GENETICS

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

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Co-localization of *Chlamydia trachomatis* incorporation membrane proteins with cell organelles was studied in HeLa cell culture after transfection by expressing vectors carrying *incA*, *incB*, *incC*, *incD*, *incE*, *incF*, *incG* genes, respectively, fused with the marker green fluorescent protein (EGFP) gene. The prokaryotic proteins were co-located with compartments of the secretory pathway of the eukaryotic cell in the course of biogenesis.

Key Words: *Chlamydia trachomatis; incorporation membrane proteins; IncA; IncB; IncC; IncD; IncE; IncF; IncG*

Chlamydia trachomatis, an agent of many human diseases, is an obligate intracellular bacterium with a vital cycle running inside a special membrane vacuole (chlamydial incorporation) providing isolated existence of the agent in the eukaryotic cell and selective relations with its compartments [10, 15]. Chlamydial incorporation carries no markers of the endocytosis system, escapes lysosomal fusion, but is capable of capture of exocytic vesicles from Golgi complex [3,5]. The Inc family chlamydial incorporation membrane proteins play the key role in the creation of a unique intracellular niche and mediate the interactions with the eukaryotic cell [12]. Precise molecular mechanisms and the role of each of the Inc proteins (except incA) remain unknown up to the present time [11].

Location of the protein after expression of the gene encoding for it in an eukaryotic cell will appreciably reduce the range of probable partners of molecular interactions [2]. The first data on location of *C. trachomatis* Inc proteins after expression of their genes fused with marker green fluorescent protein (EGFP) gene in HeLa cell culture have been reported [1].

Here we studied co-location of *C. trachomatis* IncA, IncB, IncC, IncD, IncE, IncF, and IncG with cell organelles in HeLa cell culture.

MATERIALS AND METHODS

Cloning of genes encoding *C. trachomatis* Inc proteins in pEGFP-N1 and pEGFP-C1 plasmid vectors (Clontech) was carried out as described previously [1]. Cells HeLa 229 were cultured in Eagle's medium (PanEco) with 10% FCS (PanEco). Transfection of HeLa cells by plasmid DNA was carried out using Lipofectamine 2000 (Invitrogen) as described in the instruction. The period of transfected cells' observation was determined by their life span. Viability of transfected cells was evaluated using the LIVE/DEAD^R Viability/Cytotoxicity Kit (Invitrogen Corporation). Mitochondria, endoplasmic reticulum

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(EPR), external membrane (EM), F-actin, α-tubulin, Golgi complex were detected in the transfected monolayer using fluorescent probes and antibodies MitoTracker™ Red CMXRos, ER-Tracker™ Red, WGA-Alexa 594, Alexa 594-phalloidine (Invitrogen Corporation); anti-α-tubulin mouse IgG_1, monoclonal 236-10501; anti-Golgin-97 mouse IgG_1 monoclonal CDF4 antibodies; Alexa Fluor 594 goat anti-mouse conjugate (Invitrogen Corporation). Cell nuclei were stained with DAPI (Invitrogen Corporation) in a concentration of 0.1 μg/ml for 10 min. For immunofluorescent staining the preparations were fixed for 5 min in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 10 min, treated with blocking buffer (2% goat serum in Tris-buffer (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% Twin-20) for 40 min, incubated with primary antibodies and then with the conjugate overnight at 4°C. After washing and drying, the preparations were embedded in moviol and examined under a microscope. Fluorescent and confocal laser scanning microscopy (CLSM) were carried out on an ECLIPSE E800 epifluorescent microscope and Nikon ECLIPSE C1 confocal modulus (Nikon) with Ag (λ=488) and He-Ne (λ=594) lasers. Confocal images were obtained by using 40× 1.25 NA and 63× 1.4 NA objectives with resolution of 1024× 1024 pixels and processed in EZ-C1 2.00 software. The Z-projections were mounted from a series of optic sections by ImageJ software. Co-localization parameters were determined for each optical section by Manders' method [7]. Fluorescence distribution and quantitative evaluation of fluorescent labels co-localization were carried out in 100 cells selected at random for each experiment.

