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EFFECT OF THE T-DOMAIN ON INTRACELLULAR TRANSPORT OF DIPHTHERIA TOXIN

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Subunit B of diphtheria toxin (DT), which consists of two domains: R (receptor-binding) and T (transmembrane), plays an important role in toxin-receptor binding on the cell-targets and in transportation of catalytic subunit A to the cell cytosol. Recombinant analogues of the subunit B are promising representatives in the unique class of transporting proteins, able to deliver different types of biologically active molecules to cell cytosol. In the development of these protein constructs understanding of the role of each of the DT fragments in determination of transporting pathways of endocytosed complex toxin-receptor is urgently required.

We have studied in this work the T-domain effect on intracellular transport of recombinant fragments of DT. We have compared intracellular transport of the R-domain and the subunit B, the last one consisted of both R-domain and T-domain. Recombinant fragments of DT used in this work were labeled with fluorescent proteins, which allowed applying colocalization technique for our study. Application of confocal microscopy technique revealed differences in transportation of recombinant derivates of DT in Vero cells: R-domain moved faster than subunit B to tubular compartments. Analysis of R-domain and subunit B transportation confirmed almost linear increase of their colocalization with the time regarding to Pearsons correlation coefficient (PCC). However, amount of colocalized with R-domain subunit B were not linearly increased with time according to Manders coefficient (M_1), this could indicate the ability of subunit B to transport to such compartments that R-domain do not reach. Possible role of the T-domain in intracellular transportation and compartmentalization of the toxin may be associated with the ability of the T-domain to form a proton channels and its ability to interact with COPI complex.

K e y w o r d s: diphtheria toxin, T-domain of diphtheria toxin, endocytosis, fluorescent proteins, confocal microscopy, intracellular transport.

Diphtheria toxin (DT) is a major virulence factor of the diphtheria causative agent bacteria *Corynebacterium diphtheriae*. DT belongs to the family of bacterial AB toxins, featured by existence in their structure of two functionally distinct subunits of subunit A (Enzymatically Active) and subunit B (Receptor Binding). Subunit B (SubB) is responsible for binding with membrane-anchored receptor and participates in a translocation of subunit A (SubA) into cell cytosol. Subunit A is ADP-ribosyltransferase, which modifies intracellular target – eukaryotic translation elongation factor 2 (eEF-2). Accumulation of the large amount of modified eEF-2 leads to termination of protein biosynthesis and further cell death. Detailed scheme of toxin action was covered in reviews [1, 2].

DT is made by one polypeptide chain, but to reach cytotoxic activity proteolytic cleavage of the toxin into separate fragments (subunits) is necessary. DT subunits are connected by polypeptide linker, which cleavages by membrane-anchored proteinase from the serine endoproteases family furin. It is known that other proteinases [3], like proteinase PACE4 [4, 5], may activate DT. Furin make DT active by cleavage of polypeptide bond between residues of Arg193 and Ser194 in a loop region consisting of 14 amino acid residues, after DT subunits are linked only by disulfide bond between cysteine 186 and 201 [6, 7]. Its reduction takes place in cytosol with assistance of thioredoxin reductase [8].

Tertiary structure of DT is formed by three domains, each of which has specific structure and function. N-terminal catalytic C-domain made by amino acid residues 1-193, corresponds to subunit A (21.1 kDa). Subunit B (37.2 kDa) consists of transmembrane T-domain (Td) formed by amino acid

residues 205-378 and of C-terminal receptor-binding R-domain (Rd), formed by residues 386-535 [9]. T-domain possesses hydrophobic properties and have high content of spiral structures, it is responsible for DT-endosomal membrane interaction and for translocation of subunit A into the cell cytosol [9]. R-domain of subunit B possesses hydrophilic properties and have high affinity for trans-membrane form of proHB-EGF (progenitor of Heparin-Binding Epidermal Growth Factor-like Growth Factor) [10–13].

DT-sensitive cells have on their surface from 4 000 to 200 000 proHB-EGF molecules per cell [14]. DT transports into the cell through endosomes, which formed via clathrin-dependent receptor-mediated endocytosis [15]. DT changes its conformation after acidification of endosome lumen, hydrophobic domains become exponated outside the molecule and, correspondently, binding of the toxin with the membrane lipids increases, that may cause SubA translocation into the cell cytosol. Reduction of disulfide bound between subunits takes place in the cytosol and it causes release of SubA and development of its catalytic activity.

