Structure of a Bimodular Botulinum Neurotoxin Complex Provides Insights into Its Oral Toxicity

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Abstract

Botulinum neurotoxins (BoNTs) are produced by Clostridium botulinum and cause the fatal disease botulism, a flaccid paralysis of the muscle. BoNTs are released together with several auxiliary proteins as progenitor toxin complexes (PTCs) to become highly potent oral poisons. Here, we report the structure of a ~760 kDa 14-subunit large PTC of serotype A (L-PTC/A) and reveal insight into its absorption mechanism. Using a combination of X-ray crystallography, electron microscopy, and functional studies, we found that L-PTC/A consists of two structurally and functionally independent sub-complexes. A hetero-dimeric 290 kDa complex protects BoNT, while a hetero-dodecameric 470 kDa complex facilitates its absorption in the harsh environment of the gastrointestinal tract. BoNT absorption is mediated by nine glycan-binding sites on the dodecameric sub-complex that forms multivalent interactions with carbohydrate receptors on intestinal epithelial cells. We identified monosaccharides that blocked oral BoNT intoxication in mice, which suggests a new strategy for the development of preventive countermeasures for BoNTs based on carbohydrate receptor mimicry.

Introduction

The seven botulinum neurotoxin serotypes (BoNTs) produced by Clostridium botulinum are the causative agents of the neuroparalytic syndrome of botulism and pose a serious threat for bioterrorism [1]. Conversely, BoNT/A is a highly effective therapy for treating neurological disorders [2]. The naturally occurring BoNTs are released together with up to four non-toxic neurotoxin-associated proteins (NAPs) (also called associated non-toxic proteins, ANTPs) in the form of progenitor toxin complexes (PTCs) with different molecular compositions [3]. Such PTCs are highly potent food poisons, e.g., the PTC of BoNT/A displays an oral LD50 of ~55 μg/kg body weight [4]. While BoNT is sensitive to denaturation by the acidic environment and digestive proteases present in the gastrointestinal (GI) tract [5], the PTCs of different serotypes exhibit ~360-16,000-fold greater oral toxicity than free BoNT [4,6,7,8]. The NAPs are encoded together with the bont gene in one of two different gene clusters, the HA cluster or the orfX cluster [9]. Both clusters encode the non-toxic non-hemagglutinin (NTNHA) protein, which adopts a BoNT-like structure despite its lack of neurotoxicity [5]. The HA gene cluster also encodes three hemagglutinins (HA70, HA17, and HA33; also called HA3, HA2, and HA1, respectively), which together with BoNT and NTNHA constitute the large PTC (L-PTC) [10]. The structure and function of the corresponding orfX proteins are largely unknown [11].

Structural information of HAs is available for serotypes C and D, such as the crystal structures of HA33 of serotype C (HA33-C) [12,13], a complex composed of HA17 and HA33 of serotype D [14], and HA70 of serotype C (HA70-C) [15,16]. However, BoNT/C and D rarely cause human botulism but are known to cause the syndrome in cattle, poultry, and wild birds. For BoNT/A, the major cause of human botulism, only the structure of HA33 (HA33-A), which displays an amino-acid identity of ~38% to HA33-C and D, has been solved [17]. We have recently determined the crystal structure of the BoNT/A–NTNHA...
complex [5]. However, it remains largely unclear how the HAs assemble with one another and how they interact with BoNT and NTNHA.

Various structural models have been proposed for the L-PTC. One recent paper suggested a complex composed of BoNT:NTNHA:H70:HA17:HA33 in a 1:2:2:3 ratio for L-PTC/A [18], whereas earlier studies suggested a stoichiometry of 1:3:5-5:6-8-9 or 1:1:3:3:4 for L-PTC/A, or 1:1:2:4 for L-PTC/D [19,20,21]. In comparison, electron microscopy (EM) studies on L-PTC/A, B and D supported a stoichiometry of 1:1:3:3:6 [14,22].

The functional roles of NAPs are also not well defined. We have recently shown that NTNHA shields BoNT against low-pH denaturation and proteolytic attack in the GI tract by forming the minimally functional PTC (M-PTC), and releases it during entry into the general circulation [5,23]. However, it is not clear whether HAs further protect the toxin. At the same time, the L-PTC may contribute to BoNT internalization into the host bloodstream through interactions with intestinal cell surface glycans [24,25,26]. The HAs of BoNT/A and B could disrupt the human epithelial intercellular junction through species-specific interaction with E-cadherin, presumably facilitating BoNT transport via the paracellular route [27,28,29].

Defining the L-PTC structure would permit a more complete understanding of the complex's role in toxin shielding and delivery, and would help to describe the molecular mechanism underlying these important actions.

Here, we report the structure of a ~760 kDa L-PTC/A using a combination of X-ray crystallography, single-particle EM and three-dimensional reconstruction (3D-EM). We found that L-PTC/A consists of two structurally and functionally independent sub-complexes, the M-PTC and the HA complex. The HA complex is composed of HA70, HA17, and HA33 in a 3:3:6 stoichiometry and adopts an extended three-blade architecture, whereas the M-PTC is situated on top of the HA complex.

Structure of the HA complex

We next separated the HA complex into two major components: the central hub composed of homo-trimeric HA70 and the blade composed of HA70(93)-HA17–HA33. Their crystal structures were determined at 2.9 Å and 3.7 Å, respectively (Fig. 2C–D, Table S2 in Text S1, Fig. S3–S4 in Text S1). We also obtained a high-resolution structure of the blade by combining the structures of HA70(93)-HA17 and HA17–HA33, which were determined at 2.4 Å and 2.1 Å, respectively (Fig. 2A–B and Fig. S5–S6 in Text S1). Each HA adopts an almost identical conformation in the independently solved structures, despite differences in crystal packing, suggesting that they represent physiologically relevant conformations.

