Endocytic Trafficking of Cholera Toxin in Balb 3T3 Cells

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Endocytosis of the cholera toxin B subunit (CTxB) in various cell lines has shown diversity of entry paths of cell surface bound ligands. The aim of this study was to clarify internalisation mechanism and endosomal trafficking of CTxB in murine fibroblasts (Balb 3T3 cells) and human epithelial cells (HeLa). We have shown that CTxB enters Balb 3T3 cells mainly via cholesterol-dependent endocytic pathway(s), while in HeLa cells clathrin endocytosis plays a major role. Early endosomal acidic gradient is required for CTxB progression towards the Golgi in HeLa cells, whereas a majority of internalised CTxB bypasses early endosomes (EEA1+ compartments) in Balb 3T3 cells. The acidic gradient is also required for exit from the Golgi into the late endosomes and degradation of CTxB in Balb 3T3 cells. Thus, the CTxB trafficking may help in the understanding of non-clathrin endocytic mechanisms and the complexity of late endosomal compartments.

INTRODUCTION

Membrane components are internalised into the cell by the process of endocytosis into endocytic vesicles whose structure is determined by proteins required for their formation (for review see Ref.1). Clathrin and caveolin are major proteins that are involved in their organization and, accordingly, the endocytosis can be divided on clathrin- or caveolae-mediated endocytosis. However, endocytic vesicles can also arise without assistance of these proteins (non-clathrin and non-cavolae mediated endocytosis). The endocytic entry from the plasma membrane and intracellular trafficking depends on the membrane microdomain organization and also on membrane dynamics that is dependent on the cell type.[2] Thus, the same membrane component may use different routes for entry into the cell. Although, endocytic entry via clathrin-coated vesicles is relatively well characterized on several natural receptor-ligand systems, other clathrin-independent pathways are still poorly characterized, primarily due to the lack of appropriate receptor-ligand models. Bacterial protein toxins, thus, may serve as a useful tool for studying endocytic transport pathways in cells.

Vibrio cholerae enterotoxin, cholera toxin (CTx), belongs to a family of two component toxins with characteristic structure and function (AB_5 toxins). It contains five identical binding subunits (CTxB) that carry the toxin into the cell and heterodimeric A-subunit that activates adenylyl cyclase.[3–5] The B-subunits of the toxin contain five binding sites and can specifically bind up to five cell-surface glycosphingolipid GM1,[6] which serve as a receptor for CTX entry into the cell. Visualized by CTxB, GM1 has been reported to be uniformly distributed in the plasma membrane.[7] It was found to be concentrated in caveolae,[8–10] flask-shaped invaginations at the plasma membrane that are enriched in cholesterol, sphingolipids and protein caveolin, but also it can be visualized in clath-

rin-coated pits,[8,9] although to a much lesser extent than in caveolae. A majority of intracellular GM1 could be found in late endosomal vesiculo-tubular compartments.[8]

Internalisation pathway of CTX has been investigated in various cell lines and has been shown to vary dependent on the cell type examined. CTX binds to GM1 and then follows a complex endocytic route involving retrograde membrane traffic trough the Golgi complex towards the endoplasmic reticulum (ER),[11,12] where A-subunit is activated. In the absence of the A subunit, the B subunit (CTxB) traffics through the endocytic pathway towards Golgi and is rerouted from the Golgi into late endosomes for degradation.[5] Thus, the CTxB can be used as a useful tool for characterization of the endocytic trafficking.

Binding to GM1 associates CTxB with detergent-resistant membrane domains, presumably lipid rafts. Exactly how the CTxB-GM1 complex traffics from the plasma membrane to the Golgi and then to the ER remains poorly understood. Due to its association with caveolae, it is assumed that CTxB internalise via caveolar endocytosis and it is generally used as a marker for caveolar endocytosis.[4,12–14] The fact that little CTxB is detected in clathrin-coated pits[9] does not exclude the possibility that the toxin is also rapidly endocytosed via these structures, which is more difficult to visualize since they have half-life of only 1 minute at the cell surface. In fact, recent studies suggest that CTxB may enter cells via the clathrin-dependent pathway, but inhibition of this pathway did not prevent its uptake.[4,15–17] In addition, CTxB was internalised in cells devoid of caveolin,[18] suggesting that clathrin- and caveolae-independent mechanisms can be involved in toxin internalisation.[19–21] Once internalised, CTxB can be found in different intracellular compartments: early endosomes, caveosomes, recycling endosomes, perinuclear recycling compartments but also in caveolin-negative pH neutral compartments similar to caveosomes.[15]

In this study, we have compared internalisation and intracellular trafficking of CTxB in murine fibroblastic cell line Balb 3T3 and in well-characterized human epithelial cell line HeLa, known that internalise CTxB via a clathrin-dependent mechanism.[22] The experiments rule out that CTxB enters Balb 3T3 cells mainly via cholesterol dependent endocytic pathway, while in HeLa cells both clathrin and cholesterol dependent pathways play a role.

EXPERIMENTAL

Cells, Antibodies and Reagents

Balb 3T3 cells (murine fibroblastic cell line) and HeLa cells (human epithelial cell line) were grown in Petri dishes as adherent cell lines in Dulbeco's modified minimal essential medium (DMEM) supplemented with 10 % (vol. ratio) foetal bovine serum (FBS), 2 mmol dm^{-3} L-glutamine, 100 mg

ml^{-1} streptomycin and 100 U ml^{-1} penicillin (1 U ml^{-1} ≈ 1 mg ml^{-1}). All reagents were obtained from Gibco, Grand Island, NY.

Transferrin-FITC, anti-caveolin-1-Cy3, chlorpromazine, filipin, methyl-β-cyclodextrin, monensin, leupeptin, LY294002, anti-CTxB and cholera toxin B subunit conjugated with FITC or with biotin (CTxB-FITC, CTxB-biotin) were provided by Sigma-Aldrich Chemie Gmbh. NH$_4$Cl was from Kemika Zagreb. LysoSensor Green DND-189, LysoSensor Green DND-153, CTxB-Alexa Fluor 555 and 488 (CTxB-AF 555 and CTxB-AF 488), anti-mouse IgG$_{2a}$-AF 488 and 555, anti-rat IgG-AF 555 and 488, and anti-mouse IgG$_1$-AF 555 and 488, and monoclonal antibodies (mAbs) against human transferrin receptor were obtained from Molecular Probes. mAbs to Lamp-1, GM130, p115, Vti1a, GS28 and EEA1 were from Becton Dickinson & Co., San Jose, CA. mAbs against murine transferrin receptor (TfR) were produced by R17 hybridoma cells (obtained from American Type Culture Collection).

Protein A-sepharose was from Amersham Pharmacia Biotech.; polyvinylidene difluorid (PVDF) Western blotting membrane and streptavidin-POD were from Roche Diagnostics GmbH, Mannheim, Germany; Super Signal West Dura Extended Duration Substrate was from Pierce Chemical Co., Rockford, IL, USA and BioMax film was from Kodak, Rochester, NY.