RESULTS

We previously showed that the location of EGFP-Inc-composite proteins during expression of their genes in HeLa cell culture was specific of each protein and in many cases was modified depending on the orientation of EGFP molecule with respect to the amino- or carboxyl terminal of Inc protein molecule (arbitrary names of composite proteins are EGFP-Inc and Inc-EGFP, respectively) [1]. This paper presents the data on the location and co-location of composite Inc proteins, in which EGFP is attached to the NH_2 terminal of Inc-polypeptide chain (arbitrary name of the construction is EGFP-*inc*).

The results of evaluation of EGFP-Inc composite proteins co-location are presented in Table 1. No co-location with mitochondria, actin cytoskeleton, and tubulin microtubules at the level of organelles was detected for any of EGFP-Inc composite proteins.

Twelve hours after transfection EGFP-IncA was detected in cells in the form of vesicles and tubulo-vesicular formations connected to each other by a ramified network. With time, the vesicles grew in size and acquired the shape of granules of different size, oriented along tubulin microtubules and evenly distributed in the entire cell between the actin

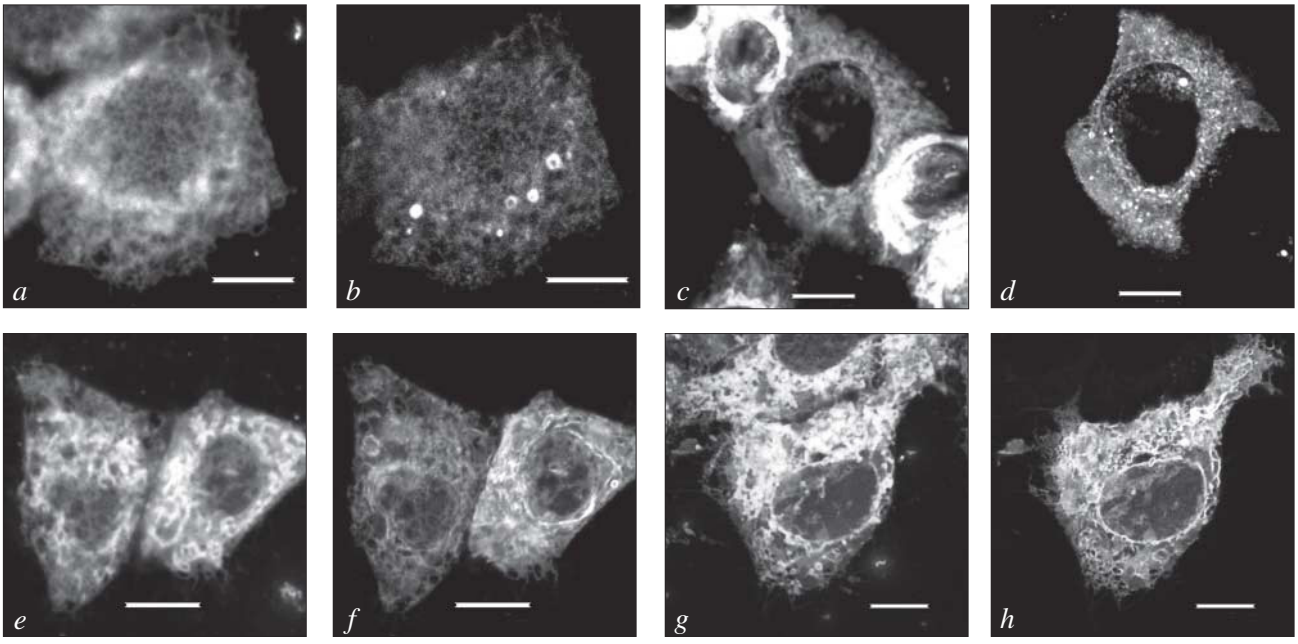

Fig. 1. Localization of EGFP-IncA, EGFP-IncD, EGFP-IncB, and EGFP-IncF. HeLa cell culture, transfected with vector: *a*, *b*: EGFP-*incA*; *c*, *d*: EGFP-*incD*; *e*, *f*: EGFP-*incB*; *g*, *h*: EGFP-*incF*. Cells stained by ER-Tracker™ Red. *a*, *c*, *e*, *g:* red channel; *b*, *d*, *f*, *h*: green channel. Here and in Figs. 2, 3: scale: 10 μ.

cytoskeleton. With accumulation of protein, the granules increased in size, ramified network, identified by the results of co-location as EPR, was more clearly seen (Fig. 1, *a*, *b*). Large EGFP-IncA granules, in which the entire protein was located after all, were not co-located with EPR.