HA70 consists of three domains (D1–3) (Fig. S3 in Text S1). The D1 and D2 domains, which adopt similar structures, mediate the trimerization of HA70 with each protomer contributing ~3,100 Å² of solvent-accessible area for interactions. The D3 domain, sitting at the tip of the trimer, is composed of two similar
jelly-roll-like β-sandwich structures. The linker between D1 and D2 (residues Thr190–Ser205) is degraded and not visible in the crystal structure, which is reminiscent of the post-translational nicking of HA70 into ~25 and ~45 kDa fragments that occurs physiologically [20].

HA17 has a compact β-trefoil fold and connects HA70 and HA33. Based on the crystal structure of the HA70D3–HA17 complex, the interactions between HA70 and HA17 bury a solvent-accessible area of ~795 Å² (per molecule) (Fig. 3A and Fig. S5 in Text S1). The structure of HA70D3 is almost identical to its equivalent domain in the full-length HA70 with a root-mean-square deviation (rmsd) of ~0.93 Å over 232 Cα atoms. The major HA70–HA17 interactions are composed of 13 pairs of hydrogen bonds and salt bridges. In addition, HA70-Phe547 is buried in a hydrophobic region in HA17 composed of Ile18, Ile92, Ala93, Thr96, and Met140 (Fig. 3A and Fig. S5 in Text S1).

HA17 simultaneously binds to two HA33 molecules that form a dumbbell-like shape composed of two β-trefoil domains linked by an α-helix. The two pairs of HA17–HA33 interfaces bury a solvent-accessible area of ~666 Å² and ~410 Å² (per molecule), respectively (Fig. 3B and Fig. S6 in Text S1). The two HA33-binding interfaces on HA17 are adjacent but non-overlapping. HA17 contributes seven and four pairs of hydrogen bonds and salt bridges to bind the two HA33 molecules, respectively. Complementing these hydrophilic interactions, the two HA33s contain a hydrophobic surface (Trp75/Leu116/Leu129) that interacts with two neighboring hydrophobic patches on the HA17 surface (Phe75/Pro125/Lys127 and Leu108/Pro130/Phe132) (Fig. 3B).

The two molecules of HA33 in each blade of the HA complex are almost identical (rmsd of ~0.35 Å over 286 Cα atoms) and bury a solvent-accessible area of ~961 Å² (per molecule) between them (Fig. 3C). All the interacting residues are in the N-terminal domain of HA33, whereas the interface consists of hydrophilic interactions on the periphery and a hydrophobic core in the center (Fig. S6C in Text S1). Due to the two-fold symmetry between the two molecules, intra-HA33 interactions are generally symmetric.

Finally, we assembled the subunit crystal structures to create a complete structure of the HA complex (Fig. 2E). The 12-subunit
HA complex is stabilized by numerous protein–protein interactions that include interactions among the HA70s of the central trimer, between HA70 and HA17, between HA17 and the two HA33 molecules, and between the two HA33s in each blade. The assembled HA complex structure could be unambiguously docked into the 3D-EM density of the native L-PTC/A (correlation coefficient, CC 87.7%) (Fig. 1), whereas a small difference was observed in the C-terminal domain of HA33 due to its structural flexibility. We also performed an independent 3D-EM reconstruction of our recombinant, in vitro-reconstituted HA complex at 31 Å resolution (CC 93.1%) (Fig. 2E), and found it to be almost identical to the HA complex present in the L-PTC.

The bimodular architecture of the L-PTC

The crystal structure of the M-PTC was unambiguously docked into the 3D-EM density of the native L-PTC (CC 87.3%), which is situated on top of the HA complex, yielding a 760 kDa 14-subunit complex (Fig. 1 and Fig. S2 in Text S1). BoNT/A interacts with the HAs only through its receptor-binding domain (HC domain). The interface is likely composed of Gly399 and Ile400 in HA70 and Val1128, Gly1129, Gln1210, Pro1212, and Asp1213 in HC (pairwise Cα–Cα distance within 15 Å) (Fig. 1D and Fig. S7A in Text S1). Gly399 and Ile400 of HA70 are located in a loop that has weak electron density in the crystal structures, suggesting high flexibility. Moreover, the potentially interacting residues in HC are located in two flexible loops and not conserved among various BoNT serotypes (Fig. S7B in Text S1). Thus, the BoNT/A–HA70 interface in the L-PTC may be formed by induced fit.

The major interface between the M-PTC and the HAs is mediated by NTNHA. The potential interface residues in NTNHA, which are within 12 Å Cα–Cα distance of the HAs, are located in loop Gly308–Gly313 and the residues flanking loop Gly116–Ala148 (nLoop) [5]. The corresponding interface residues in the HA complex are located around the center of the HA70 trimer (Fig. 1D). The nLoop displays no visible electron density in the structure of the M-PTC and is spontaneously nicked in the free NTNHA or the M-PTC [5,31,32,33,34]. However, the nLoop remains intact in the L-PTC, suggesting it may be buried by the HA complex [30,31,35]. We found that the synthetic nLoop peptide binds to HA70 with high affinity (Kd 340 nM) at a stoichiometry of one nLoop to one HA70 trimer (Table S3 in Text S1).
Intestinal absorption of BoNT, and acts by compromising the functional unit of the L-PTC that facilitates the transport of BoNT across the intestinal wall. For this study, we used the well-characterized intestinal epithelial cell line Caco-2. Although derived from a human colon adenocarcinoma, Caco-2 cells differentiate to form a polarized epithelial cell monolayer that provides a physical and biochemical barrier to the passage of ions and small molecules, resembling the uptake and barrier properties of the small intestinal epithelial layer [36, 37, 38, 39]. Caco-2 cells have been extensively used to investigate their permeability upon infection, e.g. by rotavirus [40] or enteropathogenic E. coli [41], and transcytosis upon intoxication with cholera toxin [42] or BoNT [43, 44, 45]. Furthermore, it was demonstrated that the transepithelial electrical resistance (TER) of Caco-2 cell monolayers was reduced by the L-PTC of BoNT/A and B. Although the mechanism by which this may occur is unclear, BoNT absorption has been proposed to occur via the paracellular route [27, 28, 29].