Internalisation of CTxB

Cells were incubated with fluorescent CTxB-FITC for 30 min at 4 °C, washed out with cold PBS and incubated at 37 °C. At different periods (indicated in figures), cells were collected and divided. Half was exposed to low-pH treatment (30 s; pH = 2) and the other half was untreated (control of total fluorescence intensity), and intracellular fluorescence was analysed by flow cytometry. The result is presented as mean fluorescence intensity (MFI) ratio of acid-treated and acid-untreated cells.

Endocytosis of CTxB

Cells were incubated with fluorescent CTxB-AF 555 for 30 min at 4 °C, washed with cold PBS to remove unbound marker and incubated at 37 °C for different periods (indicated in figures). After incubation, the cells were fixed with 4 % paraformaldehyde, permeabilised with 0.5 % Triton X-100 and labelled with markers of subcellular compartments using primary antibodies to Lamp1, GM130, TfR, or EEA1, and isotype-specific secondary antibody conjugated with AF 488.

Drug Treatments

Cells were pre-incubated for 30 min at 37 °C in DMEM containing 1.2 µg ml^{-1} filipin, 8 µmol dm^{-3} chlorpromazine, 10 mmol dm^{-3} methyl-β-cyclodextrin, 15 mmol dm^{-3} NH$_4$Cl, 9 µmol dm^{-3} monensin, 200 nmol dm^{-3} bafilomycin A1, 25 µmol dm^{-3} LY294002 or 1 µg ml^{-1} leupeptin. All subsequent incubations contained inhibitors in the same concentrations. Cells were cooled to 4 °C and incubated in the same

medium containing 1 μg ml⁻¹ CTxB-AF 555 or CTxB-FITC for 30 min. Labelled cells were than washed and incubated at 37 °C for indicated periods, and analysed by flow cytometry or confocal microscopy. Drug treatment did not affect cell viability.

CTxB Localization in Detergent-resistant Membrane Domains

Cells were labelled with CTxB-AF 555 and mAb against TfR for 30 min at 4 °C, washed with cold PBS and then treated with 0.5 % Triton X-100 for 1 min to elute detergent-sensitive membrane domains. After three washes with PBS, cells were fixed with 4 % paraformaldehyde and analysed by confocal microscopy.

Confocal Microscopy and Analysis of Colocalization

Images were obtained using Olympus Fluoview FV300 confocal microscope (Olympus Optical Company, Tokyo, Japan) with 60 PlanApo objectives. Presentation of figures was accomplished in Adobe Photoshop (San Jose, CA). To visualize the level of co-localization, 8–10 cells per experimental condition were randomly selected on the same cover slip among those that were well spread and showed a well-resolved pattern. Images of single cells were acquired at the same magnification, exported in a TIFF format, and processed by Fluoview, Version 4.3 FV 300 (Olympus Optical Co.).

Immunoprecipitation

One confluent 10-cm Petri dish of cells was labelled with 1 μg ml⁻¹ CTxB-biotin for 30 min at 4 °C. Unbound CTxB-biotin was washed out with cold PBS, and cells were lysed in a lysis buffer containing 50 mmol dm⁻³ Tris-Cl (pH = 8.0), 150 mmol dm⁻³ NaCl, 1 mmol dm⁻³ EDTA, 0.5 % NP40, 0.02 % NaN₃ and 2 mmol dm⁻³ PMSF immediately or incubated for additional 1 or 2 hours at 37 °C. Supernatants of cellular lysates were immunoprecipitated with anti-CTxB antibody. Immune complexes were retrieved with protein A-sepharose, washed, heated to 95 °C for 10 min and run in a 13 % SDS-PAGE gel. The gels were blotted onto the polyvinylidene difluorid (PVDF) Western blotting membrane, incubated for 1 h with 100 mU ml⁻¹ of streptavidin-POD washed and incubated with Super Signal West Dura Extended Duration Substrate for 1 min and exposed to BioMax film (Kodak, Rochester, NY). Relative intensities of the bands were measured by scanning densitometry of each of the individual bands. Relative ratio was calculated on the basis of band density.

RESULTS

Plasma Membranes of Balb 3T3 Cells Have Higher CTxB Binding Capacity than Plasma Membranes of Hela Cells

Cholera toxin B subunit (CTxB) binds to the cell surface glycosphingolipid GM1 that serve as a receptor molecule for its entry into the cell.[6] Internalisation pathway and intracellular route of CTxB may depend on the initial amount of CTxB bound to the cell surface receptors. Thus, in order to compare CTxB binding capacity of plasma membranes of Balb 3T3 and HeLa cells we determined the level of GM1 cell surface expression on these two cell lines. Cells were incubated for 30 min with CTxB-FITC at 4 °C and cell surface bound CTxB determined by flow cytometry. Fluorescence signal on Balb 3T3 cell was much stronger (15 to 20 times) than fluorescent signal on HeLa cells, indicating that GM1 receptors were abundant in the plasma membrane of Balb 3T3 cells. Flow cytometric profiles shown in Figure 1a also indicate that, in both cell lines, two populations of cells regarding to the level of GM1 expression could be distinguished: approximately 25 % of Balb 3T3 cells had lower expression and 10–15 % of HeLa cells had higher expression than majority of cell population.

In order to determine the cell surface binding capacity of plasma membranes we labelled cells with CTxB-
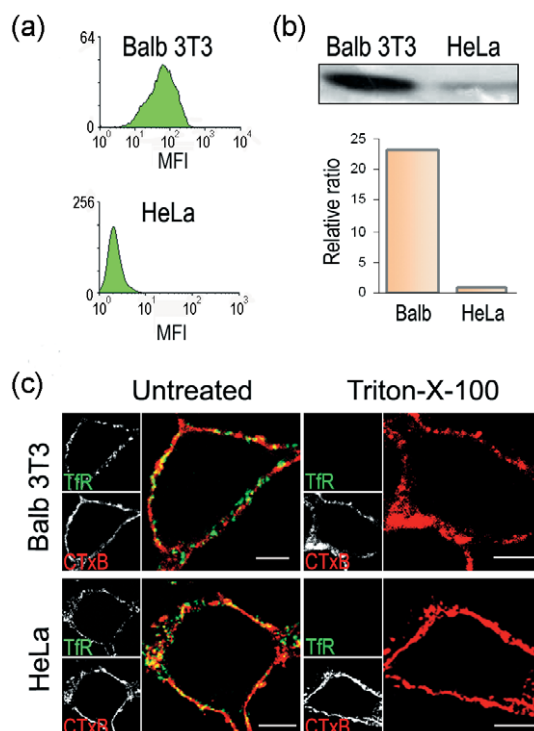

Figure 1. Expression of GM1 at the cell surface. (a) Flow cytometric profile of GM1 expression at the cell surface of Balb 3T3 and HeLa cells. Cells were incubated with CTxB-FITC for 30 min and analysed by flow cytometry. (b) Cell surface binding capacity for cholera toxin B subunit (CTxB). Cells were labelled with CTxB-biotin for 30 min and NP40 cell extracts immunoprecipitated with anti-CTxB (11 kDa band of CTxB). Relative ratio was calculated on the basis of band density. (c) Localization of GM1 in detergent resistant membrane microdomains. Cells were labelled with CTxB-AF 555 (red fluorescence) and mAb against TfR (green fluorescence), and then treated with 0.5 % Triton X-100 for 1 min. After fixation the cells were analysed by confocal microscopy on 0.5 μm sections. Scaling bar represents 5 μm. Representative of three independent experiments with comparable results are shown.

biotin and immunoprecipitated cell surface bound CTxB from cytoplasmic extracts of the same number of cells with anti-CTxB antibody (Figure 1b). Intensity of bands from immunoprecipitates of Balb 3T3 cells was much stronger than bands of HeLa cells. Densitometric analysis of bands revealed that 23 times more of biotinylated material was loaded on gels with immunoprecipitates from Balb 3T3 cells. These data indicate that Balb 3T3 cells had more GM1 receptors at the cell surface and much higher CTxB binding capacity than HeLa cells.