Composite protein EGFP-IncD was detected as a component of ramified network and in granules, which completely corresponded to EPR (Fig. 1, *c*, *d*). The granules were round, larger ones were concentrated in the perinuclear area, mainly in the microtubulin-organizing center.

Composite proteins EGFP-IncB and EGFP-IncF were detected only in the long network consisting of connected tubular elements. According to the co-location data, the areas of protein distribution corresponded to EPR distribution area (Fig. 1, *e-h*). Fluorescent analysis in different periods after transfection showed gradual accumulation of protein in the ramified network consisting from numerous cisterns and tubules crossing each other and forming an intricate structure occupying the entire space of the cell except the nucleus. Accumulation of protein in the perinuclear zone was characteristic of EGFP-IncF during the late period (24 h) after transfection, which was paralleled by degradation of the actin cytoskeleton and cell death.

The picture of EGFP-IncE, EGFP-IncG, and EGFP-IncC distribution depended on the time elapsed after transfection. After 12 h, the area of EGFP-IncE and EGFP-IncG distribution corresponded to EPR (Fig. 2, *a-d*), while 16 h after transfection, co-location with EPR decreased because of compact accumulation of EGFP-IncE and EGFP-IncG in the perinuclear zone. The form of protein accumulation morphologically corresponded to Golgi complex, which was confirmed by the results of co-location with antibodies to Golgin-97 protein (peripheral protein of Golgi complex surface facing the cytoplasm) observed in experiments with EGFP-IncE and EGFP-IncG (Fig. 2, *d-h*). Golgi complex cisterns, system of tubules originating from the cis-