We found that treatment of Caco-2 cells with the in vitro-reconstituted HA complex markedly reduced the TER. This effect was more marked when the HA complex was applied to the cell monolayer from the basolateral side than from the apical side, which needed ~17 nM and ~58 nM to achieve a 90% and 70% decrease in TER after 12 hours, respectively (Fig. S9A–B in Text S1). Remarkably, the potency of the isolated HA complex was similar to that of the intact L-PTC (Fig. 4A–B). In contrast, there was no effect on Caco-2 TER by BoNT/A, NTNHA, the M-PTC, or by the subunits of the HA complex, including HA70, HA33, the HA17–HA33 complex, and the mini-HA complex (Fig. 4C–D). Taken together, these data suggest that the fully assembled HA complex is the functional unit of the L-PTC that facilitates intestinal absorption of BoNT, and acts by compromising the integrity of the epithelial cell layer.

The HA complex interacts with carbohydrates

Many human receptors for microbial pathogens or toxins are glycoconjugates. The L-PTC is known to initiate toxin absorption by binding to intestinal cell surface glycans [24, 25, 26]. We therefore performed a comprehensive thermodynamic analysis to characterize the interactions between HAs and several common monosaccharides and oligosaccharides (Fig. S10 and Table S3 in Text S1). We found that HA33 bound to lactose (Lac), N-acetylactosamine (LacNac), and galactose (Gal) with dissociation constants ($K_d$) of ~1.0 mM, ~1.0 mM, and ~1.8 mM, respectively, and that it also bound to isopropyl β-D-1-thiogalactopyranoside (IPTG) [46], a non-metabolizable galactose analog, with a $K_d$ of ~0.8 mM. HA70 bound to α2,3- and α2,6-sialylactose (SiaLac), both with a $K_d$ of ~0.5 mM, and displayed a lower affinity for N-acetylneuraminic acid (Neu5Ac) ($K_d$ ~7.8 mM). There was no overlap between the carbohydrate selectivity of HA70 and HA33.

To determine the physiological relevance of these HA–glycan interactions during toxin absorption, we examined their ability to interfere with the HA complex-mediated disruption of Caco-2 TER. Lac, Gal, and IPTG markedly inhibited the TER reduction induced by the HA complex, and showed higher potencies when applied to the apical than to the basolateral compartment (Fig. 5A–B and Fig. S11A–D in Text S1). In contrast, α2,3- and α2,6-SiaLac, and to a lesser extent Neu5Ac, inhibited the decrease in TER only when applied apically, albeit more weakly than Lac (Fig. 5A–B and Fig. S11E–F in Text S1). We then examined the transport of the HA complex across the Caco-2 monolayer using a fluorescence-labeled HA complex (HA*) (Fig. 5C). Lac and IPTG efficiently inhibited the transport of HA* when it was applied to the apical or basolateral chamber. Blocking the transport of HA* via α2,3- and α2,6-SiaLac was more potent toward the basolateral compartment than toward the apical side. Neu5Ac at 50 mM did not inhibit transport of HA* from either side of the Caco-2 cell monolayer. These data are consistent with the ability of these carbohydrates to inhibit TER reduction induced by the HA complex. Collectively, these results suggest that the binding of HAs to Neu5Ac- and Gal-containing glycans on epithelial cells is essential for the transport of BoNT across the intestinal wall. Moreover, the carbohydrate receptors may play a more important role in the initial L-PTC absorption in the intestinal lumen, whereas other host receptors (e.g., E-cadherin) are involved once it gains access to the basolateral side.

The HA complex recognizes its carbohydrate receptors with high specificity

To fully understand the binding specificity, we determined the crystal structures of HA70 in a complex with α2,3- or α2,6-SiaLac (Fig. 6 and Table S4 in Text S1). We found that α2,3- and α2,6-SiaLac bound to the same region in the D3 domain of HA70, where the terminal Neu5Ac in both glycans mediates the majority of the HA70–glycan interactions through six pairs of hydrogen bonds (Fig. 6A and Fig. S12 in Text S1). Mutating the Neu5Ac-binding residues (e.g. T527P, R528A, or T527P/R528A) completely abolished the binding (Table S3 in Text S1). The Neu5Ac-binding mode in HA70-A is also conserved in HA70-C (Fig. S13 in Text S1) [15, 16], suggesting HA70 is unlikely to be a major determinant of the host tropism of various BoNT serotypes.