It is well know that GM1 resides in the detergent insoluble membrane microdomains,[4,5] but also that microdomain composition may vary between different cell types.[23] To confirm that GM1 in Balb 3T3 cells is located in the detergent insoluble domains of the plasma membrane, we labelled cells with CTxB-AF 555 and mAb against TfR and after labelling extracted them with Triton X-100 for 1 min at 4 °C. It had been shown that this treatment washes out less organized membrane lipid bilayers, including also proteins that reside in these areas.[24] Since it is well known that TfR belongs to this group[16] we used it as a control. The distribution of TfR and CTxB at plasma membranes was analysed by confocal microscopy (Figure 1c). Both TfR and CTxB were found at distinct regions of plasma membranes of untreated cells and they did not significantly colocalize, indicating that they are located in different areas of the plasma membrane. After extraction with Triton X-100 the bound TfR was washed out, whereas amount of the bound CTxB-AF 555 remained unchanged, confirming our previous observation that TfR and GM1 reside in different membrane microdomains. These data indicate that in both cell lines CTxB binds to GM1 receptors that are located in detergent resistant membrane microdomains.

CTxB Uses Different Internalisation Route in Balb 3T3 and HeLa Cells

To determine the internalisation capacity of Balb 3T3 cells we bound CTxB-FITC to the cell surface at 4 °C and allowed internalisation at 37 °C for different periods. Non-internalised material from half of cells was removed from the cell surface by short acidification (30 s, pH = 2) and cellular fluorescence was determined by flow cytometry. Internalisation was assessed by division of the fluorescent signal of acid-untreated (total fluorescence bound) and acid-treated cells (internalised fluorescence) and result expressed as in percents of internalised material (Figure 2a). We have found that 70 % of cell surface bound CTxB rapidly internalised within 30 min into HeLa cells, whereas internalisation into Balb 3T3 cells showed different kinetics: after 30 min only 17 % of initially bound CTxB was internalised, after 60 min 33 % and after 90 min 43 %. Thus, it appears that approximately one third of cell surface bound CTxB was internalised in an hour by a slow and constant internalisation process. This difference in internalisation kinetics indicates that in Balb 3T3 cells CTxB uses different internalisation mechanism than in HeLa cells.

It is well known that after internalisation CTxB transported into the Golgi compartments.[5,15,25] To confirm this on Balb 3T3 cells, we performed colocalization analysis
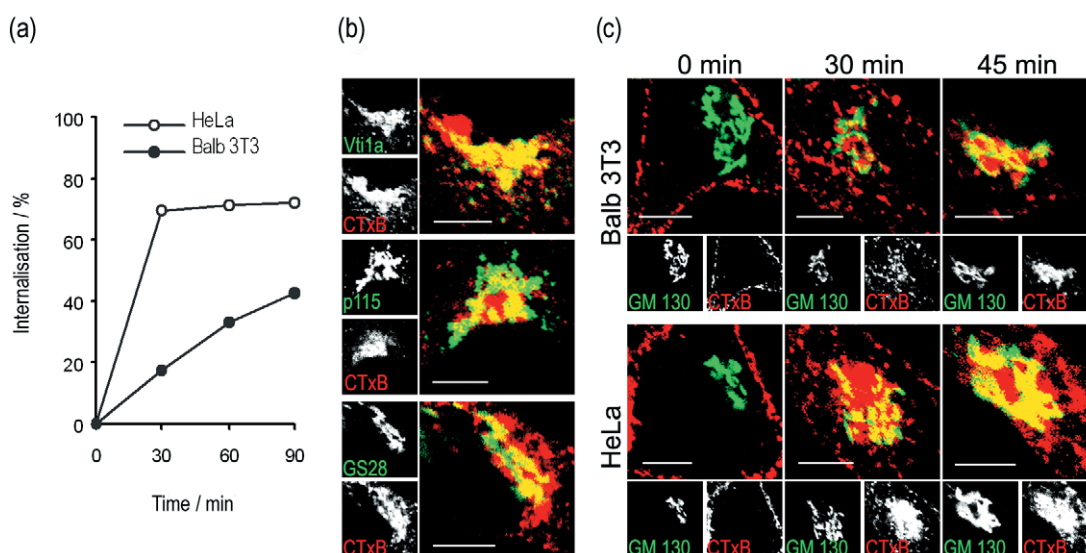

Figure 2. Kinetics of CTxB internalisation. (a) Internalisation of the cell surface bound CTxB. Balb 3T3 (closed circles) and HeLa cells (open circles) were incubated with CTxB-FITC, cell surface bound CTxB-FITC removed by short low-pH treatment (30 s; pH = 2) and intracellular fluorescence analysed by flow cytometry. The result is presented as MFI ratio of acid-treated and acid-untreated cells. (b) Colocalization of CTxB with Golgi markers (green fluorescence) in Balb 3T3 cells after 45 min of internalisation of CTxB-AF 555 (red fluorescence). (c) Kinetic of CTxB internalisation into the Golgi. CTxB-AF 555 (red fluorescence), bound to the surface at 4 °C for 30 min, was internalised at 37 °C and Golgi was labelled with mAb GM130 and anti-mouse IgG1-AF 488 (green fluorescence). Scaling bar represents 5 μm. Representative of three independent experiments with comparable results are shown.

of the internalised CTxB with different markers of Golgi compartments, such as, p115 (*cis*-Golgi and ER-Golgi trafficking including ERGIC, GS28 (*cis*-Golgi), and Vti1a (*cis*- to *trans*-Golgi). After 45 min of internalisation CTxB colocalised with all described markers, indicating that the Golgi complex is in the route of its intracellular trafficking also in Balb 3T3 cells (Figure 2b), although it uses slower internalisation route than in HeLa cells. Detailed dissection of time required for reaching the Golgi in Balb 3T3 and HeLa cells showed that CTxB needed 30 min to reach the Golgi in the majority of HeLa cells, whereas only in a small proportion of Balb 3T3 cells colocalization with the Golgi marker was found in that time. In the majority of Balb 3T3 cells, the Golgi complex was reached after 45 min (Figure 2c). These data indicate that, in addition to the different internalisation mechanism used in two cell lines, CTxB also uses different endosomal route and travels longer towards the Golgi in Balb 3T3 cells.

CTxB Enters Balb 3T3 Cells Mainly via Cholesterol Dependent Endocytic Pathway

To dissect the mechanism of CTxB internalisation we treated Balb 3T3 and HeLa cells with a set of chemicals that disrupt endocytic machinery and inhibit clathrin-dependent and cholesterol-dependent endocytosis. The sterol-binding agent filipin disrupts caveolae structure and function,[26,27] methyl-β-cyclodextrin (MβCD) disturbs structure of lipid rafts[28] and has similar effect as filipin, whereas chlorpromazine (Cp) blocks clathrin-dependent endocytosis.[29] Cells were treated with these inhibitors and internalisation of CTxB determined by flow cytometry (internalisation rate), or by confocal microscopy by its colocalization with the Golgi marker.