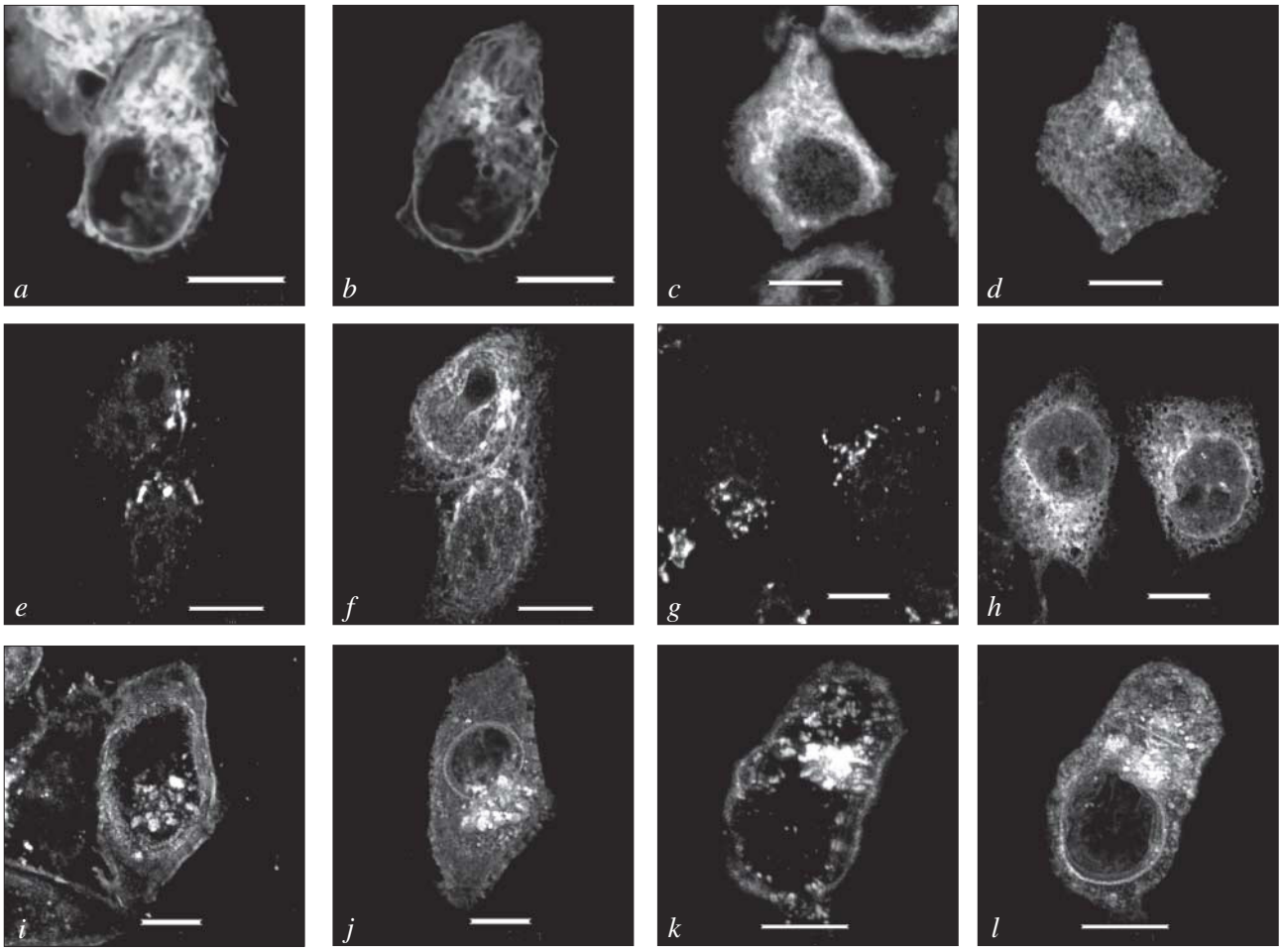

Fig. 2. Localization of EGFP-IncE and EGFP-IncG. HeLa cell culture transfected with the vector: *a*, *b*, *e*, *f*, *i*, *j*: EGFP-*incE*; *c*, *d*, *g*, *h*, *k*, *l*: EGFP-*incG*. Staining: *a-d*: ER-Tracker™ Red; *e-h*: anti-Golgin-97, Alexa 594 anti-mouse conjugate; *i-l*: WGA-Alexa 594. *a*, *e*, *i*, *c*, *g*, *k*: red channel; *b*, *f*, *j*, *d*, *h*, *l*: green channel.

TABLE 1. Parameters of Co-Location of Areas of EGFP-Inc Protein Distribution and Areas of Cell Organelle Distribution in HeLa Cell Culture According to CLSM

Protein	Actin cytoskeleton	Microtubules	Mitochondria	EPR	Golgi complex	External membrane
EGFP-IncA	Rr=0.05±0.01	Rr=0.25±0.02	Rr=0.14±0.01	Rr=0.64±0.06	N.d.	N.d.
	R=0.26±0.04	R=0.28±0.05	R=0.16±0.01	R=0.81±0.03		
EGFP-IncB	Rr=0.33±0.03	Rr=0.30±0.01	Rr=0.37±0.02	Rr=0.85±0.05	N.d.	N.d.
	R=0.37±0.05	R=0.47±0.02	R=0.40±0.01	R=0.89±0.04		
EGFP-IncC	Rr=0.14±0.02	N.d.	N.d.	Rr=0.65±0.05	Rr=0.46±0.05	Rr=0.84±0.02
	R=0.19±0.01			R=0.74±0.05	R=0.69±0.05	R=0.85±0.05
EGFP-IncD	Rr=0.16±0.01	Rr=0.36±0.03	Rr=0.32±0.01	Rr=0.71±0.02	N.d.	N.d.
	R=0.23±0.01	R=0.42±0.01	R=0.41±0.02	R=0.94±0.01		
EGFP-IncE	Rr=0.07±0.01	Rr=0.18±0.01	Rr=0.16±0.01	Rr=0.52±0.04	Rr=0.73±0.06	Rr=0.52±0.01
	R=0.14±0.01	R=0.37±0.05	R=0.35±0.01	R=0.79±0.03	R=0.93±0.01	R=0.64±0.03
EGFP-IncF	Rr=0.09±0.01	Rr=0.41±0.01	Rr=0.29±0.01	Rr=0.83±0.02	N.d.	N.d.
	R=0.24±0.01	R=0.56±0.03	R=0.32±0.01	R=0.96±0.01		
EGFP-IncG	Rr=0.24±0.02	Rr=0.39±0.02	Rr=0.28±0.01	Rr=0.66±0.04	Rr=0.76±0.04	Rr=0.29±0.01
	R=0.39±0.03	R=0.53±0.04	R=0.41±0.02	R=0.79±0.05	R=0.89±0.02	R=0.58±0.01

Note. Rr: Pearson's coefficient of correlation (*M*±*m*); R: Manders' coefficient (*M*±*m*). N. d.: not determined.