In contrast to the well-defined conformation of Neu5Ac, the Gal–Glc moiety seems to have a larger structural flexibility and is not essential to HA70–glycan recognition. Specifically, α2,3-SiaLac adopts a linear conformation, which is likely stabilized by a Glc-mediated crystal contact with its symmetry mate. However, α2,6-SiaLac binding to the same site adopts a folded conformation in which there is no crystal contact and Glc has no visible electron density (Fig. S12B in Text S1). Furthermore, these conformations are also different than that observed in the structures of α2,3- and α2,6-SiaLac when they bind to HA70-C, despite the conserved Neu5Ac-binding mode [16]. The different glycan conformations and the weak electron densities for Gal–Glc observed here are probably due to the intrinsic flexibility of SiaLac in solution [47].

The ability of HA70 to bind SiaLac with different glycosidic linkages contrasts with the binding profile of influenza virus HA. Neu5Ac binds to a deep pocket in influenza HA, which restricts the composition and topology of glycans that can bind to influenza HA [48, 49, 50]. In contrast, the Neu5Ac-binding site in HA70 is on a flat surface, allowing more freedom for additional glycan binding beyond the terminal Neu5Ac.
We also determined the crystal structures of the HA17–HA33 complex bound with Gal, Lac, or LacNAc (Table S4 in Text S1). All three bind to an identical site in HA33, where the HA33–glycan interactions are mediated only by the Gal moiety through extensive hydrogen bonding and a crucial stacking interaction between Phe278 and the hexose ring of Gal (Fig. 6B). The HA33–Gal interaction is well-defined and identical for the two HA33 molecules in one asymmetric unit (AU). The Glc or GlcNAc

Figure 4. The fully assembled HA complex markedly reduced the TER of human intestinal Caco-2 cell monolayers. Caco-2 cells were grown on transwell filter membranes into confluent polarized monolayers. (A, B) TER was measured following application of the L-PTC, the HA complex, the M-PTC, BoNT/A, or NTNHA-A to the apical (A; 58 nM) or basolateral (B; 17 nM) chambers. (C, D) TER was measured when the HA complex (HA wt), the mini-HA complex (HA70D3–HA17–HA33), HA70 trimer, HA33, or the HA17–HA33 complex were applied to the apical (C; 58 nM) or basolateral (D; 17 nM) chambers. Values are means ± SD (n = 4–12).
doi:10.1371/journal.ppat.1003690.g004

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Figure 5. The HA complex interacts with carbohydrate receptors to cross epithelial cell monolayers. (A, B) TER of Caco-2 monolayers was measured when Alexa-488-labeled HA complex (HA*) pre-incubated with Lac, IPTG, α2,3-SiaLac, or α2,6-SiaLac was applied to the apical (A; 58 nM) or basolateral (B; 17 nM) chambers. Values are means ± SD (n = 4–12). (C) HA* (with or without carbohydrates) or the Alexa-488-labeled HA33-DAFAcomplex (HA33-DAFA*) was applied to the apical (at 58 nM) or basolateral (at 17 nM) chamber. The fluorescence signals in both chambers were quantified after 24 hours and the amount of transported HA*/HA33-DAFA* was expressed as a percentage of the total HA*/HA33-DAFA* used. Values are means ± SD (n = 3–22).
doi:10.1371/journal.ppat.1003690.g005
moiety does not directly interact with HA33. One Glc/GlcNAc in the AU is involved in a crystal packing and shows clear electron density, while the density for the other copy is weakly defined; the latter is likely caused by the weak HA33–glycan binding affinity and intrinsic structural flexibility of HA33 that will be discussed later (Fig. S12D–F in Text S1). To further confirm the structural findings, we mutated the Gal-binding residues in HA33 (e.g., D263A or F278A) and found that these mutations almost completely abrogated the Lac binding (Table S3 in Text S1).

Gal binds at an equivalent site in HA33 of L-PTC/C (HA33-C) (Fig. S14 in Text S1) but with a 15-fold lower binding affinity than with HA33-A [13], which is likely caused by the replacement of Phe278 in HA33-A with Asp271 in HA33-C. In addition, HA33-C binds Neu5Ac in an adjacent binding site [51]. However, HA33-A does not bind Neu5Ac-containing sugars because the key Neu5Ac-binding residues in HA33-C, Trp176 and Arg183, are replaced in HA33-A with Tyr180 and Asn187, respectively (Table S3 in Text S1). These differences between HA33-A and HA33-C indicate that the known host susceptibility to different BoNT serotypes may be determined in part by the interaction between HA33 and host glycan receptors.

To further analyze the functional role of BoNT’s glycan receptors, we “knocked-down” specific glycan binding to the HA complex using structure-based mutagenesis. The HA33-DAFA complex (composed of the wild-type WT-HA70, WT-HA17, and HA33-D263A/F278A) did not bind to Gal, whereas the HA70-TPRA complex (composed of the WT-HA70-T527P/R528A, WT-HA17, and WT-HA33) failed to bind to Neu5Ac (Table S3 in Text S1). We found that the HA33-DAFA complex did not reduce TER when applied from either side of the Caco-2 cell monolayer. Furthermore, the loss of the Gal-binding site prevented the transport of HA33-DAFA through the Caco-2 monolayer (Fig. 5C), indicating the crucial role of the carbohydrate interaction during transcytosis. The HA70-TPRA complex maintained activity only when applied from the basolateral side, which was inhibited by Lac (Fig. 6D–E). These data suggest that there are at least two steps at which HA-glycan interactions play an important role in toxin absorption. Both Neu5Ac- and Gal-containing glycans are important for the initial L-PTC absorption in the intestinal lumen, but Gal-containing receptors on the basolateral surface of the epithelial cells may also participate, presumably in facilitating transport via the paracellular route [27,28,29].

