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Differential contribution of the residues in C-terminal half of the heavy chain of botulinum neurotoxin type B to its binding to the ganglioside GT1b and the synaptotagmin 2/GT1b complex

Tomoko Kohda[a], Hideshi Ihara[b], Yukiji Seto[a], Hiroyasu Tsutsuki[b], Masafumi Mukamoto[a], Shunji Kozaki[a,*]

[a]*Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan*
[b]*Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan*

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Abstract

Clostridium botulinum type B neurotoxin was effectively bound to synaptotagmin 2 (Stg2) associated with ganglioside GT1b, however, the molecular interaction between the neurotoxin and the Stg2/GT1b complex has not been identified. Previously, we found that infant botulism-related strain 111 generated a low activity of the neurotoxin (111/NT), which differed in some amino acid residues, especially in the carboxyl terminal half of the heavy chain (H_C), from the original neurotoxin of strain Okra (Okra/NT) associated with a food-borne botulism. In this study, we evaluated the binding capabilities of site-directed mutants of Okra/H_C to the Stg2/GT1b complex and to GT1b alone, and investigated the relationship between the toxic action and receptor binding. Replacement of K1187 and E1190 with glutamic acid and lysine, respectively, which substituted for the 111/NT residues, caused a reduction of binding affinity to the Stg2/GT1b complex, suggesting that both these residues contribute to the different binding affinity between Okra/NT and 111/NT. Substitution of four residues, H1240, S1259, W1261 and Y1262, which form a ganglioside pocket, drastically decreased the binding of H_C to the Stg2/GT1b complex and to GT1b. Mutation in the residues, K1186, E1189, K1191 and K1260 reduced the binding of H_C to GT1b alone, but not to the Stg2/GT1b complex. Analyses of effects of mutant toxins on toxicity of BoNT/B to cerebellar granule cells suggest the association of cell toxicity with binding to Stg2/GT1b complex but not that to GT1b alone.

1. Introduction

Clostridium botulinum produces highly potent neurotoxins (BoNTs), which inhibit neurotransmitter release from the presynaptic neuronal cells and cause flaccid paralysis. The BoNTs, classified into seven types (A–G), are released from the bacteria as a single polypeptide chain of 150 kDa, and are cleaved by protease into a 100 kDa heavy chain and a 50 kDa light chain. The heavy chain is composed of at least two functionally different domains, an N-terminal (H_N) and a C-terminal (H_C). The H_N domain plays a critical role in the translocation of the catalytic light chain across the vesicle membrane into the cytosol, while the H_C domain is responsible for neurospecific binding at the presynaptic membrane [1,2], which is a crucial first step in the specific and potent neurotoxicity. The light chain, which belongs to a zinc-dependent protease family, catalyzes the proteolysis of a specific [soluble NSF (N-ethyl maleimide sensitive fusion protein) attachment protein receptor] (SNARE) proteins at a unique site involved in synaptic vesicle membrane fusion to the presynaptic membrane [3,4]. The early studies concerning toxin-binding molecules present on the membrane have been focused on their interaction with the ganglioside. In fact, BoNTs exhibited the binding affinities in the high-nM range for the isolated ganglioside. However, such direct interaction between BoNT and ganglioside rarely occurs under physiological conditions [5]. Furthermore, the binding of BoNTs to the neuronal membranes showed

*Corresponding author. Tel.: +81 72 254 9504; fax: +81 72 254 9499.
E-mail address: kozaki@center.osakafu-u.ac.jp (S. Kozaki).

