Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1763 (2006) 120–128
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Cell internalization and traffic pathway of *Clostridium botulinum* type C neurotoxin in HT-29 cells

Nobuo Uotsu [a], Atsushi Nishikawa [a,d,*], Toshihiro Watanabe [c], Tohru Ohyama [c], Takashi Tonozuka [a], Yoshiyuki Sakano [a], Keiji Oguma [b]

[a] *Department of Applied Biological Science, and Department of Biotechnology, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, 183-8509 Tokyo, Japan*
[b] *Department of Bacteriology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, 700-8558 Okayama, Japan*
[c] *Department of Food Science, Faculty of Bioindustry, Tokyo University of Agriculture, 196, Yasaka, 099-2422 Abashiri, Japan*
[d] *CREST, Japan Science and Technology Agency, Japan*

Received 9 August 2005; received in revised form 31 October 2005; accepted 17 November 2005
Available online 27 December 2005

Abstract

The bacterium *Clostridium botulinum* type C produces a progenitor toxin (C16S toxin) that binds to O-linked sugar chains terminating with sialic acid on the surface of HT-29 cells prior to internalization [A. Nishikawa, N. Uotsu, H. Arimitsu, J.C. Lee, Y. Miura, Y. Fujinaga, H. Nakada, T. Watanabe, T. Ohyama, Y. Sakano, K. Oguma, Biochem. Biophys. Res. Commun. 319 (2004) 327–333] [21]. Based on this, it was hypothesized that the C16S toxin is internalized via clathrin-coated pits. To examine this possibility, the internalized toxin was observed with a fluorescent antibody using confocal laser-scanning microscopy. The confocal images clearly indicated that the C16S toxin was internalized mainly via clathrin-coated pits and localized in early endosomes. The toxin was colocalized with caveolin-1 which is one of the components of caveolae, however, implying the toxin was also internalized via caveolae. The confocal images also showed that the neurotoxin transported to the endosome was transferred to the Golgi apparatus. However, the non-toxic components were not merged with the Golgi marker protein, TGN38, implying the neurotoxin was dissociated from progenitor toxin in endosomes. These results suggested that the C16S toxin was separated to the neurotoxin and other proteins in endosome and the neurotoxin was further transferred to the Golgi apparatus which is the center for protein sorting.

Keywords: Botulinum toxin; Endocytosis; Mucin; Clathrin; Caveolae; HT-29 cell

1. Introduction

Clostridium botulinum has been classified into seven types, A to G, which are differentiated by the immunological specificity of the neurotoxin (NT) produced. The molecular masses of all types of these neurotoxins are approximately 150 kDa. In the type C strain, two different sized progenitor toxins with molecular masses of 500 kDa (C16S) and 300 kDa (C12S) are produced. The C12S toxin consists of a NT and a non-toxic component having no hemagglutinin (HA) activity that is designated as non-toxic non-HA. The C16S toxin consists of C12S toxin and four kinds of different subcomponents (HAs): HA1, HA2, and HA3a, and HA3b provided processing by protease after translation of HA3. Hemagglutinin activity of HAs was shown in previous reports [1–6].

NT is a high potent inhibitor of the neurotransmitter release from the peripheral nerve terminus. Once localized in the cytoplasm of nerve cells, the NT functions by specifically cleaving one of three different SNARE proteins essential for synaptic vesicle fusion [7,8]. The non-toxic components (non-toxic non-HA, HA1, HA2, HA3a and HA3b) are presumed to have the role of protecting the neurotoxin against acidity and

Abbreviations: C16S toxin, *Clostridium botulinum* type C progenitor toxin; HA, hemagglutinin; NT, neurotoxin; mβ-CD, methyl-β-cyclodextrin; TGN, trans-Golgi network

* Corresponding author. Department of Applied Biological Science, and Department of Biotechnology, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, 183-8509 Tokyo, Japan. Tel.: +81 42 367 5905; fax: +81 42 367 7162.
E-mail address: nishikaw@cc.tuat.ac.jp (A. Nishikawa).

0167-4889/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2005.11.014

proteases in the digestive tract [9]. The HAs function as an adhesion in the attachment of 16S toxin to the microvilli of the intestine of guinea pig [10]. In type A and C, HA1 and HA3b bind to each terminal galactose and sialic acid moieties of sugar chain [9,11]. However, the internalization pathway into epithelial cells and its mechanism are still unknown.

Oligosaccharides are involved in a wide range of biological processes, for example, bacterial and viral infection, cancer metastasis, the blood-clotting cascade and many other crucial intercellular recognition events. To express their function, oligosaccharides occur as glycoconjugates attached to proteins or lipids that are found on the surface of the cells [12]. Mucins are high molecular-weight glycoproteins that consist of oligosaccharides attached to serine or threonine residues of the mucin core protein backbone by O-glycosidic linkages. Mucin peptides are biosynthesized in the endoplasmic reticulum (ER) and transported to the Golgi apparatus. N- and O-glycosylated mucins are secreted into the plasma membrane via the trans-Golgi network. Mucin on the cell surface that is not completely glycosylated, referred to as premature mucin, is internalized via clathrin-mediated endocytosis and again localized in the Golgi apparatus. After further O-glycosylation, the mucin is resecreted onto the apical membrane [13,14]. Mucins are expressed on the surface of the gastrointestinal track in the human body to protect epithelial cells from pathogens and digestive enzymes [15–17]. Human adenocarcinoma HT-29 cells are epithelial-like and moderately well-differentiated cells [18] and express several kinds of mucins [19,20]. We previously reported that the receptor on the HT-29 cell surface targeted by the C16S toxin is an O-linked sugar chain of mucin-like glycoproteins [21]. Therefore, it is hypothesized that the C16S toxin is internalized with mucin-like glycoprotein via clathrin-mediated endocytosis. This paper reports the identification of the endocytic pathway of C16S toxin into HT-29 cells through confocal laser-scanning microscopy using antibodies specific to the clathrin heavy chain, EEA1 and caveolin-1. It was found that the C16S toxin is internalized into HT-29 cells via both clathrin-coated pits and caveolae. Furthermore, neurotoxin is transferred to Golgi apparatus.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle Medium (DMEM), minimum essential medium (MEM), Dulbecco's phosphate-buffered saline (PBS), methyl-β-cyclodextrin (mβ-CD) and transferrin were all purchased from Sigma. Bafilomycin A_1 was purchased from Wako Pure Chemical Industries (Japan). Acridine orange was purchased from Molecular Probes. The ECL Plus Western Blotting Detection System was obtained from Amersham Biosciences. All other chemicals were purchased from Sigma.