Carbohydrate receptor mimics could inhibit BoNT/A oral toxicity

To determine whether the glycans could interfere with BoNT absorption in vivo, we examined the effect of the monosaccharides Neu5Ac, Gal, and IPTG on the oral toxicity of L-PTC/A in mice [4]. Concomitant oral administration of L-PTC/A and IPTG at 500 mM significantly extended the median survival time (MST) of animals to ~91 hours compared with ~39 hours for the control group. Furthermore, IPTG was effective when it was administered one hour prior to treatment with L-PTC/A with an increase of MST to ~62 hours. Some improvement in survival was also evident with IPTG treatment one hour after intoxication with L-PTC/A, with an increase of MST to ~55 hours (Fig. 6F).
IPTG does not affect the neurotoxicity of BoNT/A based on the mice phrenic nerve hemidiaphragm assay, this finding suggests that receptor mimics could block BoNT/A intestinal absorption at an early point of oral intoxication. Gal and Neu5Ac (up to ~500 mM) did not confer significant protection, most likely due to their low binding affinity and/or metabolism (Fig. S15 in Text S1).

Discussion

Here, we report the complete structure of a 14-subunit ~760 kDa L-PTC/A, which is achieved by building novel crystal structures of each subunit into 3D-EM reconstruction. To our knowledge, this is the largest bacterial toxin complex known to date. The L-PTC/A adopts a unique bimodular architecture, whereas BoNT/A and NTNHA form a compact M-PTC and three HA proteins adopt an extended three-arm shape. Our results conclude the same stoichiometry and a similar overall architecture as suggested by recent EM studies of L-PTC/A, B, and D [14,22]. Furthermore, our complementary crystallographic, EM, and biochemical studies have revealed for the first time that both BoNT/A and NTNHA are involved in interactions with the HA complex, and that the two modules associate through two small interfaces, in contrast to numerous protein–protein interactions within each module.

Aside from a small interface involving the BoNT/A receptor-binding domain, the majority of the interactions between the M-PTC and the HAs are mediated by the NTNHA nLoop. In spite of the overall structural similarity between BoNT/A and NTNHA, the nLoop is a unique feature of NTNHA, which is fully exposed on the M-PTC surface [5]. The nLoop is conserved in the NTNHA homologues that shield BoNT/A1, B, C, D, and G, and assemble with HAs to form the L-PTC. However, the nLoop is missing in NTNHA homologues that assemble with BoNT/A2, E, and F, which do not form binding the HA-negative M-PTC [11,52,53]. We have found that one molecule of the synthetic nLoop peptide binds to the trimeric HA70 with a high affinity, clearly suggesting that the nLoop is bridging the M-PTC and the HA complex. This is consistent with previous reports that the nLoop is intact in the context of the L-PTC but spontaneously nicked in the free NTNHA or the M-PTC [5,30,31,32,33,34].

Structural and sequence analyses suggest that the 12-subunit architecture of the HA complex is likely conserved across different BoNT serotypes [14,22]. For example, pairwise structural comparisons yield rmsd of ~1.28 Å (582 Cα atoms) and ~1.20 Å (137 Cα atoms) for HA70-A/HA70-C and HA17-A/HA17-D, respectively; they are ~0.87 Å (129 Cα atoms) and ~1.23 Å (134 Cα atoms) for the two domains of HA33 between serotypes A and D and similarly between HA33-A and HA33-C [13,14,15,16]. Moreover, the protein–protein interactions within the HA70 trimer and between HA17 and HA33 are largely conserved among our crystal structures of serotype A and the known crystal structures of serotypes C and D.

Despite the largely rigid structure of the HA complex, HA33 seems to have an intrinsic structural flexibility. The N-terminal domain of HA33 is fixed in the HA complex through extensive inter-HA33 and HA17–HA33 interactions, but its C-terminal domain is largely unrestricted. When comparing two HA33-A structures that were determined in different crystal forms, we found that the N- and C-terminal domains of HA33-A twist against each other by ~14° (Fig. S16 in Text S1) [17]. A more significant conformational change is observed between HA33-A and C (~61°) and HA33-A and D (~65°) (Fig. S16 in Text S1) [13,14]. In the context of the assembled HA complex, such a conformational change leads to a shift up to ~23 Å for the C-terminal Gal-binding site in HA33. We suggest that HA33 could require such structural flexibility to achieve its multivalent host-receptor binding in the intestine.

The loose linkage between the M-PTC and the HA complex clearly suggests divided functions. We previously reported that the M-PTC’s compact structure protects BoNT against digestive enzymes and the extreme acidic environment of the GI tract [5,23]. We now show that the HA complex is mainly responsible for BoNT absorption in the small intestine, through binding to specific host carbohydrate receptors. This new finding permitted the identification of IPTG as a prototypical oral inhibitor that extends survival following lethal oral BoNT/A intoxication of mice. Multivalent interactions involving nine binding sites for Neu5Ac- and Gal-containing glycans increase the overall avidity of binding between the L-PTC and glycans on the epithelial cell surface, and thus compensate for the modest glycan-binding affinities at individual binding sites (Fig. 6C). Similarly, the potency of carbohydrate receptor mimics could be improved by optimizing the HA–glycan interactions as revealed here or by introducing new HA-inhibitor interactions at individual binding sites based on rational design, as well as by designing multivalent inhibitors. Although such inhibitors cannot be used to treat fully developed food-borne botulism, they could provide temporary protection upon pre-treatment and could also be useful for cases of intestinal colonization with C. botulinum spores such as in cases of infant or adult intestinal botulism. Our results also suggest that the L-PTC could be exploited for alternative applications. For example, protein-based therapeutics could be coupled to the modified non-toxic L-PTC to allow oral delivery by improving drug stability, absorption efficiency, and bioavailability.

