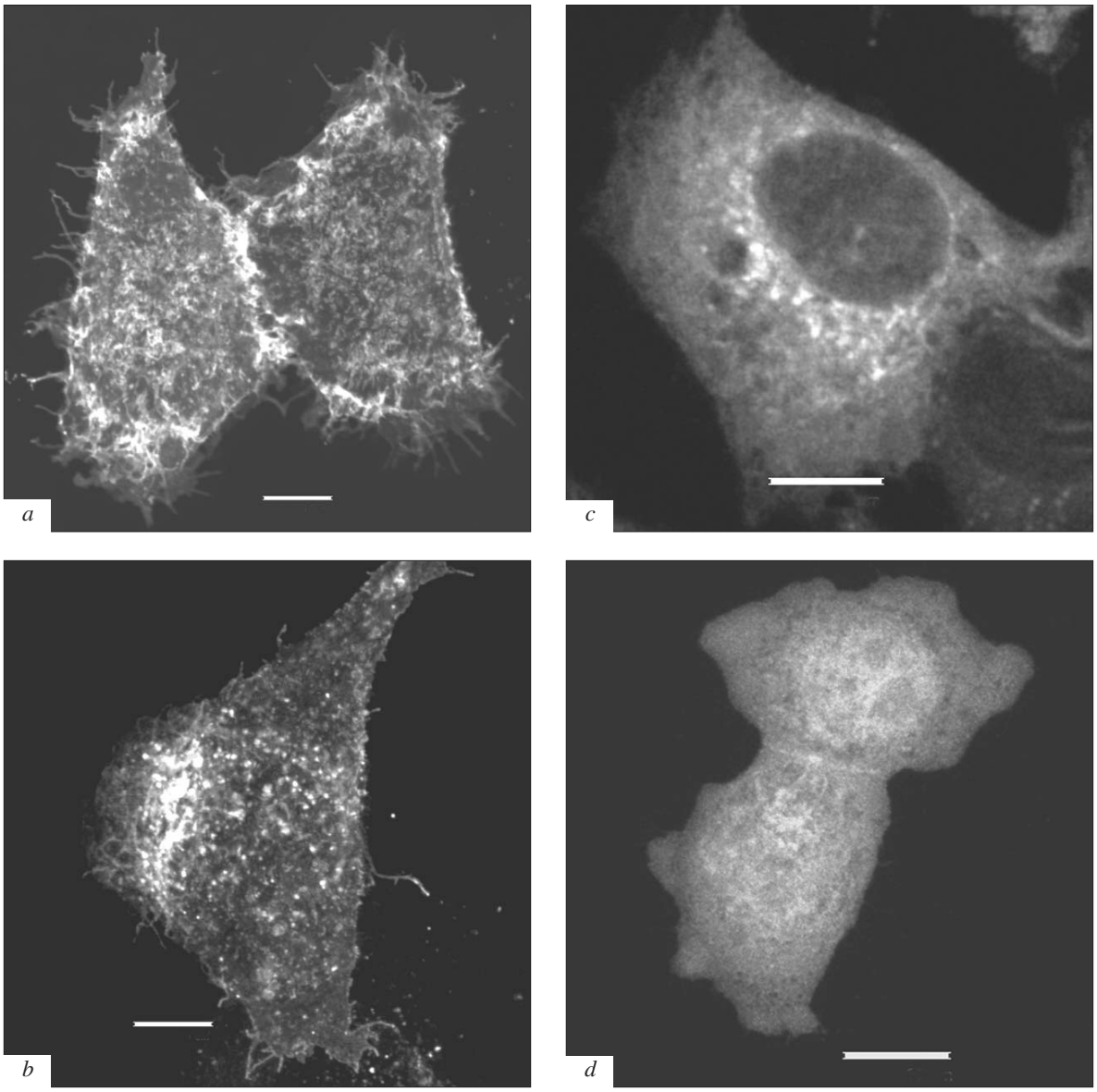

Fig. 3. Confocal microscopy of HeLa cells transfected with obtained plasmid constructs 24 h after transfection (×60): Z-projection of protein localization. *a*) fil(IncB)-fob(IncC); *b*) fil(IncE)-fob(IncC); *c*) fil(IncG)-fob(IncC).

identified in the corresponding genes and cloned in recombinant plasmid vectors pEGFP-N1 and pEGFP-C1. These constructs carry reporter gene *gfp* under immediate-early human cytomegalovirus (CMV) promoter [11]. The genes cloned in plasmid pEGFP-N1 were located between the CMV promoter and *gfp* gene and were expressed in the form of proteins carrying CFP at the carboxyterminal end of the molecule (Fig. 1, *a*). The genes cloned in plasmid pEGFP-C1 were located between *gfp* gene and the polyadenylation signal and were expressed in the form of proteins carrying CFP at the aminoterminal end of the molecule (Fig. 1, *b*). Thus, were obtained paired plasmid constructs allowing expression of genes encoding hydrophilic (fil) and hydrophobic (fob) domains of IncC and IncG of *C. trachomatis* in the form of proteins carrying GFP at aminoterminal (IncXfil(fob)-N1) or carboxyterminal (IncXfil(fob)-C1) end of the molecule.

