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Conformational divergence in the HA-33/HA-17 trimer of serotype C and D botulinum toxin complex

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ABSTRACT

Clostridium botulinum produces a large toxin complex (L-TC) comprising botulinum neurotoxin associated with auxiliary nontoxic proteins. A complex of 33- and 17-kDa hemagglutinins (an HA-33/HA-17 trimer) enhances L-TC transport across the intestinal epithelial cell layer *via* binding HA-33 to a sugar on the cell surface. At least two subtypes of serotype C/D HA-33 exhibit differing preferences for the sugars sialic acid and galactose. Here, we compared the three-dimensional structures of the galactose-binding HA-33 and HA-33/HA-17 trimers produced by the C-Yoichi strain. Comparisons of serotype C/D HA-33 sequences reveal a variable region with relatively low sequence similarity across the *C. botulinum* strains; the variability of this region may influence the manner of sugar-recognition by HA-33. Crystal structures of sialic acid- and galactose-binding HA-33 are broadly similar in appearance. However, small-angle X-ray scattering revealed distinct solution structures for HA-33/HA-17 trimers. A structural change in the C-terminal variable region of HA-33 might cause a dramatic shift in the conformation and sugar-recognition mode of HA-33/HA-17 trimer.

1. Introduction

Food-borne botulism is caused by the botulinum neurotoxin (BoNT), produced by the gram-positive bacterium *Clostridium botulinum*. The BoNT is antigenically classified into seven serotypes, labeled A–G [1]. Serotypes A, B, E, and F are predominantly associated with human diseases, whereas serotypes C and D cause botulism in birds and other animals [2]. In bacterial culture and in contaminated food, the BoNT is bound to nontoxic proteins; this assemblage constitutes the toxin complex (TC). Orally ingested TC passes through the gastrointestinal tract, and reaches the small intestine, where it enters the bloodstream. Ultimately, BoNT dissociates from TC and penetrates nerve cells, where it uses zinc-dependent proteolysis to cleave specific sites on a target protein. These target sites are involved in the release of neurotransmitters, a process that causes muscular paralysis in humans and other animals [3].

Free from TC, BoNT is susceptible to digestive degradation into small inactive fragments; unbound BoNT exerts relatively low oral toxicity [4]. In contrast, TC-associated BoNT is stable under digestive conditions, and exhibits heightened oral toxicity. *C. botulinum* produces several types of TCs with different molecular sizes. The M-TC complex is a single molecule of BoNT bound to a single molecule of nontoxic nonhemagglutinin (NTNHA). Further, M-TC association with 70, 33, and 17-kDa hemagglutinin (HA) molecules (HA-70, HA-33, and HA-17, respectively) produces L-TC (Fig. 1A) [5]. Initially, M-TC association with three HA-70 molecules yields M-TC/HA-70. Further M-TC/HA-70 association with HA-33/HA-17 trimers, comprised of two HA-33 proteins and one HA-17 protein, produces 1-arm L-TC (M-TC/HA70 + single HA-33/HA-17 trimer), 2-arm L-TC (M-TC/HA-70 + two HA-33/HA-17 trimers), and finally, 3-arm L-TC

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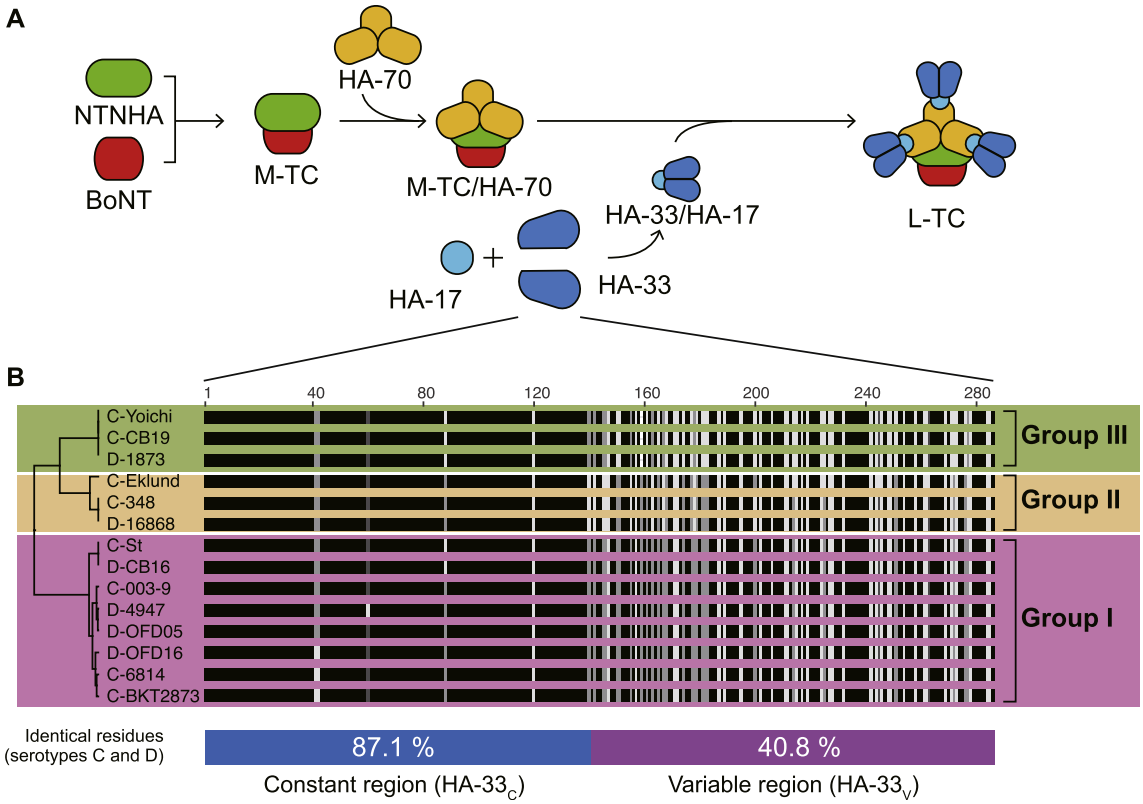