Filipin and MβCD treatment inhibited accumulation of CTxB-FITC inside Balb 3T3 cells, although some more CTxB entered into MβCD treated cells. However, treatment of HeLa cell by filipin had no effect on CTxB internalisation and little inhibitory effect of MβCD could be seen (Figure 3a). When filipin and MβCD were used in combination, no internalised CTxB could be detected inside Balb 3T3 cells (data not shown). On the contrary, treatment of Balb 3T3 cells with chlorpromazine did not inhibit CTxB internalisation, whereas almost completely prevented its entry into HeLa cells (Figure 3a).

When internalisation of CTxB was followed by confocal microscopy, we have found that filipin prevented entry of majority of CTxB into endosomal structures of Balb 3T3 cells and thereby its delivery into the Golgi (Figure 3b). As expected, filipin did not inhibited entry of CTxB into the Golgi of HeLa cells, although a lot of internalised CTxB remained in endosomal structures. Similar to filipin, MβCD inhibited internalisation of CTxB and its delivery into the Golgi of Balb 3T3 cells, but in majority of cells internalised CTxB could be found in
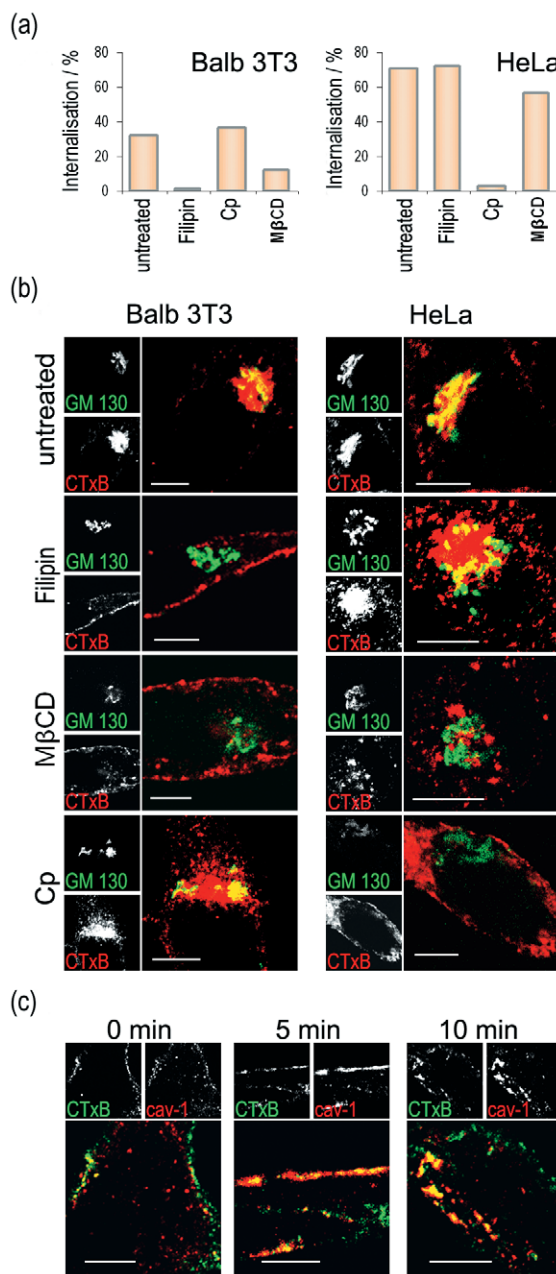

Figure 3. Effect of inhibitors of caveolar and clathrin endocytosis on internalisation of CTxB. (a) Internalisation of CTxB in the presence of inhibitors. Cells were pre-incubated for 30 min with filipin (F), chlorpromazine (Cp) and methyl-β-cyclodextrin (MβCD) or control medium (O) and incubated in the same medium containing CTxB-FITC for 30 min at 4 °C. Labelled cells were than incubated at 37 °C for additional 45 min in medium containing inhibitors. Surface bound CTxB was removed by short low-pH treatment (30 s; pH = 2.0) and intracellular fluorescence analysed by flow cytometry. The result is presented as MFI ratio of acid-treated and acid-untreated cells. (b) Internalisation of the CTxB-AF 555 (red fluorescence) into the Golgi (green fluorescence) in the presence of inhibitors. Cells were treated as described above, fixed and labelled with anti-GM130/anti-mouse IgG1-AF 488 for confocal analysis (c) Translocation of cytoplasmic caveolin into sites of CTxB binding. CTxB-AF 488 (green fluorescence) was bound to the surface of Balb 3T3 cells at 4 °C for 30 min and internalised at 37 °C for indicated periods. After that cells were fixed and stained with anti-caveolin-1-Cy3 (red fluorescence). Scaling bar represents 5 μm. Representative of three independent experiments with comparable results are shown.

peripheral endosomal vesicles. However, in MβCD treated HeLa cells a lot of internalised CTxB was found in endosomal structures, including large juxtanuclear endosomal vesicles, but very little of internalised CTxB was found colocalising with the Golgi marker. As expected, chlorpromazine had no effect on internalisation of the CTxB into the Balb 3T3 cell, including its delivery to the Golgi complex. In contrast to Balb 3T3 cells, in Cp treated HeLa cells CTxB was not found in the Golgi, although some internalised material could be detected in subplasmalemal endosomal vesicles. Thus, our data indicate that CTxB enters Balb 3T3 cells mainly via cholesterol-dependent (caveolar) endocytosis, while in HeLa cell line dominant endocytic pathway is clathrin-dependent.

To test whether CTxB enters into the Balb 3T3 cells via caveolae we performed colocalization analysis with caveolin-1 (cav-1), a major protein required for formation of caveolae. Immediately after CTxB binding at 4 °C, caveolin-1 was not found at the plasma membrane but rather dispersed in the cytosol (Figure 3c). When cells were shifted to 37 °C already after 5 min a lot of cav-1 was found recruited to the internal part of plasma membrane. In these cells, colocalization with CTxB was found (Figure 3c). After 10 min, CTxB was found in cytoplasmic endosomal vesicles and many of them colocalised with cav-1. In contrast to Balb 3T3 cells, cav-1 did not recruit to the plasma membrane of HeLa cells after CTxB binding and remained dispersed in the cytosol. Endosomal vesicles containing internalised CTxB also did not colocalize with cav-1 (data non shown).

From these experiments we can conclude that CTxB enters into the Balb 3T3 cells via caveolar endocytosis that requires intact cholesterol rich membrane microdomains.