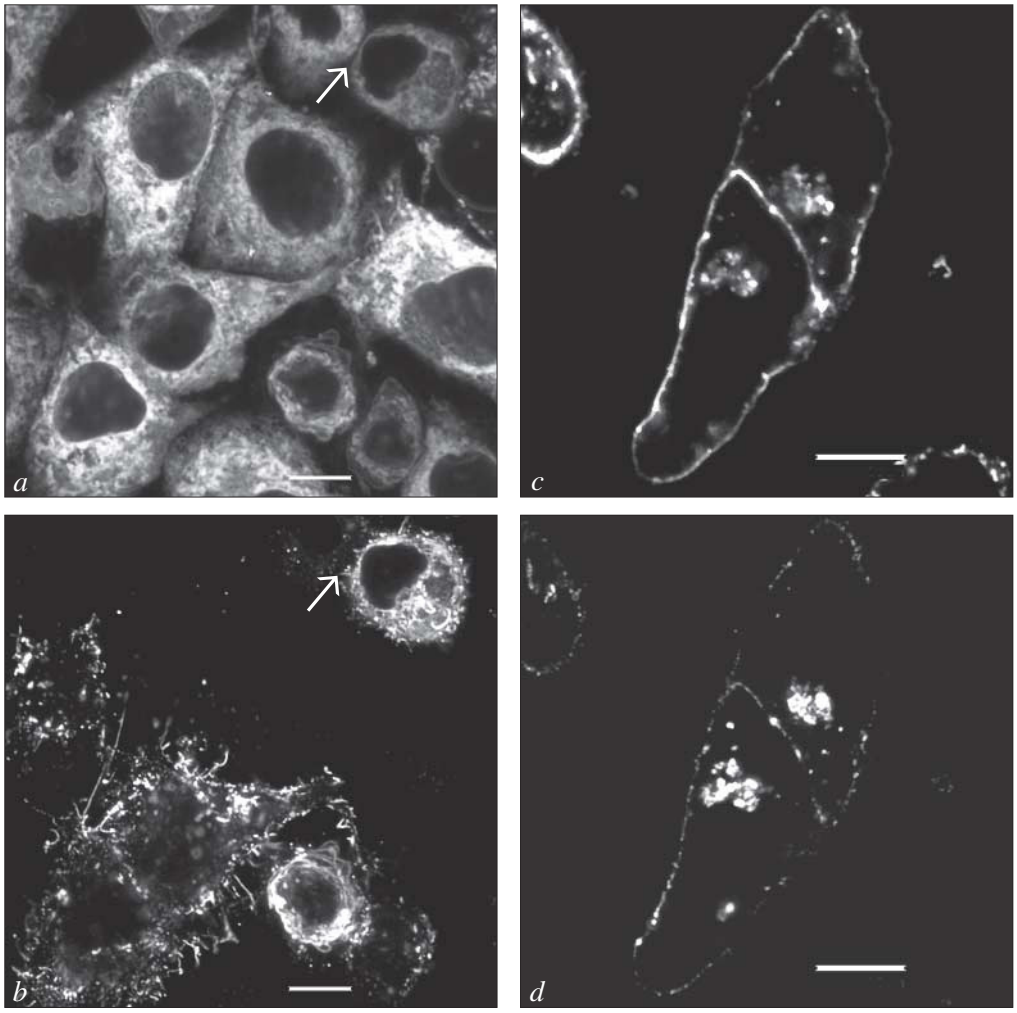

Fig. 3. Localization of EGFP-IncC. HeLa cell culture transfected with EGFP-*incC* vector. Cells stained by: *a*, *b*: ER-Tracker™ Red; *c*, *d*: WGA-Alexa 594. *a*, *c*: red channel; *b*, *d*: green channel. Arrows show co-location areas.

terns and forming an intricate network, and vesicles, mainly small, closing the terminal compartments of the tubules, were very well discernible at late stages of transfection (24-48 h). The area of protein distribution during this period after transfection also corresponded to the area of distribution of Golgi complex marker (WGA-Alexa 594; Fig. 2, *i-l*).