SubA translocation throughout endosome lipid bilayer is mediated by the T-domain [16, 17]. But DT receptor proHB-EGF and the R-domain, possibly, participates in this process as well [18–20]. Few regions of the R-domain (380-421, 422-441 i 442-483) plunge deeply into the membrane, this process is accompanied by formation of α-spiral transmembrane structure [20]. One of those three regions (442-483) possesses phosphate-binding P-site (456-458-460-472-474) [21], which, possibly, plays some role in the process of translocation.

Transmembrane domain consists of 9 helixes, gathered in 3 layers. The first layer is created by 2 hydrophobic C-terminal helixes TH8 and TH9, second layer was created by 3 hydrophobic helixes TH5-TH7, and the third one is created by 4 helixes TH1-TH4, which possess strong hydrophilic features. Helixes TH8-TH9, which are bound by loop TL5, create the nucleus of the domain and participate in subunit A translocation into cytosol [9]. It is known that Td is able to interact with proteins which have intrinsically disordered structure and are in the condition of "molten globule" [22]. One of the conditions of successful recognition of protein by Td is its mild hydrophobicity. Thus, Td in the process of translocation shows properties similar to chaperon proteins [23].

Despite detailed mechanism of SubA translocation is not clearly known, the major requirement for successful translocation is acidification of endosome lumen what causes conformation changes in the toxin molecule. It is known that both DT subunits plunge into the membrane, though SubA plunges less deep and its groups less exponated to lipids due to contact with SubB, which partially covers SubA and prohibits its contact with membrane lipids [24].

That is why further detailed study of mechanisms of SubA translocation into cytosol and mechanisms of resistance to DT needs new experimental models and new instruments for research of these processes. One of such instruments may be fluorescent truncated DT analogues, in particular, its SubB and R-domain, fused with different fluorescent proteins, for example, with red fluorescent protein mCherry and with green fluorescent protein EGFP [25]. Thus, the aim of this work is to investigate features of transportation of subunit B of diphtheria toxin and its R-domain on cell line Vero with recombinant fluorescent truncated DT forms mCh-SubB and EGFP-Rd.

Materials and Methods

Recombinant proteins expression. Recombinant proteins EGFP, mCherry, mCh-SubB and EGFP-Rd have been obtained from *Escherichia coli* BL 21 (DE3) Rosetta (Novagen, USA) host strain, transformed by genetic constructions based on plasmid vector pET-24a(+) (Novagen, USA).

Bacterial culture has been grown at 37 °C under aeration conditions (250 rpm) up to A_{600} – 0.5-0.7 in the 2xYT medium with 50 mg/l of kanamycin and 170 mg/l of chloramphenicol. Expression of the proteins has been inducted by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Target proteins expression has been performed during 3 hours at 30 ºC under strong aeration conditions (250 rpm), after what cells have been precipitated by centrifugation at 3300 g during 10 min.

Purification of recombinant proteins with metal-affinity chromatography on Ni^{2+}-NTA-agarose. Recombinant proteins has been purified with metal-affinity chromatography on the column with Ni^{2+}-NTA-agarose. Column containing affine sorbent has been equilibrated with buffer E (50 mM Na_2HPO_4, 0.5 M NaCl, 10 mM imidazole, pH 8.0) with 8 M of urea. Centrifuged bacterial cells have been resuspended in buffer E containing 8 M urea (1 ml of buffer solution per 10 ml of cell culture). Next, samples have been sonicated by ultrasonic homogenizer LabsonicM (Sartorius, Germany). Cell residues have

been precipitated by centrifugation under 10 000 g 15 min, and a column has been filled by supernatant.

Renaturation by washing the column with gradational decrease of urea concentration (8 M → 6 M → 4 M → 2 M → 0 M) in buffer E has been performed for obtaining soluble recombinant proteins. Protein was eluted by buffer E containing 400 mM imidazole. Protein for further procedures was dialyzed against PBS (0.14 M NaCl, 0.03 M KCl, 0.011 M Na_2HPO_4, 0.002 M KH_2PO_4, pH 7.2).

Electrophoretic separation of proteins. Electrophoresis in polyacrylamide gel with SDS has been performed in compliance with modified methodology of Schagger H. [26].

Cultivation of Vero cell line. Vero cell line, originating from green monkey kidney epithelium (*Cercopithecus aethiops*) [27], was obtained from the cell lines bank of the R.E. Kavetskiy Experimental Pathology, Oncology and Radiobiology Institute of NAS of Ukraine. Vero cell line has been cultivated on the nutrient medium RPMI-1640 containing L-glutamine, with addition of 5% FCS (fetal calf serum), streptomycin (100 mg/l), penicillin (10 000 U) and amphotericin B (250 μg/l) under conditions of increased concentration of CO_2 in atmosphere.

