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Intracellular trafficking of *Clostridium botulinum* C2 toxin

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ABSTRACT

Clostridium botulinum C2 toxin is a binary toxin composed of an enzymatic component (C2I) and binding component (C2II). The activated binding component (C2IIa) forms heptamers and the oligomer with C2I is taken up by receptor-mediated endocytosis. We investigated the intracellular trafficking of C2 toxin. When MDCK cells were incubated with C2I and C2IIa at 37 °C, C2I colocalized with C2IIa in cytoplasmic vesicles at 5 min, and C2I then disappeared (15 min incubation and later), and C2IIa was observed in the vesicles. Internalized C2I and C2IIa were transported to early endosomes. Some of both components were returned to the plasma membrane through recycling endosomes, whereas the rest of C2IIa was transported to late endosomes and lysosomes for degradation. Bafilomycin A1, an endosomal acidification inhibitor, caused the accumulation of C2IIa in endosomes, and both nocodazole and colchicine, microtubule-disrupting agents, restricted C2IIa's movement in the cytosol. These results indicated that an internalized C2I and C2IIa complex was delivered to early endosomes, and that subsequent delivery of C2I to the cytoplasm occurred in early endosomes. C2IIa was either sent back to the plasma membranes through recycling endosomes or transported to late endosomes and lysosomes for degradation.
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1. Introduction

Clostridium botulinum produces botulinum C2 toxin, which recruits a binding component (C2II) to deliver the enzymatic component (C2I) to the interior of eukaryotic cells (Barth et al., 2004; Aktories and Barbieri, 2005; Aktories et al., 2012). Each protein has been reported to lack toxic activity when injected alone (Barth et al., 2004). These proteins act in binary combinations to produce toxic, cytotoxic, and lethal effects, and influence vascular permeability (Barth et al., 2004). C2I ADP-ribosylates monomeric actin at arginine-177 in the cytosol (Aktories et al., 1986; Wiegers et al., 1991). This ADP-ribosylation causes the breakdown of F-actin, leading to cell rounding and death. C2II cleavage by trypsin was shown to remove the N-terminal 20 kDa fragment, leading to the activation of C2II (C2IIa) (Barth et al., 2004). C2 toxin belongs to a family of binary actin-ADP-ribosylating toxins that includes *Clostridium perfringens* iota-toxin (Ia, an enzymatic component and Ib, the binding component), *Clostridium spiroforme* iota-like toxin, *Clostridium difficile* ADP-ribosyltransferase, and vegetative insecticidal protein from *Bacillus cereus* (Barth et al., 2004; Aktories and Barbieri, 2005; Aktories et al., 2012).

Abbreviations: Bafilomycin A1, BFA; DAPI, 4′,6-diamidino-2-phenylindole; DEME, Dulbecco's modified Eagle medium; EEA1, early endosome antigen 1; ER, endoplasmic reticulum; GFP, green fluorescent protein; Lamp2, lysosomal-associated membrane protein 2; MDCK cell, Madin–Darby canine kidney cell.

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C2IIa recognizes asparagine-linked carbohydrates on the surface of target cells and forms heptamers that bind C2I (Eckhardt et al., 2000). The toxin-receptor complex was shown to be internalized by receptor-mediated endocytosis and translocated to early endosomes (Barth et al., 2000; Pust et al., 2010). At the acidic pH of the endosomal compartment with vesicular H^{+}-ATPase, the C2IIa oligomer was reported to be inserted into the endosomal membrane and formed pores, through which bound C2I was then translocated into the cytosol (Barth et al., 2000). C2IIa pores are essential for the translocation of C2I across endosomal membranes (Blöcker et al., 2003a, 2003b) and C2I translocates in an unfolded conformation through these pores into the cytosol (Haug et al., 2003). Membrane translocation of C2I was shown to be facilitated by the chaperone heat shock protein (Hsp) 90 (Haug et al., 2003) and folding helper enzyme cyclophilin A (CyPA) (Kaiser et al., 2009). CyPA interacts with C2I in intact cells (Kaiser et al., 2009) and Hsp90 directly binds to C2I (Kaiser et al., 2011). Kaiser et al. (2012) also demonstrated that the FK506-binding protein plays a role during membrane translocation of C2I. After translocation to the cytosol, C2I ADP-ribosylates G-actin in the cytosol. This event subsequently causes the depolymerization of actin filaments, breakdown of the actin cytoskeleton, and rounding of the cells (Barth et al., 2004; Aktories and Barbieri, 2005; Aktories et al., 2012). C2IIa and iota-toxin b were shown to induce endocytosis of their receptors via a lipid raft-mediated process (Nagahama et al., 2004, 2009, 2012). However, the endosomal trafficking pathway of internalized C2 toxin is unknown. MDCK cells provide a good model system to study the binding and internalization of C2 toxin (Nagahama et al., 2009, 2012). In this study, we examined the intracellular trafficking of C2 toxin using MDCK cells.