Internalised CTxB Bypass EEA1 Early Endosomal Compartments

When the distribution of internalised CTxB-AF 555 was analysed by confocal microscopy, CTxB could be found in endosomal vesicles that were dispersed throughout the cytosol, migrating from the cell periphery towards the juxtanuclear pericentriolar area, before reaching Golgi (Figure 4a). A smaller part of these vesicles were EEA1+ (early endosome antigen 1), indicating that CTxB was transported to the juxtanuclear area generally bypassing the EEA1+ early endosomes. Thus, it appears that internalised CTxB in Balb 3T3 cells enters also the endocytic route that is used by transferrin and other cell surface proteins that are internalised by clathrin endocytosis or through the bulk internalisation pathway. This is supported by finding that internalised CTxB was found in vesicle containing transferrin receptor (TfR). At earlier stages after internalisation, many of CTxB containing endosomes were TfR- and the highest degree of colocalization was found in the juxtanuclear region (Figure 4b).

As expected, colocalization with TfR and EEA1 was more pronounced in HeLa cells since CTxB enters these cells via the same route as TfR (Figure 4b and unpublished data). These data indicated that, in both cell lines, internalised CTxB could be sorted into early endosomal vesicles that acquire EEA1 which are than used for transport of the internalised material into the juxtanuclear region. This was expected for HeLa cells. However, colocalization of the internalised CTxB with cav-1 in Balb 3T3 cells would indicate that it is transported into the Golgi via caveosomes that do not require recruitment of EEA1.

To test whether EEA1 early endosomal pathway was required for delivery of the internalised CTxB into the Golgi we used LY294002, an inhibitor of PI(3)-kinase that is required for recruitment of EEA1 to the early endosomal vesicles and thereby blocks transport through early endosomes.[30] In LY294002 treated cells CTxB reached the Golgi with indistinguishable kinetics (30 min) although clear perinuclear accumulation of CTxB containing vesicles appeared earlier (after 20 min, Figure 4c). Many of these vesicles were found in the vicinity of the Golgi stacks (Figure 4c, 20 min) but at that time very little of the internalised CTxB colocalised with the Golgi marker GM130. Clear colocalization was found after 30 min of internalisation (Figure 4c, 30 min). Although CTxB trafficking towards the Golgi was not inhibited in LY294002 cells, many internalised CTxB material remained retained in subplasmalemal vesicles (Figure 4c, 40 min) indicating that at least a part of internalised CTxB needed to enter the EEA1+ endosomes to be transported in the juxtanuclear region.

To verify the inhibitory effect of LY294002 on early endosomal trafficking in Balb 3T3 cells, we used well-characterized model of TfR endocytosis. In contrast to CTxB, LY294002 treatment reduced cell surface expression of TfR (Figure 4d) because of interference with many steps in TfR trafficking, including TfR recycling. Internalised TfR was found colocalised with EEA1 after 15 min in untreated cells (Figure 4e). Early endosomal vesicles containing endocytosed TfR fuse and recruit EEA1, as a result of PI(3)-kinase activity on Rab5 protein.[30] Since PI(3)-kinases are target for inhibitory effect of LY294002, recruitment of EEA1 to early endosomal vesicles and their transport along microtubules is the first step inhibited in the endosomal pathway.[30] In LY294002 treated cells we found many TfR+ vesicles separated from EEA1+ vesicles (Figure 4e), suggesting that LY294002 inhibited early stages in endosomal trafficking in Balb 3T3 cells. Thus, our data indicate that internalised CTxB in Balb 3T3 cells used both caveolar and EEA1 early endosomal vesicles for transport into the juxtanuclear region. When recruitment of EEA1 to early endosomes was inhibited, a proportion of the internalised CTxB remains stacked in subplasmalemal vesicles waiting appropriate cargo vehicles for transportation.
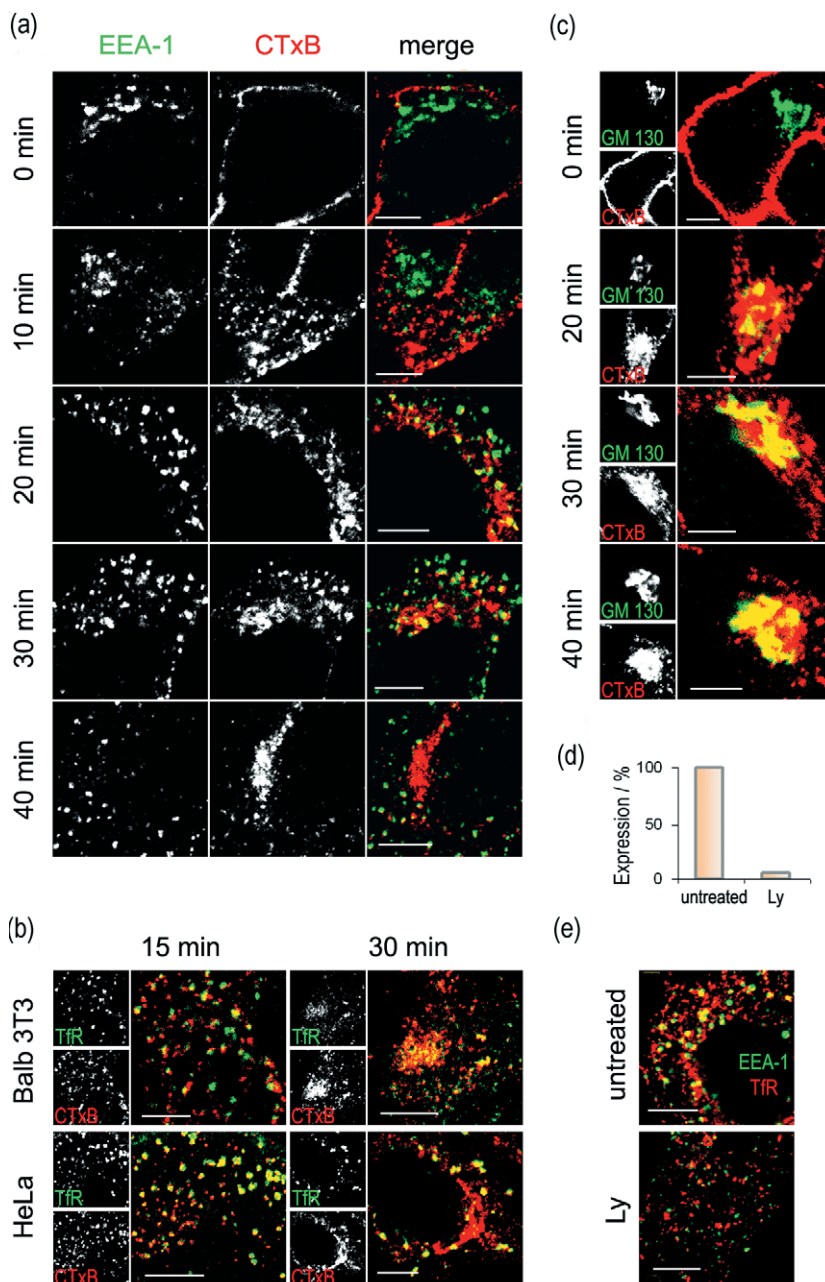
Figure 4. Endocytic trafficking of CTxB through early endosomes in Balb 3T3 cells. (a) Colocalization of internalised CTxB with early endosomal antigen 1 (EEA1) on Balb 3T3 cells. CTxB-AF 555 was internalised as described above and colocalised with EEA1 by using anti-EEA1 and anti-chicken IgG-AF 488. Scaling bar represent 5 μm. (b) Colocalization of internalised CTxB with steady-state transferrin receptor (TfR). TfR was visualized with anti-TfR mAb R17 and anti-rat IgG-AF 488. (c) Internalisation of CTxB in LY294002 treated Balb 3T3 cells. 25 μmol dm^{-3} LY294002 was added 30 min prior and kept through the internalisation assay. Golgi was stained with anti-GM130 and anti-mouse IgG1-AF488. (d) Cell surface expression of TfR in Ly 294002 treated Balb 3T3 cells determined by flow cytometry. (e) Internalisation of TfR into early endosomes in Ly 294002 treated Balb 3T3 cells. Externally bound mAb to TfR (R17) was colocalised with EEA1 after 15 min of internalisation. Scaling bar represents 5 μm. Representative of three independent experiments with comparable results are shown.