Flow cytometry. Cells have been detached from flask by addition of 20 mM EDTA in PBS. Optimal quantity of cells for staining was $0.3\text{-}0.5\times10^6$ per probe. Cells have been stained by incubation with 725 nM of proteins in 200 μl of BSA/PBS solution (1% BSA in PBS) for 15 min at 4 °C.

For washing of non-bound proteins, 1 ml of BSA/PBS was added, and then cell pellet carefully resuspended and centrifuged (200-300 g, 10min). Next, cell pellet was resuspended in 1 ml of BSA/PBS. Further solution of stained cells were transferred into the test-tubes for cytometer.

Determination of cells fluorescence intensity were performed on flow cytometer Coulter Epics XL (Beckman Coulter, USA). There are parameters beneath, used in the protocol: forward light scatter (FS), side light scatter (SS) and logarithm of the fluorescence level on channel FL1 (515-535 nm) for probes stained with EGFP and channel FL3 (610–630 nm) for probes stained with mCherry. Two graphs have been calculated with these parameters: dot plot of cells morphology (FS vs SS) and histogram of fluorescence intensity according to channel FL1 or FL3. Quantity of events per graph was 10 000.

Preparation of cell specimens for confocal microscopy. Vero cells grew up on cover glass to semiconfluent state and then washed up by solution RPMI-1640 (pH 7.3). Fluorescent proteins in concentration of 145 nM (EGFP-Rd) and 390 nM (mCh-SubB) and 10 μM of cell nuclei stain Hoechst 33342 in RPMI-1640 incubated at 37 °C from 5 to 75 min. Non bound proteins have been washed up by a solution RPMI-1640 after 15 min of incubation and only solution 10 μM Hoechst 33342 in RPMI-1640 have been used for further incubation. After incubation cells were fixed by solution 4% of paraformaldehyde in 0.1 M phosphate buffer during 40 min at 4 °C . Cover glass with cells has been mounted on a slide in mounting medium based on polyvinyl alcohol

Cell specimens were analyzed on confocal microscope Zeiss LSM 510 Meta (Germany). Oil immersion objective Plan-Apochromat 63x/1.4 Oil DIC was used. EGFP chromophore was excited with 488 nm laser and chromophore mCherry chromophore was excited with 543 nm laser, information about fluorescence was collected on channels 505–530 nm for EGFP and 560–615 nm for mCherry. Nuclei stain Hoechst 33342 was excited with 405 nm laser and detected on 420–480 nm channel.

Results and Discussions

Obtaining of recombinant fluorescent proteins. Genes, which encode fused proteins mCherry – subunit B DT and R-domain DT - EGFP (mCh-SubB and EGFP-Rd) was created by merging of corresponding nucleotide sequences into one readin frame [25]. Protein expression was performed in the culture *E. coli* BL 21 (DE3) Rosetta (Novagen, USA), transformed by pET-24a(+)-based (Novagen, USA) genetic constructs. Purification of recombinant proteins EGFP, mCherry, mCh-SubB and EGFP-Rd performed with metal affinity chromatography on Ni^{2+}-NTI column. Taking into the account that products of interest were insoluble, procedure of refolding was performed. Analysis of protein fractions after the refolding was performed on 12% PAAGE. (Fig. 1).

Flow cytometry. Determination of ability of fluorescent DT fragments mCh-SubB and EGFP-Rd to the specific interaction with cell DT receptor performed on Vero cells, on the surface of which proHB-EGF represented in considerable quantity (around $1–2\times10^5$ per cell).

Results of binding analysis presented in a histogram of fluorescence intensity distribution of Vero cells on channel FL1 (515-535 nm), which represents fluorescence EGFP, and on channel FL3 (610-

630 nm), which represents fluorescence mCherry (Fig. 2). As that appears from the presented data, obtained fluorescent proteins mCh-SubB and EGFP-Rd effectively bind to Vero cells in comparison with control proteins mCherry and EGFP, what indicates on specificity of fluorescent DT derivates interaction of with those sells.

To compare traffic of different DT derivates under condition of their simultaneous adding to Vero cells, molar ratio of these chimera proteins for their binding in equivalent quantity with cells needed to be determined first. To determine such ratio, Vero cells were stained with two proteins under condition that concentration of mCh-SubB has been changed from 100 to 857.14 nM, but concentration of EGFP-Rd was constant (500 nM). That is molar concentrations ratio of mCh-SubB and EGFP-Rd changed in probes from 0.2 : 1 to 2 : 1.