Materials and Methods

Ethics statement

The Institutional Animal Care and Use Committee of the United States Department of Agriculture, Western Regional Research Center approved the experimental and husbandry procedures used in these studies (protocol # 11-2). All animal experiments were conducted under the guidelines of the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training.

Construct design and cloning

The sequences corresponding to full-length HA70 (residues M1–N626), HA70D3 (residues P378–N626), full-length HA17 (residues M1–I146), and full-length HA33 (residues M1–P293) from BoNT/A1-producing C. botulinum strain 62A were cloned into expression vectors pQE30, pGEX-6p-1, pRSFDuet-1, and pET28a, respectively. In addition, HA17 and HA33 were cloned into the bicistronic pRSFDuet-1 for co-expression.

To facilitate protein purification, a 6xHis tag followed by a thrombin cleavage site was introduced to the N-termini of HA70, HA17, and HA33. HA70D3 was cloned into pGEX-6p-1 following the N-terminal GST and a PreScission cleavage site. For HA17 and HA33 in the pRSFDuet-1 vector, HA17 was produced with an N-terminal 6xHis tag followed by a PreScission cleavage site, while HA33 had no affinity tag. All HA33 or HA70 mutations were generated by QuikChange site-directed mutagenesis (Stratagene).

Protein expression and purification

Four different protein expression schemes were used to produce the individual HAs or HA complexes. (1) HA70 (pQE30), HA70D3
and HA33 (pET28a) were expressed alone; (2) HA70 (pQE30) and HA17 (pRSFDuet-1) were co-transformed into bacteria and co-expressed; (3) HA70D3 (pGEX-6p-1) and HA17 (pRSFDuet-1) were co-transformed into bacteria and co-expressed; and (4) HA17 and HA33 were co-expressed using the biarcistric pRSFDuet-1 vector.

All recombinant proteins were expressed in the E. coli strain BL21-RIL (DE3) (Novagen). Bacteria were grown at 37 °C in LB medium in the presence of the appropriate selecting antibiotics. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when OD600 had reached 0.7. The temperature was then decreased to 18 °C and expression was continued for ~16 hours. The cells were harvested by centrifugation and stored at −20 °C until use.

For purification of His-tagged proteins (HA70, HA33, the HA70-HA17 complex, and the HA17-HA33 complex), proteins were bound to a Ni-NTA (nitrilotriacetic acid, Qiagen) affinity column in a buffer containing 50 mM Tris (pH 8.0) and 400 mM NaCl, and subsequently eluted in the same buffer containing 300 mM imidazole. The eluted fractions of each protein were added to Superdex 200 chromatography with 20 mM Tris (pH 7.6) and 50 mM NaCl, then the His-tag was removed with thrombin (for HA70, HA33, and the HA70–HA17 complex) or PreScission protease (for the HA17–HA33 complex). GST-tagged HA70D3 and the HA70D3-HA17 complex were purified using Gltathione Sepharose 4B resins (GE Healthcare) in phosphate-buffered saline, and eluted from the resins after on-column cleavage using PreScission protease.

The following three schemes were used to further purify the proteins. (1) HA70 and the HA70–HA17 complex were purified by MonoQ ion-exchange chromatography (GE Healthcare) in a buffer containing 20 mM Tris (pH 8.0) and eluted with NaCl gradient, followed by Superdex 200 size-exclusion chromatography (GE Healthcare) in 20 mM Tris (pH 8.0) and 50 mM NaCl. (2) HA33 and the HA17–HA33 complex were purified by MonoS ion-exchange chromatography in a buffer containing 20 mM sodium acetate (pH 5.0) and eluted with a NaCl gradient, followed by Superdex 200 chromatography in 20 mM Tris (pH 8.0) and 50 mM NaCl. (3) HA70D3 and the HA70D3–HA17 complex were purified by MonoQ ion-exchange chromatography in 20 mM Tris (pH 8.0) followed by Superdex 200 chromatography in 20 mM Tris (pH 8.0) and 50 mM NaCl for HA70D3 or 20 mM sodium citrate (pH 5.0) and 100 mM NaCl for the HA70D3-HA17 complex. Each protein or protein complex was concentrated to ~3–6 mg/ml using Amicon Ultra centrifugal filters (Millipore) and stored at ~80 °C until used for further characterization or crystallization.

The purified HA70 was labeled with Alexa Fluor® 488 carboxylic acid, succinimidyl ester (Life Technologies) according to the manufacturer’s instructions. The labeled HA70 was further purified by Superdex 200 chromatography in 20 mM Tris (pH 8.0) and 50 mM NaCl. The calculated dye to protein ratio was ~2 moles of dye per mole of monomeric HA70.

**In vitro reconstitution of the HA complex**

The HA17–HA33, the HA70–HA17, and the HA70D3-HA17 complexes were produced by co-expression and co-purification as described above. To assemble the mini-HA complex (HA70D3–HA17–HA33), the purified HA33 and the HA70D3-HA17 complex were mixed at a molar ratio of ~2:5:1 and incubated at 4 °C overnight. The excess HA33 was removed by Superdex 200 chromatography with 20 mM Tris (pH 7.6) and 50 mM NaCl. The fully assembled HA complex was reconstituted by mixing the purified HA70 and the HA17–HA33 complex at a molar ratio of ~1:1.3. The mixture was incubated at 4 °C overnight and the excess HA17–HA33 complex was removed from the mature HA complex by Superdex 200 chromatography with 20 mM Tris (pH 7.6) and 50 mM NaCl. The fluorescelabeled HA complex was prepared with Alexa Fluor® 488-labeled HA70 and unlabeled HA17–HA33 complex (HA*) or HA17–HA33D3 complex (HA33D3*) using a similar protocol.