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much higher affinities and their binding appeared to be toxin type specific. From these findings, it has been considered that the protein receptor substance mediates type-specific toxin binding. Eventually, we revealed that the specific protein receptor for BoNT/B was synaptotagmins (Stg) 1 and 2, which was able to display the toxin-binding activity in the presence of ganglioside GT1b and GD1b [6]. In addition, confirmative data have been accumulated that Stg2 directly interacts with BoNT/B and mediates the toxin entry into the neural cells [7]. Furthermore, BoNT/G was found to bind to both Stg1 and 2 in a ganglioside-independent manner [8]. Recent studies on crystal structures of clostridial neurotoxins show that the H_C's of tetanus neurotoxin (TeNT) [9], BoNT/A [10], and BoNT/B [11] can be further divided into two distinct subdomains: the N-terminal subdomain (H_{CN}) exhibiting a jelly-roll motif and the C-terminal subdomain (H_{CC}) with a β-trefoil motif. Several experiments with the TeNT/H_C mutants provided the information on the key residues of the H_{CC} subdomain that played a significant role in the formation of the receptor recognition site [12–14]. Binding sites on BoNT/B molecules for ganglioside GT1b have been characterized by the cocrystallization of the toxin with sialyllactose [11]. They involved the specific peptide motif, which formed the ganglioside-binding pocket. The motif was also conserved in other BoNTs and TeNT [15]. However, these observations did not show any data concerning the interaction of type-specific protein receptor with BoNT on the molecular basis. We have previously reported that a BoNT/B variant produced by strain 111 (111/NT), which has been associated with infant botulism, showed biological properties different from that of strain Okra (Okra/NT) involved in food-borne botulism. The toxicity of 111/NT was lower than that of Okra/NT because of the differences between their receptor binding capabilities [16]. The hybrid H_C, in which the Okra/H_{CC} was replaced by the homologous half of 111/H_{CC}, showed the same decreased binding capability as 111/H_C, which confirmed the idea that the H_{CC} was involved in the binding to the receptor [17]. The amino acid sequences of both BoNTs revealed that the 23 residues in 111/H_{CC} were substituted in comparison with Okra/H_{CC}; however, there is no data available on the relationship between the replacement of amino acid residues and the binding affinity. The aim of this study is to evaluate the contribution of the H_{CC} of BoNT/B to binding to its receptor, particularly of the amino acid residues differed between Okra/NT and 111/NT and of those forming the ganglioside-binding pocket.

2. Results

2.1. Preparation of site-direct mutants

We examined the structural difference of two recombinant H_C's, Okra/H_C and 111/H_C, which was obtained as described previously [17] by circular dichroism (CD) spectrum analysis. No significant differences in the CD spectra of the two recombinant H_C's were observed (data not shown), suggesting that they retained a very similar conformation. It seems likely that the binding capability of BoNT/B to its receptor is influenced directly by substitutions of amino acid residues observed for 111/H_C (Fig. 1). In the BoNT/B molecule, E1188 and E1189 were reported to form hydrogen bonds with sialic acid and galactose, respectively, based on the result from the cocrystal structure of BoNT/B with sialyllactose. The residues 1189–1191 formed a loop, which is exposed to the surface of the toxin molecule [11]. We prepared the mutants, termed loop mutants, involving the residues surrounding the loop along with several neighboring residues. In addition, we constructed site-directed mutants for some residues surrounding W1261, which have been identified as a binding site for sialyllactose [11]. They were designated as W1261 surrounding mutants. Table 1 shows the mutated residues and primers used in this study.

2.2. Binding activity of loop mutants

Binding efficiency of the mutants to the Stg2/GT1b complex incorporated into lipid vesicles was evaluated by competitive binding experiments with ^{125}I-labeled H_C (Table 2, Fig. 2a). The binding of ^{125}I-labeled H_C was competed by unlabeled wild-type H_C in a concentration-dependent manner, whose IC_{50} (50% inhibition concentration) was 0.5 nM. The result was consistent with that of ^{125}I-labeled BoNT/B as described before [17]. Alanine mutants of K1186, K1187, E1188, E1189, E1190 and K1191 inhibited the binding of ^{125}I-labeled H_C to the Stg2/GT1b complex at the same level, as the wild-type H_C. The results indicated that these residues did not play a significant role in the toxin binding. Two mutants, K1187E and E1190K, which replaced the residues of 111/Hc from Okra/H_C, had a slight effect on the binding of ^{125}I-labeled H_C to the Stg2/GT1b complex (IC_{50} were 7.7 and 6.2 nM, respectively) (Table 2, Fig. 2a).