EGFP-Rd and mCh-SubB binding with cells has been determined according to channels FL1 and FL3, correspondingly. Obtained results of fluorescence intensity of these cells is shown on dot plot (Fig. 3), where axis x demonstrates intensity of fluorescence on channel FL1 (EGFP-Rd fluorescence), and axis y is intensity of fluorescence on channel FL3 (mCh-SubB fluorescence). Four regions are gated in the diagram with usage of corresponding control probes, which were stained with fluorescent proteins EGFP, mCherry, EGFP-Rd and mCh-SubB. Gate A on the Fig. 3 (upper left dot plot). Gate A includes the unstained cells, according to channels FL1 and FL3 (double-negative staining), gate B includes the cells stained according to channel FL1, but not stained according to channel FL3, gate C gates cells, stained according to channel FL3, but not stained according to channel FL1, and gate D includes cells, stained according to both channels (double-positive staining).

Fluorescent signal from mCh-SubB increased according to increase of concentration of this protein in the probe (Fig. 3). As mCh-SubB concentration increased, under stable EGFP-Rd concentration, fluorescence on channel FL1 slowly decreases, but stays in the gates B and C, but intensity of fluorescence on channel FL3 gradually increases and transfers from negative gate B to a positive gate C, what proves steady binding with cell receptors of both fluorescent DT derivates. In compliance with the experimental data, molar ratio of EGFP-Rd to mCh-SubB 1:2 was used in the next studies on confocal microscope. Best staining of cells by both DT fluorescent

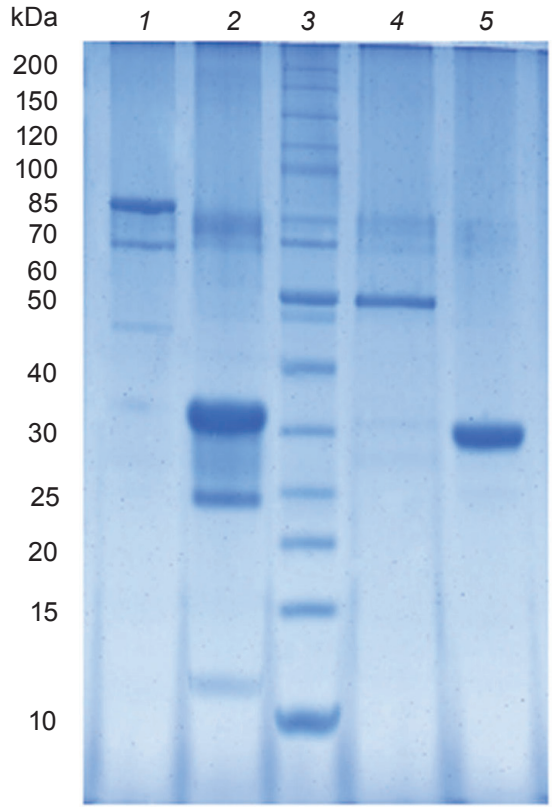

Fig. 1. Electroforegram of recombinant proteins 1 – mCh-SubB; 2 – mCherry; 3 – molecular weight ladder; 4 – EGFP- Rd; 5 – EGFP

derivates was observed under this ratio (Fig. 3, low right dot plot).

Confocal microscopy. Details of fluorescent DT derivates transport inside the cells were studied on Vero cell line. Cells were stained with proteins EGFP-Rd and mCh-SubB in molar ratio 1 : 2. Colocalization between those proteins on the confocal images were detected with RG2B_Colocalization plugin for FIJI software [28].

As it appeared from Fig. 4, these proteins had different localization on the 15th minute of observation: mCh-SubB located closer to the cell surface at the time when EGFP-Rd is localized closer to the cell nucleus; colocalization between these proteins was low. Similar state also observed on the 30th and 45th minutes of observation, however colocalization between them gradually increased. Both proteins started significantly colocalize only on the 60th min of observation, however EGFP-Rd has been situated already in tubular structures, at the time when mCh-SubB, predominantly, was situated in vesicular structures. mCh-SubB and EGFP-Rd had a high level of colocalization in tubular struc-