**Analytical ultracentrifugation (AUC)**

Sedimentation equilibrium (SE) experiments were performed in a ProteomeLab XL-I (BeckmanCoulter) analytical ultracentrifuge. Purified HA samples were dialyzed extensively against a buffer containing 50 mM Tris (pH 7.6) and various NaCl concentrations, or 50 mM citric acid (pH 2.3) and various NaCl concentrations. Protein samples at concentrations of 0.4, 0.2, and 0.1 unit of OD280 were loaded in 6-channel equilibrium cells and centrifuged at 20 °C in an An-50 Ti 8-place rotor at the first speed indicated until equilibrium was achieved and thereafter at the second speed. HA33 was analyzed at rotor speeds of 19,000 and 22,000 rpm. The HA17–HA33 and the HA70D3–HA17–HA33 complexes were analyzed at 12,000 and 14,000 rpm. The HA70–HA17 and the HA70–HA17–HA33 complexes were run at speeds of 6,000 and 8,000 rpm. For each sample, data sets for the two different speeds were analyzed independently using Hetero-Analysis software (by J.L. Cole and J.W. Lary, University of Connecticut). Three independent experiments were performed for each sample.

The AUC data showed that HA33 is predominantly monomeric in solution at pH 2.3 or pH 7.6. HA17–HA33 forms a tight complex at pH 2.3 or pH 7.6, and the data were best fit to a model composed of one HA17 and two HA33 molecules.

The HA70–HA17 complex precipitated at pH 2.3 and was therefore analyzed only at pH 7.6. The best fits for HA70–HA17 clearly suggested a complex composed of three HA70 and three HA17 molecules. The data for the HA70D3–HA17–HA33 complex were best fit to a model composed of one HA70D3, one HA17, and two HA33 molecules. HA70–HA17–HA33 forms a tight complex containing three HA70, three HA17, and six HA33. Weak dimerization was observed for the mini-HA complex (Kd of ~23.1 μM) and the full HA complex (Kd of ~10.9 μM) at pH 7.6 in the presence of 100 mM NaCl, but was not observed at higher ionic strength. The weak oligomerization Kd suggests that the mini-HA and the full HA complex are monomeric under physiological conditions.

**3D-EM of the L-PTC and the HA complex**

The L-PTC of BoNT/A was obtained from List Biological Laboratories, Inc. (Campbell, California) and Miprolab GmbH (Göttingen, Germany). The recombinant HA complex was reconstituted in vitro as described above. Negatively stained EM specimens were prepared following a previously described protocol [54]. Briefly, 3 μl of the L-PTC (~0.02 mg/ml in 20 mM MES, pH 6.2, and 100 mM NaCl) or the HA complex (~0.01 mg/ml in 20 mM Tris, pH 7.6, and 50 mM NaCl) was placed on a freshly glow-discharged carbon-coated EM grid, blotted with filter paper after 40 seconds, washed with two drops of deionized water, and then stained with two drops of freshly prepared 1% uranyl formate, which also served to fix the proteins.

Particle images were acquired using a 4k×4k TVIPS CCD camera on a Tecnai F20 electron microscope (FEI) equipped with a field emission electron source operated at 200 kV, at a nominal magnification of ~70,000, resulting in a calibrated pixel size of 4.28 Å/pixel on the object scale after binning. The defocus values were set in the range of 1.5–3.2 μm. The electron dosage was ~40
Structure of the Botulinum Neurotoxin Complex

Isothermal titration calorimetry (ITC)

The calorimetry titration experiments were performed at 23°C on an ITC200 calorimeter from Microcal/GE Life Sciences (Northampton, MA). The HA samples were used as the titrant in the cell and the carbohydrates were used as titrants in the syringe. To control for heat of dilution effects, protein samples were dia lyzed extensively against the titration buffer (50 mM Tris, pH 7.6, and 100 mM NaCl) prior to each titration. Carbohydrates and nLoop peptide were dissolved in the same buffer. The pH of the acidic Neu5Ac solution was carefully adjusted to pH 7.6. The following concentrations were used for pair-wise titrations: HA33 (200 μM) vs. carbohydrates (Gal, Lac, LacNac, IPTG, or α2,6-SiaLac) (50 mM); HA70\textsuperscript{D3} (200 μM) vs. α2,3-, or α2,6-SiaLac (40 mM); HA70\textsuperscript{D3} (160 μM) vs. Neu5Ac (80 mM); and HA70 (30 μM) vs. nLoop (400 μM). The data were analyzed using the Origin software package provided by the ITC manufacturer. The thermodynamic values reported are the average of three independent experiments (Table S3 in Text S1).
Proteolysis with trypsin or pepsin

The recombinant HA70–HA17–HA33 complex, HA70, the HA17–HA33 complex, and the M-PTC were subjected to limited proteolysis with trypsin and pepsin overnight at room temperature. The trypsin digestions were performed at two different pHs in buffers containing 50 mM sodium phosphate (pH 6.0 or 7.5) and 300 mM NaCl, or in the Krebs-Ringer’s solution (119 mM NaCl, 2.5 mM KCl, 1.0 mM NaH2PO4, 2.5 mM CaCl2, 1.3 mM MgCl2, 20 mM Heps, and 11 mM D-glucose). The trypsin samples contained 1:10 (pH 6.0) or 1:20 (pH 7.5). The digestions were stopped by adding 1 mM PMSF and boiling the samples in reducing SDS-loading buffer for 10 minutes. The pepsin digestions were performed at a 1:10 ratio (w/w) of pepsin:sample in a buffer containing 50 mM citrate acid (pH 2.6, an optimal pH for the pepsin reaction) and 300 mM NaCl. Pepsin digestions were terminated by addition of a 1 M Tris-HCl (pH 8.0) stock solution to give a final concentration of 200 mM and samples were then boiled in the reducing SDS-loading buffer. All samples were subjected to SDS-PAGE.