2.2. Toxins and antibodies

Type C 16S toxin was prepared from the culture fluid of *C. botulinum* type C strain Stockholm according to a procedure described previously [10]. Rabbit antiserums against the type C non-toxic (anti-C non-toxic) component [22], type C NT (anti-C ST HC) [23] and transferrin, all previously prepared, were used. Mouse monoclonal antibody against the type C HA1 component (CHA1-489) and HA3b component (CHA3b-6) was prepared previously [24]. Rabbit polyclonal antibody against caveolin and mouse monoclonal antibody against human clathrin heavy chain, caveolin-1 and EEA1 were purchased from BD Transduction Laboratories. Monoclonal antibody against LAMP-1 (CD107a) was purchased from BD Bioscience Pharmingen. Monoclonal antibody against human PDI and TGN38 were purchased from Affinity BioReagents. Rabbit polyclonal antibody against Src-family kinases (c-Src) was obtained from Santa Cruz Biotechnology. Tetramethylrhodamine anti-rabbit IgG (H+L) and Alexa Fluor 488 anti-mouse IgG (H+L) were purchased from Molecular Probes.

2.3. Cell culture

Human colon carcinoma cell line HT-29 was purchased from the American Type Culture Collection. The cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 0.1 mg/ml kanamycin (FBS/DMEM) at 37 °C in a 5% CO_2 atmosphere.

2.4. Cholesterol depletion

HT-29 cells were grown in 8-well chamber slides (Nalge Nunc International). The cells were washed twice with fresh MEM without FBS and incubated with 10 mM mβ-CD in MEM at 37 °C for 30 min in a 5% CO_2 atmosphere [25].

For the examination of endocytosis recovery, the mβ-CD-treated cells were washed twice with fresh MEM without mβ-CD and incubated in DMEM with 10% FBS at 37 °C for 90 min in a 5% CO_2 atmosphere [26].

2.5. Confocal laser-scanning microscopy

HT-29 cells grown in 8-well chamber slides were washed twice with fresh DMEM (pH 7.0) and incubated with C16S toxin (final concentration, 40 nM) or transferrin (final concentration, 0.017 mg/ml) in DMEM (pH 7.0) at 37 °C for 5 min in a 5% CO_2 atmosphere. After incubation, the cells were immediately washed with DMEM (pH 7.0) and incubated at 37 °C in a 5% CO_2 atmosphere for various times. The cells were then washed with PBS twice, fixed with 3.7% formaldehyde for 15 min, quenched with 50 mM NH_4Cl for 15 min, and then permeabilized with 0.1% saponin in 2% BSA and PBS for 1 h at room temperature. All antibodies were diluted with 0.1% saponin, 2% BSA in PBS (dilution buffer) and incubated at room temperature. The permeabilized cells were incubated with primary antibodies (anti-C non-toxic, anti-C ST HC, anti-transferrin, anti-caveolin, or c-Src; diluted 1:200, CHA1-489; 1.6 mg/ml, CHA3b-6; 2.4 mg/ml, anti-clathrin heavy chain, anti-ceveolin-1 or anti-EEA1; 25 μg/ml, anti-TGN38 and anti-PDI; diluted 1:10, anti-LAMP-1; 12.5 μg/ml) for 1 h and washed three times with dilution buffer. The cells were then incubated with a secondary antibody, tetramethylrhodamine-conjugated anti-rabbit IgG (H+L) (0.01 mg/ml), for 1 h and then washed three times. After treatment with a third antibody, Alexa Fluor 488-conjugated anti-rabbit IgG (H+L) (0.01 mg/ml), the cells were mounted with Vectashield mounting medium (H-1000). Confocal images were collected a lot from the top to the bottom of the cells using a Leica TCS 4D laser-scanning confocal microscope. The images used in this study were from the middle of the cells.

2.6. Detection of toxin receptor by far Western blotting analysis

HT-29 cells were grown to about 90% confluency in a dish, some of which were then treated with 10 mM mβ-CD for 30 min. After this treatment, the cells were washed twice with PBS, harvested, and lysed on ice for 20 min with 1% Triton X-100 in 25 mM MES buffer (pH 6.5) containing 1 mM PMSF and 150 mM NaCl (MES buffer), followed by homogenization with a Potter-type homogenizer. The homogenized cells were then mixed with an equal volume of 80% sucrose. The homogenate was then overlaid with 1.5 ml of 30% sucrose and 0.5 ml of 5% sucrose in MES buffer and centrifuged through a step density gradient at 200,000×*g* for 18 h at 4 °C in a TLA-100.3 rotor (Beckman). After centrifugation, each 0.3 ml fraction was collected from the top. The Triton X-100-soluble and -insoluble fractions were