As early as just 12 h after transfection EGFP-IncC remained bound to EPR in just solitary cells (Fig. 3, *a*, *b*). A characteristic picture of this period of observation was protein concentration in vesicles, Golgi complex, and tubulovesicular formation outside EPR and directed to the cell periphery. Sixteen hours posttransfection, EGFP-IncC was detected at the cell periphery, where is was delivered in vesicles. During this period IncC was co-located with the outer membrane (Fig. 3, *c*, *d*). After 19 h, the protein was almost completely distributed on the cell surface. Co-location with the external membrane and protein concentration on cell surface suggested that IncC was tropic to external membrane. After mechanical fractionation with subsequent differential centrifugation of cells transfected with EGFP-*incC* plasmid vector (19 h after transfection), the protein was detected only in the external membrane fraction.

Biogenesis of eukaryotic proteins conforms to the regularities of topogenesis, and protein transport routes are determined by their structure and function: proteins containing signal sequences pass via the signal route, while other proteins pass via the cytoplasmic route. By the present time, the secretory pathway organelles were visualized by CLSM. These organelles include EPR, EPR-Golgi intermediate compartment, Golgi complex, trans-Golgi network, and external membrane [7]. Protein transport between the secretory pathway compartments is realized with participation of transport vesicles and/or vesiculotubular elements and is supported by the accessory protein system, providing the formation of transport vesicles (SNARE family), transportation (motor proteins), and addressed delivery (Rab family GTPases) [6].

All proteins in our study during biogenesis were related to EPR of the eukaryotic cell and were transported via the secretory route. Throughout the entire study IncA, IncD, IncB, and IncF were detected in EPR; IncG and IncE were first detected in EPR, but were very rapidly transported into Golgi complex. At later terms after transfection (24-48 h), the proteins were located in Golgi complex and numerous vesicles related to trans-Golgi reticulum. Protein IncC was transported from EPR through Golgi complex to the external membrane and was delivered to the cell surface in vesicles. The relationship with the secretory pathway elements during biogenesis of chimeric Inc proteins after expression of their genes in the eukaryotic cell was characteristic of constructions in which EGFP did not shield the hydrophobic domain of Inc proteins. This observation suggested that hydrophobic domains could contain signal/sorting sequences recognized by the eukaryotic cell.

Experimental data accumulated by the present time indicate that chlamydial proteins can react with the signal route proteins or perform their function. It was shown that 40-50% synthesized sphingomyelin from exogenous NBD ceramide were transported from Golgi complex into chlamydial incorporation and it was hypothesized that *C. trachomatis* possessed a unique instrument for recognition and capture of exocytic vesicles [5]. Transposition of immature *C. trachomatis* incorporation towards the perinuclear area is realized with participation of dinein (motor protein) and $p150^{glued}$ [4]. Chlamydial incorporation is co-located with the early endosome Rab 4 and Rab 11 GTPases, with Golgi complex Rab 1 and Rab 6 GTPases [14]. One of Inc proteins (CT 229) reacts with Rab 4A acting as its false effector [13]. Chlamydial incorporation of *C. trachomatis* L2 is co-located with EGFP-Rab 6 active GTP-related form and its EGFP-BICD1 effector, delivery of EGFP-BICD1 to incorporation being a Rab 6-independent process [9].

Our findings confirm the capacity of *C. trachomatis* incorporation membrane proteins to react with the secretory pathway proteins. Addressed delivery of IncE and IncG to Golgi complex and trans-Golgi network vesicles when these genes are expressed in the eukaryotic cell can indicate that IncG and IncE react with Rab 1, Rab 6, and their effectors during the infection cycle. Detection of IncC in vesicles circulating from Golgi complex to the external membrane and of IncG and IncE in vesicles of the trans-Golgi network, as well as co-location of these proteins with WGA hemagglutinin bound to glycosylated proteins and lipids in Golgi complex suggest that IncC, IncG, and IncE are components of a unique molecular mechanism for recognition, sorting, capture, and delivery of exocytic vesicles with glycoproteins and glycolipids, transported from Golgi complex to the external membrane.

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