Transwell assay

Cell culture: Caco-2 cells were obtained from the German Cancer Research Center (Heidelberg, Germany). Cells were cultured in Dulbecco modified Eagle medium (DMEM, Gibco® | Life Technologies, Darmstadt) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml for up to six months. The cells were subcultured twice a week and seeded on BD Falcon Cell Culture Inserts (#553494, growth area 0.9 cm², pore size 0.4 μm) at a density of approximately 10⁵ cells cm⁻² for flux studies and determination of transepithelial electrical resistance (TER).

Measurement of TER: All TER experiments were conducted in 0.5 ml and 1.5 ml of Iscoves Modified Dulbecco Medium without phenol red (IMDM, Gibco® | Life Technologies, Darmstadt) in the apical and basolateral reservoir, respectively. TER was determined with an epithelial volt-ohm meter (World Precision Instruments, Berlin, Germany) equipped with an Endohm 12 chamber for filter inserts. Filters with cell monolayers were used at day 11 after seeding which is seven days of post confluency. Only filters with an initial resistance of ≥300 Ω cm⁻² were used. For analysis of independent experiments subsequent results were expressed as percentages of the corresponding resistance of each data set determined immediately after administration of samples. Values are expressed as means of ≥3 independent experiments with duplicate samples ± standard deviations.

Carbohydrate inhibition assays: Lac, Gal, IPTG, Neu5Ac, α2,6- and α2,3-SiaLac were dissolved in IMDM, sterile filtered and stored at −20°C. Neu5Ac stock solution was adjusted to pH 7.4. The wild type HA complex (HA wt), fluorescence-labeled HA complex (HA*), or the HA17–HA33 complex were pre-incubated with the corresponding carbohydrate over night at 4°C in IMDM and diluted to the final concentration with IMDM prior to administration. The TER upon administration of each carbohydrate in the highest concentrations used was checked in the absence of HA and was virtually identical to that of the control without sugars.

Transport measurement: For paracellular transport studies, filters were incubated in IMDM added to the apical (0.5 ml) and basolateral (1.5 ml) reservoir. As marker substance Alexa Fluor® 488 labeled HA* or HA17–HA33–Lac was administered to the apical or basolateral reservoirs at final concentrations of 58 nM and 17 nM, respectively. After 24 hour of incubation, 200 μl of samples were taken from the apical and the basolateral reservoir. The marker substance was measured in a BioTek Synergy 4 fluorescence spectrophotometer at 495 nm excitation and 519 nm emission wavelengths.

Mouse protection assay

The mouse protection assay was performed following a previously described protocol [4]. Briefly, random sets of 10–20 female Swiss Webster mice (20–23 g) were used per dose. Mice were treated by oral gavage with 100 μl containing 1.9 μg of L-PTC/A (Metabionics) in phosphate-gelatin buffer (10 mM phosphate buffer, pH 6.2, and 2% gelatin), with or without the indicated concentrations of IPTG, Neu5Ac, or Gal. Mice were also administered 100 μl of 500 mM IPTG by gavage 1 hour prior or after treatment with 100 μl containing 1.9 μg of L-PTC/A in phosphate gelatin buffer. The acidic Neu5Ac was adjusted to pH 6.2 for administration. Mice were monitored for botulism symptoms for up to 14 days post-intoxication. Median survival and p-values were determined with the GraphPad Prism 5 program (San Diego, CA).

Accession numbers

Atomic coordinates and structure factors for HA70, HA70P31–HA17–HA33, HA70P31–HA17, HA17–HA33, HA17–HA33–Lac, HA17–HA33–Gal, HA17–HA33–LacNeu5Ac, HA70–α2,3-SiaLac, and HA70–α2,6-SiaLac have been deposited with the Protein Data Bank under accession codes 4LO4, 4LO7, 4LO8, 4LO0, 4LO2, 4LO1, 4LO3, 4LO5, 4LO6, respectively. EM 3D reconstructions for the L-PTC and the HA complex have been deposited with the Electron Microscopy Data Bank (EMDB) under accession codes EMD-2417 and EMD-2416, respectively.

Supporting Information

Text S1 | File contains Figures S1–S16 and Tables S1–S4, with legends.

(PDF)

Acknowledgments

We thank Drs. Ralf Gerhard, Andrey Bobkov, and Thomas Henderson II for technical supports, and Dr. Thomas Binz for reading of the manuscript. We thank Dr. Z. Hong Zhou for the use of the EM facility at the Electron Imaging Center for NanoMachines at UCLA, and Dr. Timothy S. Baker for the use of the UCSD Cryo-EM Facility.

Author Contributions

Conceived and designed the experiments: AR RJ. Performed the experiments: KL SG GY LJ TTNL JS AMK LWC KP AR RJ. Analyzed the data: KL SG GY LJ TTNL JS AMK IWC KP AR RJ. Wrote the paper: AR RJ.

References


PLOS Pathogens | www.plospathogens.org 11 October 2013 | Volume 9 | Issue 10 | e1003690