In order to identify the residues involved in GT1b binding, the binding of mutants was assessed by competitive binding assays of ^{125}I-labeled H_C to GT1b immobilized on a plastic plate. E1188A and E1190A retained the inhibitory effect on the binding of ^{125}I-labeled H_C and wild-type H_C to GT1b. K1186A, K1187A, K1187E, E1189A and K1191A did not inhibit the ganglioside binding (IC_{50} was over 1000 nM) (Table 2). E1190K slightly inhibited the binding to GT1b (IC_{50} was 885 nM) (Fig. 3a).

2.3. Binding activity exhibited by mutants of W1261 surrounding residues

Alanine mutants of H1240 and K1260 were found to have little inhibitory effect on ^{125}I-labeled H_C binding to the Stg2/GT1b complex, but did inhibit binding to GT1b (Table 2, Fig. 3b). Mutants of S1259, W1261 and Y1262

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Okra 1138 LYIGEKFIIRRKSNSQSINDDIVRKEDYIYLDFFNLNQEWRVYTYKYFKKEEEKLFLAPI 1197
111  1138 LYIGEKFIIRRKSNSQSINDDIVRKEDYIYLDFFNSNREWRVYAYKDFKEEEKKLFLANI 1197
          ********************************** * ***** ** ** ** ***** *

Okra 1198 SDSDEFYNTIQIKEYDEQPTYSCQLLFKKDEESTDEIGLIGIHRFYESGIVFEEYKDYFC 1257
111  1198 YDSNEFYKTIQIKEYDEQPTYSCQLLFKKDEESTDEIGLIGIHRFYESGIVLKDYKNYFC 1258
           ** *** ****************************************   ** ***

Okra 1258 ISKWYLKEVKRKPYNLKLGCNWQFIPKDEGWTE                        1291
111  1259 ISKWYLKEVKRKPYNPNLGCNWQFIPKDEGWIE                        1291
          ***************  ************** *
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Fig. 1. Amino acid sequence alignment of Okra and 111 H_{CC} fragments from GenBank accession numbers M81186 for Okra/NT and AB084152 for 111/NT. An asterisk indicates strict sequence conservation. Residues of mutation analysis in this study are in white box.

Table 1
Mutants of H_C constructed

Mutant name		Primers[a]
K1186A	Fw	TATAAATATTTCGCGAAAGAGGAAG
	Rv	CTTCCTCTTTCGCGAAATATTTATA
K1187A	Fw	AATATTTTAAAGCAGAGGAAGAAAAATTGT
	Rv	ACAATTTTTCTTCCTCTGCTTTAAAATATT
K1187E	Fw	AATATTTTAAGGAAGAGGAAGAAAATTGTTCTTAGCTCCTA
	Rv	TAGGAGCTAAGAACAATTTTTCTTCCTCTTCCTTAAAATATT
E1188A	Fw	CTATAAATATTTTAAAAAAGCGGAAGAAAAATTGT
	Rv	ACAATTTTTCTTCCGCTTTTTTAAAATATTTATAG
E1189A	Fw	AAAATATTTTAAAAAAGAGGCAGAAAAATTGT
	Rv	ACAATTTTTCTGCCTCTTTTTTAAAATATTT
E1190A	Fw	AATATTTTAAAAAAGAGGAAGCAAAATTGTTTTTAG
	Rv	CTAAAAACAATTTTGCTTCCTCTTTTTTAAAATATT
E1190K	Fw	TAAGAAAGAGGAAAAAAAATTGTTCTTAGCTCCTATAAGT
	Rv	ACTTATAGGAGCTAAGAACAATTTTTTTTCCTCTTTCTTA
K1191A	Fw	TATATTTTAAAAAAGAGGAAGAAGCATTGTTTTTAG
	Rv	CTAAAAACAATGCTTCTTCCTCTTTTTTAAAATATA
H1240A	Fw	GGATTGATTGGTATTGCTCGTTTCTACGAATC
	Rv	GATTCGTAGAAACGAGCAATACCAATCAATCC
S1259A	Fw	GATTATTTTTGTATAGCTAAATGGTACCTAAAAGAGGTAAAAA
	Rv	TTTTTACCTCTTTTAGGTACCATTTAGCTATACAAAAATAATC
K1260A	Fw	TGTATAAGTGAATGGTACCTAAAAGAGGT
	Rv	ACCTCTTTTAGGTACCATTCACTTATACA
K1260E	Fw	TATTTTTGTATAAGTGCATGGTACCTAAAAGAGGTAAAAA
	Rv	TTTTTACCTCTTTTAGGTACCATGCACTTATACAAAAATA
K1260R	Fw	ATTTTTGTATAAGTAAAGCGTACCTAAAAGAGGTAAAAAGGA
	Rv	TCCTTTTTACCTCTTTTAGGTACGCTTTACTTATACAAAAATA
W1261A	Fw	TGTATAAGTAGATGGTACCTAAAAGAGGT
	Rv	ACCTCTTTTAGGTACCATCTACTTATACA
Y1262A	Fw	TGTATAAGTAAATGGGCCCTAAAAGAGGTAAAAA
	Rv	TTTTTACCTCTTTTAGGGCCCATTTACTTATACA
L1263A	Fw	ATAAGTAAATGGTACGCAAAAGAGGTAAAAAG
	Rv	CTTTTTACCTCTTTTGCGTACCATTTACTTAT