Early Endosomal Acidic Gradient Is Not Required for CTxB Progression towards the Golgi in Balb 3T3 Cells

Early endosomes trafficking of many macromolecules is dependent on the endosomal pH that is established by an ATP-dependent proton pump (vacuolar H$^+$-ATPase or V-ATPase). To determine if the endosomal acidic gradient is required for CTxB transport to the Golgi, we treated cells with inhibitors of vesicular transport (acidification) to achieve neutral pH in early endosomal compartments. For that purpose we treated cells with ionophore monensin

that neutralizes internalised vesicles,[31] lysosomotropic amine NH_4Cl that neutralize endosomal pH[32,33] and the V-ATPase inhibitor bafilomycin A1,[34] and determined delivery of CTxB into the Golgi in Balb 3T3 and HeLa cells (Figure 5a). These inhibitors did not prevent CTxB internalisation and CTxB transport to the Golgi in Balb 3T3 cells, although many vesicles that did not reach juxtanuclear area could be seen. In contrast, inhibitors of endosomal acidification retarded CTxB transport and prevented its delivery into the Golgi in HeLa cells (Figure 5a). Since we have shown that CTxB uses both caveosomal and early endosomal vesicles for transport in Balb 3T3 cells (Figures 3 and 4) we tested whether disruption of the endosomal pH gradient has quantitative effect on overall CTxB internalisation. We have found that Balb 3T3 cells internalised similar amounts of CTxB in the presence of inhibitors of endosomal acidification (Figure 5b). Therefore, we can conclude that endosomal pH gradient is not required for CTxB internalisation and intracellular trafficking in Balb 3T3 cells. This is supported by the observation that a majority of internalised CTxB localized in the neutral endosomal compartments (after 15 min of internalisation), as demonstrated by the colocalization with the LysoSensor 153, a fluid phase marker that stacks in the endosomal compartments with pH above 6.8 (Figure 5c). As expected, after 15 min internalised CTxB did not colocalize with the LysoSensor 189, indicating that at this point it did not reach acidic endosomal vesicles with pH below 6.0 (Figure 5c). LysoSensor 189 stained perinuclear endosomal compartments corresponding to the late endosomal vesicles.

Acidic Gradient Is Required for Exit from the Golgi and Degradation of CTxB in Balb 3T3 Cells

It is well known that acidification of the Golgi stacks is required for their maturation and the delivery of the cargo towards secretory vesicles and late endosomal compartments.[35] We have found that in Balb 3T3 cells CTxB reached late endosomal compartments with indistinguishable kinetics to HeLa cells, as determined by colocalization with the late endosomal marker Lamp1. However, when pH gradient of the Golgi and endosomal system was disrupted by monensin, very little of CTxB reached Lamp1 compartments, even 90 min after internalisation (Figure 6a). In addition, monensin treatment disrupted late endosomal vesicles (Figure 6a, 90 min). These data indicate that exit of CTxB from the Golgi and its delivery into the degradation pathway is dependent upon the acidic gradient established in this part of the vesicular route. To test whether this treatment affects degradation of the CTxB we first determined kinetics of its degradation by Western blotting (Figure 6b) and quantified it in the uptake experiment (Figure 6c). A substantial proportion of the CTxB-biotin bound to the cell surface in th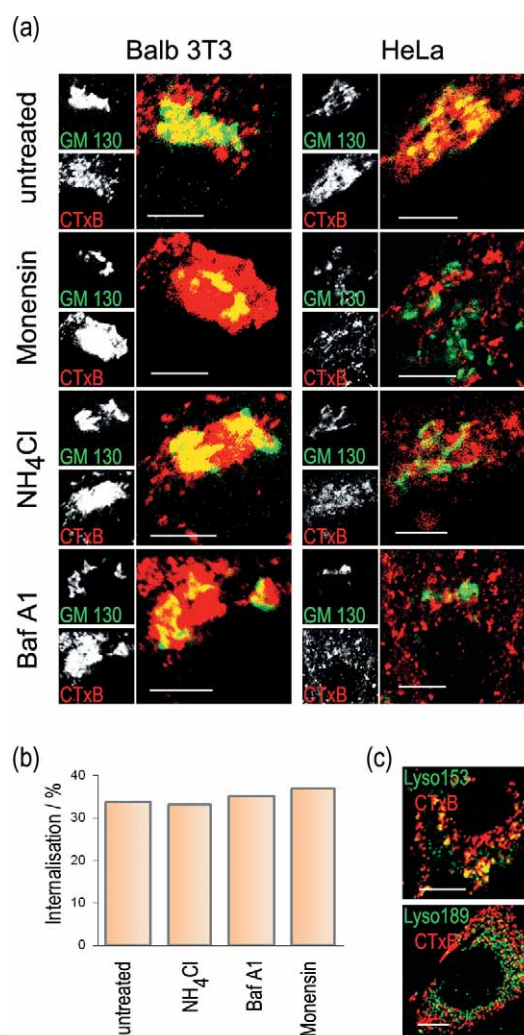e pulse-chase experiment was degraded 60 min after internalisation, indicating for rapid progression of internalised CTxB from the cell surface toward lysosomes. After 3 hours, less than 5 % of internalised material remained inside the cell (Figure 6b). Similar conclusion can be drawn by pulse-chase internalisation of the fluorescent CTxB: 40 % of initially cell surface bound material was degraded or removed from the cell after 90 min of internalisation (Figure 6c). However, pre-treatment of cells with monen-