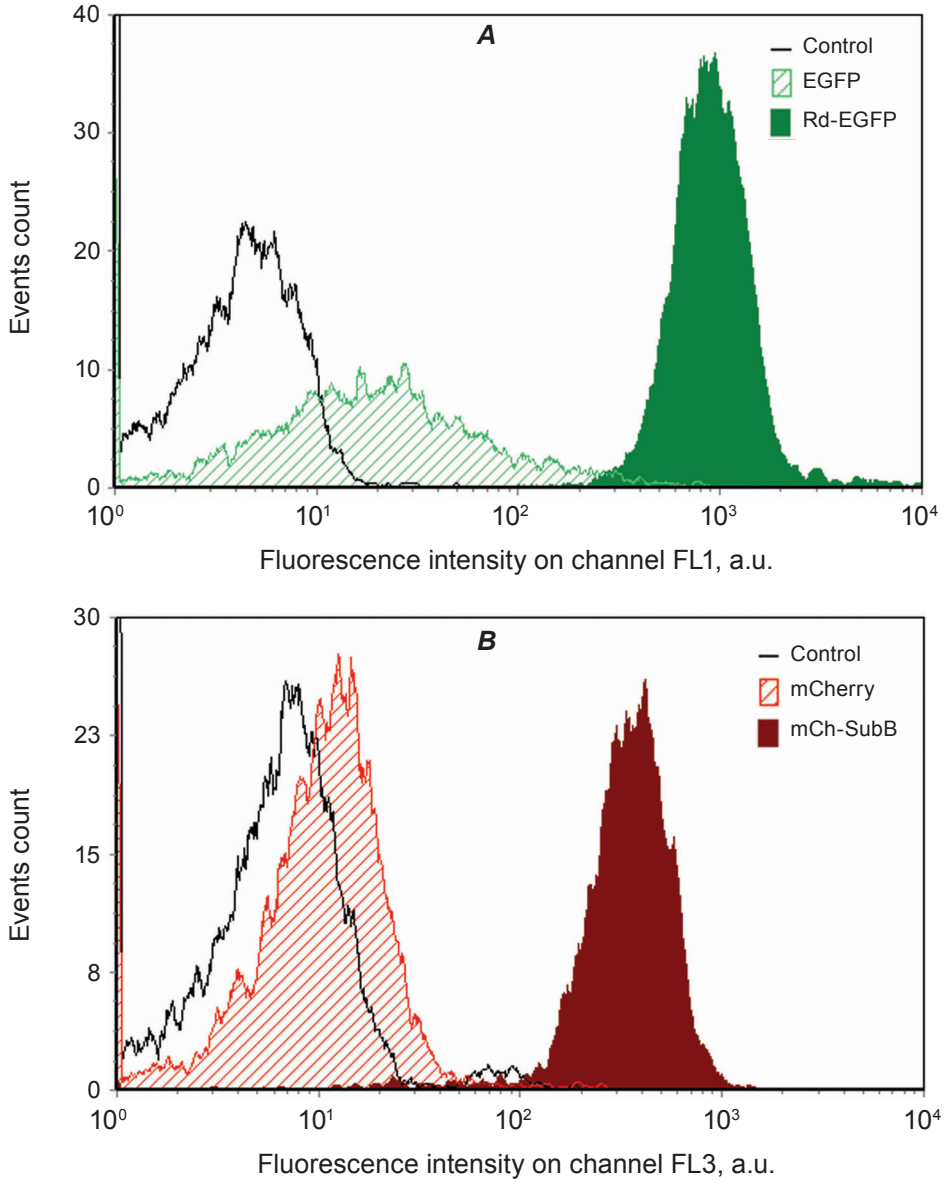

Fig. 2. Comparative histograms of fluorescence intensity of Vero cells, which were stained with recombinant fluorescent proteins or unstained (control). ***A*** *– Fluorescence intensity distribution of cells according to channel FL1 for control probe and probes, stained with EGFP and EGFP-Rd.* ***B*** *– Fluorescence intensity distribution of cells according to channel FL3 for control probe and probes, stained with mCherry and mCh-SubB*

tures on the 75th min of observation, however a part of mCh-SubB stayed not colocalized with EGFP-Rd. Thus, the obtained results point to possible DT T-domain influence on the intracellular toxin transport.

FIJI software with JACoP plugin was used for quantification analysis of colocalization between those proteins [28, 29]. Obtained pictures were analyzed to calculate two parameters: Pearson correlation coefficient and Manders correlation coefficient (M coefficients) for mCh-SubB and EGFP-Rd. Pearson correlation coefficient (PCC) in this case shows probability that proteins mCh-SubB and EGFP-Rd are colocalized, where meaning «0» corresponds with absence of colocalization, meaning «1» corresponds with full colocalization, and meaning «-1» means reverse dependency.