Fig. 1. Serotype C and D HA-33 proteins in botulinum toxin complex (TC), and their primary structures. (A) Assembly pathway of botulinum TC. BoNT and NTNHA bind, forming M-TC. Attachment of HA-70 to M-TC yields M-TC/HA-70. Otherwise, association of a single HA-17 protein and two HA-33 proteins forms a HA-33/HA-17 trimer. Binding of HA-33/HA-17 trimer to M-TC/HA-70 forms mature 14-mer L-TC. (B) Multiple alignments of the amino acid sequences of serotype C and D HA-33 proteins. This alignment was generated using ClustalW. The black boxes indicate identical residues among all sequences. Light gray and dark gray boxes indicate 60%–80% similarity and 80%–99% similarity among all sequences, respectively. The phylogenetic tree was generated using the Jukes-Cantor genetic distance model, and the UPGMA tree building method. Based on the amino acid sequence similarities, the N- and C-terminal halves of the protein were designated *constant region* (HA-33c) and *variable region* (HA-33v), respectively.

(M-TC/HA-70 + three HA-33/HA-17 trimers) [6]. In a cell-layer transport test using IEC-6 rat intestinal epithelial cells, all TC intermediates, including free BoNT, transport from the apical to the basal side of the cell layer [7]. However, TC transport efficiency through the cell layer depends on the number of HA-33/HA-17 trimers. Although not strictly necessary, the HA-33/HA-17 trimer facilitates transport of TC across the intestinal epithelial cell layer.

Among serotypes C and D, the nontoxic TC proteins are highly similar in structure and function; although BoNT exhibits only 52% amino acid sequence identity and cleaves a distinct target protein in nerve cells [8–11]. Both C and D serotype L-TC causes equine erythrocytes to aggregate. This erythrocyte aggregation is inhibited by the presence of *N*-acetylneuraminic acid (Neu5Ac) [12]. Hemagglutination is also inhibited by the pre-treatment of erythrocytes with neuraminidase (NDase), which removes their sialic-acid moieties. Serotype C and D HA-33 binds to erythrocyte and IEC-6 cells; this binding is inhibited by Neu5Ac, but not galactose or lactose. Therefore, it appears that C and D serotype L-TC preferentially recognizes sialic acid on cell surfaces *via* its HA-33 molecules.

We recently identified unusual HA-33 proteins produced by serotype C strain Yoichi [13] and D strain 1873 [14] that preferentially recognize galactose, rather than sialic acid, on erythrocyte and IEC-6 cells. *In silico* screening using the amino acid sequences of the C-Yoichi and D-1873 HA-33 indicated that the unique HA-33 is spread among the serotype C and D strains independently of their BoNT serotype [14]. The three-dimensional structures for sialic acid-binding HA-33 [15] and HA-33/HA-17 trimer [6] had been previously determined using serotype C and D strains. Here, we compared the three-dimensional structures of the galactose-binding HA-33 and HA-33/HA-17 trimers produced by the C-Yoichi strain.

2. Materials and methods

2.1. Production and purification of botulinum L-TC and isolation of HA-33/HA-17 trimer

C. botulinum serotype C strain Yoichi (C-Yoichi) was cultured using a dialysis method reported previously [13]. L-TC complex was precipitated from culture supernatant with 60% saturation of ammonium sulfate. The precipitate was dialyzed against 50 mM acetate buffer (pH 7.4) containing 0.2 M NaCl, and applied to a dialysate-equilibrated TOYOPEARL SP-650S column (Tosoh, Tokyo, Japan). Bound protein was eluted using a linear gradient of NaCl (0.2–0.8 M). The eluent containing L-TC (detected by SDS and native PAGE) was collected, concentrated with 80% saturation of ammonium sulfate, and further purified on a HiLoad 16/60 Superdex 200 pg column (GE Healthcare, Little Chalfont, UL). This column had been equilibrated with 50 mM acetate buffer (pH 5.0) containing 0.15 M NaCl. The elution fraction containing L-TC was precipitated with 80% saturation of ammonium sulfate.

Isolation of HA-33/HA-17 trimer from L-TC was performed as previously reported [16]. Purified L-TC (250 mg) was incubated in 20 mM Tris–HCl (pH 7.8) containing 4.0 M guanidine hydrochloride (Gdn buffer) at 21 °C for 4 h. Treated sample was applied to a HiLoad Superdex 200 pg 16/60 gel-filtration column equilibrated

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