2. Materials and methods

2.1. Materials

Recombinant C2I and C2II were expressed, fused with glutathione S-transferase (GST) in *Escherichia coli* BL21, as described previously (Nagahama et al., 2009). To obtain C2IIa, C2II was activated by incubation with trypsin, as described previously (Nagahama et al., 2009). Rabbit anti-C2I and anti-C2II antibodies were prepared as described previously (Nagahama et al., 2009). Bafilomycin A1 (BAF), nocodazole, colchicine, mouse anti-Golgi 58K antibody, and anti-mouse IgG–fluorescein isothiocyanate (FITC) were obtained from Sigma (St. Louis, MO). Mouse anti-early endosome antigen 1α (anti-EEA1α) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the mouse anti-lysosome-associated membrane protein 2 (anti-Lamp2) antibody was obtained from AbD Serotec (Oxford, United Kingdom). Dulbecco's modified Eagle's medium (DEME) and Hanks' balanced salt solution (HBSS) were obtained from Gibco BRL (New York, NY). Alexa Fluor 568-conjugated goat anti-rabbit IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG, CellLights lysosome-green fluorescent protein (GFP), CellLights endoplasmic reticulum (ER)-GFP, and 4′,6′-diamino-2-phenylindole (DAPI) were obtained from Molecular Probes (Eugene, OR). The expression vector for GFP-Rab11 was prepared as described previously (Nagahama et al., 2012).

2.2. Cell culture and assay of cytotoxicity

Madin–Darby canine kidney (MDCK) cells were obtained from the Riken Cell Bank (Tsukuba, Japan). Cells were cultured in DEME supplemented with 10% fetal calf serum (FCS), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine (FCS-DEME). All incubation steps were carried out at 37 °C in a 5% CO_2 atmosphere. Cells for cytotoxicity assays were inoculated in FCS-DEME on 48-well tissue culture plates (Falcon, Oxnard, CA). Various concentrations of C2I and C2IIa were mixed in FCS-DEME and inoculated onto cell monolayers. Cells were observed for morphological alterations 4 h after inoculation, as described previously (Nagahama et al., 2002). To measure the effects of BAF, nocodazole, and colchicine on the cytotoxicity of C2 toxin, MDCK cells were preincubated with these agents at 37 °C for 1 h and then incubated with C2I and C2IIa at 37 °C for 4 h.

2.3. Fluorescence labeling of C2I and C2IIa and confocal fluorescence microscopy

Fluorescent C2I and C2IIa were labeled with Cy3 (GE Healthcare) and Alexa488 (Invitrogen) according to the manufacturer's recommendations. No significant loss of biological activity was observed with fluorescent C2I and C2IIa as discerned by the cytotoxicity assay. MDCK cells were plated on a polylysine-coated glass-bottomed dish (Matsunami, Osaka, Japan) and incubated at 37 °C in a 5% CO_2 incubator overnight in FCS-DEME. Cells were washed twice with PBS and then stained with Hoechst 33258 solution (5 μg/ml) at 37 °C for 10 min. After cells were washed again twice with PBS, they were incubated with Cy3-C2I and Alexa488-C2IIa for the indicated time periods at 37 °C. These cells subsequently were fixed with 4% paraformaldehyde at room temperature for 10 min, and free radicals were quenched by incubation with 50 mM NH_4Cl in PBS. After washing in PBS, cells were observed using a Nikon A1 laser scanning confocal microscope (Tokyo, Japan).

2.4. Immunofluorescence analysis

Cells were plated on a polylysine-coated glass-bottomed dish as described above. To study the internalization of C2IIa, C2IIa (1 μg/ml) was incubated with cells at 4 °C for 1 h in FCS-DEME. After three washes in cold FCS-DEME, cells were transferred to FCS-DEME prewarmed to 37 °C and incubated at the same temperature for various periods. They were washed four times with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature. Dishes were incubated at room temperature for 15 min in 50 mM NH_4Cl in PBS and in PBS containing 0.1% Triton X-100 at room temperature for 20 min for antibody labeling. After being washed with PBS containing 0.02% Triton X-100, the dishes were incubated at room temperature for 1 h with PBS containing 4% BSA, followed by the

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