[a]Oligonucleotides used for site-direct mutagenesis.

did not hinder the binding of ^{125}I-labeled H_C to the Stg2/GT1b complex or to GT1b (Fig. 3b). Mutant of L1263 did not affect the binding of ^{125}I-labeled H_C to the Stg2/GT1b complex or to the GT1b. The role of K1260 in binding to GT1b was characterized. The mutants of glutamic acid (K1260E) and arginine (K1260R) inhibited the binding of ^{125}I-labeled H_C to the Stg2/GT1b complex while K1260E only decreased the binding activity to GT1b (Table 2).

2.4. Effect of mutants on toxicity of BoNT/B to cerebellar granule cells

BoNT/B binds to the receptor, which results in the functional internalization of the toxin and subsequent blocking of the neurotransmitter release [23]. To determine whether the binding activity of mutant H_C to the Stg2/GT1b complex reflected the characteristics of a functional domain of BoNT/B molecule, we examined the inhibition

Table 2
Binding activities of HC mutants

Recombinant H_C	Stg2/GT1b[a] (nM)	GT1b[a] (nM)
H_C	0.5	190
K1186A	1.0	>1000
K1187A	1.1	>1000
K1187E	7.7	>1000
E1188A	0.3	156
E1189A	1.3	>1000
E1190A	0.3	300
E1190K	6.2	885
K1191A	1.9	>1000
H1240A	2.4	>1000
S1259A	34.9	>1000
K1260A	0.7	>1000
K1260E	1.4	>1000
K1260R	0.4	537
W1261A	>100	>1000
Y1262A	>100	>1000
L1263A	0.5	203

[a]Data are the concentrations for 50% binding inhibition and presented as the mean ± S.D. ($n = 3$).

of the depolarization-evoked glutamate release from primary rat cerebellar granule cells by BoNT/B in the presence or absence of the H_C mutants. BoNT/B inhibited the glutamate release to 25% of control at the concentration of 1 nM. However, glutamate release was 80% of the control in the presence of 500-fold excess of H_C. The mutants of K1187E, E1190 K, W1261A and Y1262A, which were unable to bind to the Stg2/GT1 complex, did not affect the blockade of glutamate release by BoNT/B. These observations suggest that their mutations resulted in a loss of the functional binding capability to the receptor on neural cells. In the presence of K1260A, blockade of glutamate release by BoNT/B was recovered to 60% of the control (Fig. 4).