Figure 5. Dependence of the CTxB internalisation on the acidic gradient in endosomal compartments. (a) Effect of endosomal alkalisation on endocytic trafficking of CTxB in Balb 3T3 and HeLa cells. Cells were pre-incubated for 30 min with monensin, NH_4Cl and bafilomycin A1 (Baf A1), or treated with control medium (∅), and internalisation of CTxB-AF 555 (red fluorescence) in the Golgi (green fluorescence) determined after 30 min, as described above. (b) Internalisation of CTxB in Balb 3T3 cells in the presence of alkalisation agents was determined in the internalisation assay (45 min) by flow cytometry, as described above. (c) Internalisation of CTxB in neutral and acidic endosomal compartments of Balb 3T3 cells. CTxB-AF 555 (red fluorescence) was cointernalised for 15 min with lysosensors (green fluorescence): Lyso 153 (neutral compartments) or Lyso 189 (acidic compartments). Scaling bar represents 5 μm. Representative of three independent experiments with comparable results are shown.
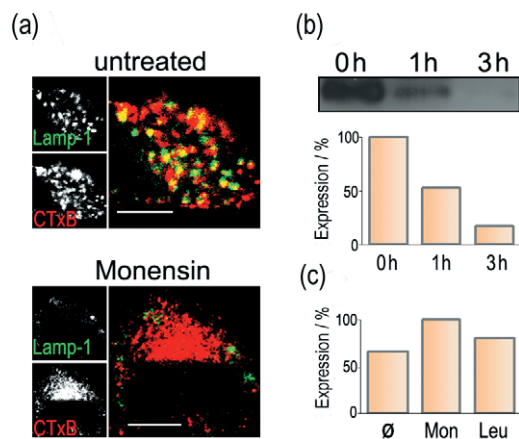
Figure 6. Effect of vesicular alkalisation on degradation of CTxB in Balb 3T3 cells. (a) Internalisation of CTxB into late endosomes. Internalisation assay in untreated Balb 3T3 cells and in the presence of monensin (30 min pre-treatment) was performed as described above. (b) Degradation kinetics of cell surface bound CTxB. Balb 3T3 cells were incubated with CTxB-biotin for 30 min at 4 °C and incubated at 37 °C. Cytoplasmic extract were separated by SDS-PAGE, blotted and stained by streptavidin-peroxidase. Percentage of expression was calculated on the basis of band density. (c) Effect of inhibitors of endosomal acidification and lysosomal degradation on degradation of internalised CTxB. Balb 3T3 cells were pre-treated with 9 μmol dm^{-3} monensin (Mon) and 1 μg ml^{-1} leupeptin (Leu), and CTxB-FITC internalised for 90 min in the presence of inhibitors. Fluorescence intensity of intracellular CTxB-FITC was determined by flow cytometry. The result is presented as MFI ratio (expressed in percents). Bar represents 5 μm. Representative of three independent experiments with comparable results are shown.

sin completely prevented degradation of the fluorescent CTxB, supporting our previous observation that CTxB do not reach late endosomes after disruption of endosomal pH gradient. Similarly, leupeptin, an inhibitor of lysosomal proteases prevented degradation of internalised fluorescent CTxB, although not completely (Figure 6c). Thus, we can conclude that acidic pH either in the Golgi or in late endosomal compartments is required exit of CTxB from the Golgi and its entry into the degradative pathway.

DISCUSSION

Given that there are numerous contradictory published data about CTxB endocytosis, the aim of this study was to clarify internalisation mechanism and intracellular trafficking of CTxB along the endocytic pathway in murine fibroblasts (Balb 3T3 cells), and to compare it with human epithelial cells (HeLa cell line). We have shown that caveolar/lipid-raft dependent endocytosis plays dominant role in Balb 3T3 cells, while in HeLa cells CTxB is internalised mainly in clathrin-dependent manner. Internalised CTxB reaches the Golgi complex in both cell lines, but with different kinetics, and transport to the Golgi requires functional acidic early endosomes only in HeLa cell line. From the Golgi CTxB is targeted to the

late endosomal compartment and into lysosomes for degradation.

Recent data indicate that CTxB can be internalised via different endocytic mechanisms in different cell types,[3,15,28] although in many cells internalisation is dominantly mediated by caveolae, and CTxB internalisation is often used as a model for caveolar endocytosis.[13,36,37] To determine mechanism of CTxB endocytosis we used filipin, a cholesterol-binding drug, and methyl-β-cyclodextrin, a cholesterol-depleting agent. These drugs disrupt detergent insoluble membrane microdomains and caveolae,[38,39] and therefore preclude caveolar and lipid raft-dependent endocytosis[13,33]. Treatment of cells with filipin and methyl-β-cyclodextrin inhibited CTxB internalisation in Balb 3T3 cells but not in HeLa cells (Figure 3a and 4a), indicating that lipid rafts are a major pathway for CTxB endocytosis in Balb 3T3 cells. Cell surface expression of GM1, which serve as receptor for CTxB, was much higher in Balb 3T3 cells than in HeLa cells (Figure 1) indicating that the endocytic pathway for CTxB entry could be dependent upon the initial level of the cell surface receptor. This is in accordance with the observation that high cell surface expression of GM1 induces caveolar and lipid raft-dependent endocytosis[28] and that CTxB is internalised via lipid raft-dependent mechanism in cells that have high GM1 expression, and lipid raft-independent in cells with low GM1 expression.[40] The reduced levels of GM1 on the cell surface of HeLa cells apparently did not influence the ability to recruit CTxB to rafts domains loaded with GM1, as the low levels of GM1 are still associated with Triton X-100 insoluble raft domains (Figure 1a).

Since is known that detergent insoluble membrane domains are essential for both caveolar and non-caveolar lipid raft-dependent endocytosis,[1] we colocalised CTxB with caveolin-1, protein known to be associated with caveolae and caveosomes.[41,42] Immediately after CTxB binding caveolin-1 was found dispersed in the cytosol, but after couple of minutes caveolin-1 was mobilized to the plasma membrane (Figure 3) and colocalised with the CTxB. This provides the second line of evidence that CTxB enter into the Balb 3T3 cells via caveolae. In the later stages many of CTxB containing endosomal vesicles colocalised with caveolin-1 indicating for its localizations in caveosomes (Figure 3c). However, cell surface binding and internalisation of CTxB in HeLa cells did not mobilize caveolin-1 to the plasma membrane and no caveolin-1 could be found associated with endosomal vesicles containing internalised CTxB. Clathrin endocytosis did not play a significant role in the internalisation of CTxB in Balb 3T3 cells since chlorpromazine, drug known to inhibit clathrin endocytosis,[33,43,44] did not influence the internalisation of CTxB (Figure 3a). In contrast to Balb 3T3 cells, internalisation of cell surface bound CTxB was almost completely prevented in HeLa cells treated with chlorpromazine, leading to the conclusion

that CTxB is internalised via clathrin mediated route in HeLa cells.

Irrespective on mechanism used to enter the cell, either caveolar endocytosis in Balb 3T3 or clathrin-dependent endocytosis in HeLa cells, internalised CTxB was delivered into the juxtanuclear Golgi stacks in both cell lines, although with slower kinetics in Balb 3T3 (45 min) then in HeLa (30 min) cells. This result is apparently not in correlation with some previous studies which shown that only non-clathrin pathways lead to CTxB delivery to the Golgi complex.[17,45] Conclusions in these studies were based on the inhibitory effect of filipin,[17] although we have found that filipin inhibits early endosomal trafficking, and on detection of the extracellular cAMP level,[45] whereas our observations are based on colocalization of internalised CTxB with Golgi markers.

Intracellular trafficking of the complete cholera toxin involves the entry into the Golgi for delivery of the A subunit into the endoplasmic reticulum where A and B subunit segregate; A subunit enters the cytosol and the B subunit is directed into the late endosomes and degraded (CTxB).[5,46] To clarify whether CTxB enters the Golgi in Balb 3T3 cells we performed colocalization studies with four markers that are distributed in all parts of the Golgi (Figure 2). We have found that internalised CTxB colocalize with all markers in the juxtanuclear region, in Golgi stacks that are concentrated near the microtubule-organizing centre. However, we have also found that substantial amount of the internalised CTxB could be found in the similar cisternal structures in the vicinity of the Golgi stacks. These structures could represent collecting multivesicular intermediate cisternal compartments that are on the crossroad of early and late endosomal route, and function as sorting organelle for cargo delivered with early endosomal membranes, either for its rerouting into the juxtanuclear recycling endosomes or for loading of late endosomes.[47,48] These parts of CTxB endocytic trafficking remain unclear and further studies are required for precise characterization of the CTxB entry into the late endosomes.