Transfection of cultured HeLa cells with these plasmid constructs and subsequent CLSM analysis of the location of fused proteins showed that recombinant proteins IncCfil-N1, IncCfil-C1 (Fig. 2, *b*), IncGfil-N1, and IncGfil-C1 (Fig. 2, *e*) did not demonstrate specific tropism to cell compartments,

but were equally distributed in the cytoplasm and nucleus, similarly to native GFP (Fig. 3, *d*). Recombinant IncCfob-C1 was located in vesicles, tubulovesicular formations directed towards the cell periphery, in Golgi apparatus, and near the outer plasma membrane (Fig. 2, *c*). IncGfob-C1 was located near the endoplasmic reticulum (EPR), Golgi apparatus, and trans-Golgi network (Fig. 2, *f*). This morphological picture largely corresponded to the location of full-length IncC (Fig. 2, *a*) and IncG (Fig. 2, *d*) during the expression of the corresponding genes in cultured HeLa cells.

Tropism of full-length IncC, IncG, and their hydrophobic domains to different membrane compartments of the eukaryotic cell excludes nonspecific hydrophobic nature of their interactions.

These results can attest to the presence of signal/sorting sequences encoded in hydrophobic domains of Inc proteins, but the analysis of the primary structure of these proteins *in silico* revealed no signal motifs typical of eukaryotic cell, which attests to unique mechanism of interaction of Chlamydia with the host cell. Moreover, the mechanisms of transport of recombinant hydrophobic domains of these proteins in eukaryotic cells through EPR, Golgi apparatus, and vesicles of the exocytic pathway suggest that IncG and, especially, IncC can interact with proteins of this transport system in the cell.

For verification of this hypothesis we synthesized recombinant constructs encoding chimeric proteins, in which hydrophilic domains of some Inc proteins (IncB 1-88 b.p., IncE 1-106 b.p., IncG 271-504 b.p.) were located in the same reading frame with hydrophobic domain IncC and GFP (located at the carboxyterminal and aminoterminal (fil(IncX-fob(IncC)) ends of chimeric protein, respectively, Fig. 1, *c*). The choice of these hydrophilic domains was determined by the results obtained in the analysis of localization of full-length Inc proteins in cultured HeLa cells [1].

The location of recombinant protein fil(IncB)-fob(IncC) was similar to that of full-length IncC (Fig. 3, *a*). Fil(IncE)-fob(IncC) was located near the Golgi apparatus and trans-Golgi network, but the major part of the protein was located near the outer plasma membrane (Fig. 3, *b*). Chimeric protein fil(IncG)-fob(IncC) was located primarily near EPR and Golgi apparatus, which corresponded to the location of full-length IncG (Fig. 3, *c*).

These data confirm the tropism of hydrophobic domain IncC to membrane compartments (primarily to the outer plasma membrane), the major components of the exocytotic pathway, which also confirms the presence of a signal/sorting sequence recognized by proteins of this transporting system. The absence of the tropism of chimeric protein fil(IncG)-fob(IncC) to the outer plasma membrane and its location in the EPR, Golgi apparatus, and trans-Golgi network probably indicates the presence of signal motifs in hydrophilic domain IncG. This distribution of the chimeric protein in the cell is not surprising in view of the fact that hydrophilic domain IncG is located at the carboxyterminal end of full-length IncG, is phosphorylated by host kinases, and interacts with eukaryotic protein 14-3-3β acting as a regulator of signal pathways and intracellular trafficking [7].

Thus, we determined localization of hydrophilic and hydrophobic domains of some Inc proteins fused with GFP and demonstrated tropism of their hydrophobic domains to compartments constituting the exocytotic pathway in the cell. Location of signal/sorting sequences responsible for specific localization was determined. This study can provide the basis for further study and deciphering of signal motifs located in hydrophobic domains of Inc proteins and for better understanding of their role in Chlamydia life-cycle.

REFERENCES

1. E. S. Kostryukova, A. V. Korobova, V. N. Lazarev, *et al., Byull. Eksp. Biol. Med*., **139**, No. 5, 600-604 (2005).
2. J. P. Bannantine, R. S. Griffiths, W. Viratyosin, *et al., Cell Microbiol.*, **2**, No. 1, 35-47 (1999).
3. R. J. Belland, G. Zhong, D. D. Crane, *et al., Proc. Natl. Acad. Sci. USA*., **100**, No. 14, 8040-8042 (2003).
4. D. Holmes and M. Quigley, *Anal. Biochem*., **114**, No. 1, 193-197 (1981).
5. J. W. Moulder, *Microbiol. Rev*., **55**, 143-190 (1991).
6. S. P. Salcedo and D. W. Holden, *Curr. Opin. Mol. Ther*., **8**, No. 1, 92-98 (2005).
7. M. A. Scidmore and T. Hackstadt, *Mol. Microbiol.*, **39**, No. 6, 1638-1650 (2001).
8. M. A. Scidmore-Carlson, E. I. Shaw, C. A. Dooley, *et al., Mol. Microbiol.*, **33**, No. 4, 753-765 (1999).
9. J. L. Sisko, K. Spaeth, Y. Kumar, and R. H. Valdivia, *Mol. Microbiol.*, **60**, No. 1, 51-66 (2006).
10. L. M. Traub and S. Kornfeld, *Cur. Opin. Cell. Biol.*, **9**, No. 4, 527-533 (1997).
11. F. Yang, L. G. Moss, G. N. Phillips Jr., *Nat. Biotechnol*., **14**, No. 10, 1246-1251 (1996).