Although it was shown that CTxB might travel different routes after internalisation, its progression along early endosomal membranes and delivery into the juxtanuclear collecting cisternae is not clear. It is generally accepted that after internalisation CTxB is directed into the acidic early endosomes, either from caveosomes if it is internalised via caveolar endocytosis, from clathrin vesicles if it is internalised in clathrin dependent manner or from glycosphingolipid enriched early compartments (GEECs) if it is internalised via non-clathrin non-caveolar route.[17] Therefore early endosomes are considered as the most important subcellular structures involved in CTxB transport from plasma membrane to the Golgi.[15,25] It has been shown in many studies that early endosomal intermediates bearing EEA1 (EEA1+ early endosomal vesicles) are required for cargo delivery to the recycling

endosomes and maturation of early endosomes into multivesicular (ECV/MVB) intermediates in the juxtanuclear region.[30,46] These vesicles are loaded with internalised TfR and TfR trafficking is considered as model for early endosomal route.[30,49] We have found that internalised CTxB did not significantly colocalize either with EEA1 or TfR in Balb 3T3 cells, in contrast to strong colocalization with both markers in HeLa cells (Figure 4d). Thus, we can conclude that CTxB bypass EEA1+ transport intermediates in Balb 3T3 cells on its route to the Golgi. Colocalization with TfR in perinuclear region on Balb 3T3 cells indicate that both TfR and CTxB are collected in the same juxtanuclear compartment before CTxB translocates into the Golgi.

It has been reported that low pH in early endosomes is required for takeover of CTxB from caveosomes as well as from clathrin coated vesicles,[50] and its subsequent sorting into the Golgi. However, we have shown that depletion of the endosomal acidic gradient by monensin, NH_4Cl, and bafilomycin A1 almost completely prevented CTxB transport to the Golgi in HeLa cells but not in Balb 3T3 cells (Figure 5). In addition, treatment of cells with LY294002, an inhibitor of PI(3)-kinase[30] did not prevent CTxB transport from plasma membrane to the Golgi in Balb 3T3 cells (Figure 4c). This drug affects a number of points in the endosomal pathway that are controlled by PI(3)-kinase, and the first step that is inhibited is recruitment of EEA1 to the Rab5+ early endosomal vesicles and generation of EEA1+ early endosomes that are required for cargo transport along microtubular network toward the juxtanuclear collecting cisternae.

In contrast to Balb 3T3 cells, the EEA1+ early endosomal transport intermediates are irreplaceable on HeLa cells. The role of early endosomes in CTxB transport to the Golgi in HeLa cells is also supported by effect of cholesterol depleting drug MβCD (Figure 3b). Although CTxB was internalised in MβCD treated cells, it did not reach the Golgi since early endosomal compartments are rich in lipid rafts and MβCD treatment disrupted their function.[51–53] We cannot exclude the possibility that CTxB does, at some stage, pass trough EEA1+ compartments also in Balb 3T3 cells, inasmuch some part of internalised CTxB can be seen »trapped« in vesicles after inhibition of acidification or treatment with LY294002, but most of CTxB is nevertheless transported to the Golgi.

Our data demonstrate that in Balb 3T3 cells CTxB is internalised via lipid raft in caveolin-1 positive structures (caveosomes) from which it is sorted into pH neutral vesicles negative for caveolin-1 and EEA1 before being translocated into the Golgi. These structures are similar to structures described for SV40 intracellular trafficking.[31] However, very little is known about cargo delivery from caveosomes and communication between caveosomes and other early endosomal compartments. Further investigations on CTxB trafficking will be necessary to clarify how cargo form caveosomes, and other neutral early endoso-

mal compartments that are negative for caveolin-1 (*i.e.* those described for SV40 trafficking), is delivered into the juxtanuclear multivesicular sorting cisternae.

Almost half of cell-surface bound and internalised CTxB disappeared after one hour, and more than 90 % after three hours (Figure 6). Colocalisation with Lamp1 in Balb 3T3 cells and with CD63 in HeLa cells, markers of late endosomes and lysosomes,[8,53] and inhibition with leupeptin indicate that a majority of internalised CTxB is degraded in lysosomes. However, other ways of CTxB discard from the cell cannot be excluded. For example, it is known that exocytosis could be a way in which the cell discards the excess of material from late endosomal compartments.[54] Incomplete inhibitory effect of leupeptin (Figure 6) and rather rapid loss of the internalised CTxB would support this option. In addition, alkalisation of endosomal compartments by monensin inhibited CTxB exit from the Golgi and completely prevented disappearance of internalised CTxB from cells. Apparently low pH in the *trans*-Golgi (pH less than 6.0) and acidic gradient in late endosomes[55] is required for entry of CTxB into the degradative pathway. Additional studies are required to clarify whether low pH is required for CTxB exit from the Golgi or for its entry into the late endosomal pathway. It has been shown that alkalisation of endosomal compartment, including also late endosomes, does not prevent entry of fluid phase markers and many other cell surface receptors and ligands into the late endosomes, including TfR, MHC class I molecules and misfolded proteins.[29] Since we have found that intracellular trafficking of lipid-raft linked proteins is not altered after endosomal alkalisation (data not shown) it is very likely that acidic milieu of the Golgi is required for CTxB exit.

Although several aspects of CTxB endocytic trafficking have been extensively studied, still many questions remained open. Recent progresses in understanding complexity of the endosomal pathway will also contribute to the understanding endosomal trafficking of CTxB, particularly transition of CTxB into the late endosomes. Indeed, CTxB trafficking may help in understanding complexity of late endosomal compartments.

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SAŽETAK

Endocitozni putevi toksina kolere u stanicama Balb 3T3

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Endocitoza B podjedinice toksina kolere (CTxB) u različitim staničnim linijama upućuje na raznolikost putova za ulazak liganada sa stanične površine. U ovom radu istražujemo mehanizme internalizacije i endosomalnog transporta CTxB u mišjim fibroblastima (stanice Balb 3T3) i ljudskim epitelnim stanicama (HeLa). U Balb 3T3 stanice CTxB ulazi uglavnom endocitoznim putovima koji su ovisni o kolesterolu dok je ulazak u HeLa stanice ovisan o klatrinskoj endocitozi. Napredovanje internaliziranog CTxB prema Golgijevu aparatu treba kiseli gradijent u ranim endosomima, a u Balb 3T3 stanicama CTxB zaobilazi rane endosome (EEA1^{+} odjeljke). Isto tako, u Balb 3T3 stanicama je kiseli endosomalni gradijent potreban za izlazak CTxB iz Golgijeva kompleksa u kasne endosome i degradaciju CTxB. Dakle, putovanje CTxB kroz endosomalne odjeljke može pomoći u razumijevanju ne-klatrinskih mehanizama endocitoze te složenosti kasnih endosomalnih odjeljaka.