3. Discussion

We investigated the differences between the binding capabilities of Okra/H_C and 111/H_C. Among the substitutions in H_{CC} differentiating Okra/NT from 111/NT, we focused on K1187 and E1190 of Okra/NT, because both the residues were located near or in the loop and were considered important for ganglioside recognition [11]. K1187 was substituted by glutamic acid of 111/NT. The mutation of K1187E resulted in a decrease in the binding to the Stg2/GT1b complex and to GT1b, while K1187A clearly reduced the binding ability to the ganglioside, but retained the binding capability to the Stg2/GT1b complex. The results indicated that a negative charge at this residue observed for 111/NT greatly reduces the binding affinity to the complex. The E1190 in Okra/NT was substituted with lysine of 111/NT. E1190K significantly decreased the binding to the Stg2/GT1b complex and moderately to GT1b, while E1190A barely reduced the binding to either receptor. The results suggest that a positive charge at this

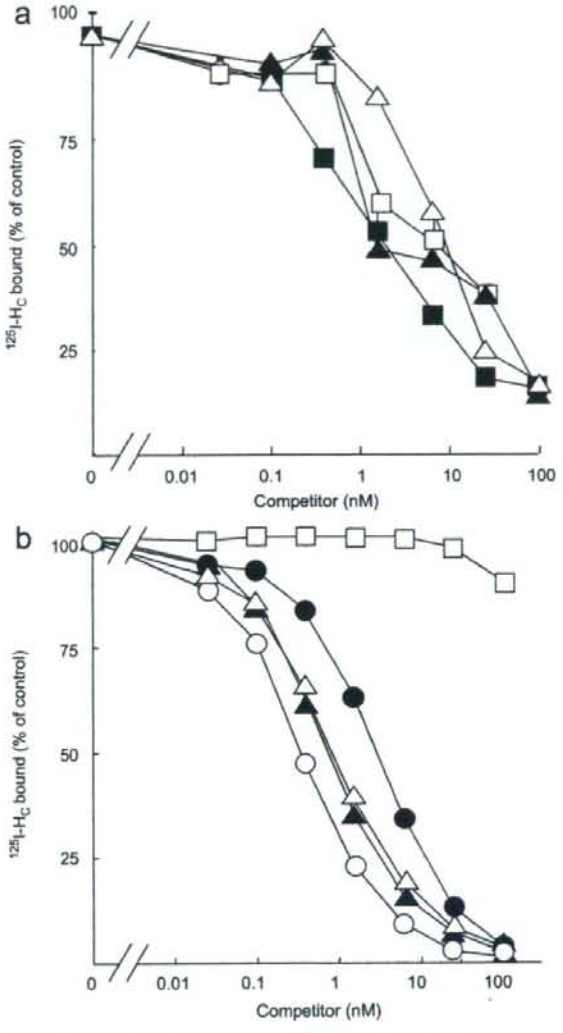

Fig. 2. Competition binding assays of ^{125}I-labeled H_C to the Stg2/GT1b with unlabeled representative H_C mutants. ^{125}I-labeled H_C (0.5 nM) was added to the recombinant Stg2 (5 ng protein) reconstituted into lipid vesicles with ganglioside GT1b (2.5 ng NeuAc) with presence of increasing concentrations of unlabeled H_C mutants. Each point represents the mean of three determinations. The bar shows standard error. (a) Loop mutants: K1187A (closed triangle); K1187E (open triangle); E1190A (closed square); E1190K (open square). (b) Mutants of W1261 surrounding residues: Wild-type H_C (open circle); H1240A (closed circle); K1260A (open triangle); L1263A (closed triangle); W1261A (open square).

residue doses the binding affinity. Thus, the electric charge at both residues is crucial for the receptor recognition and it is likely that the difference in the binding affinity of Okra/NT and 111/NT to the Stg2/GT1b complex is partly due to the substitution of K1187 to E and/or E1190 to K.

The involvement of the residues forming the ganglioside-binding pocket has been examined. S1259 and W1261 are

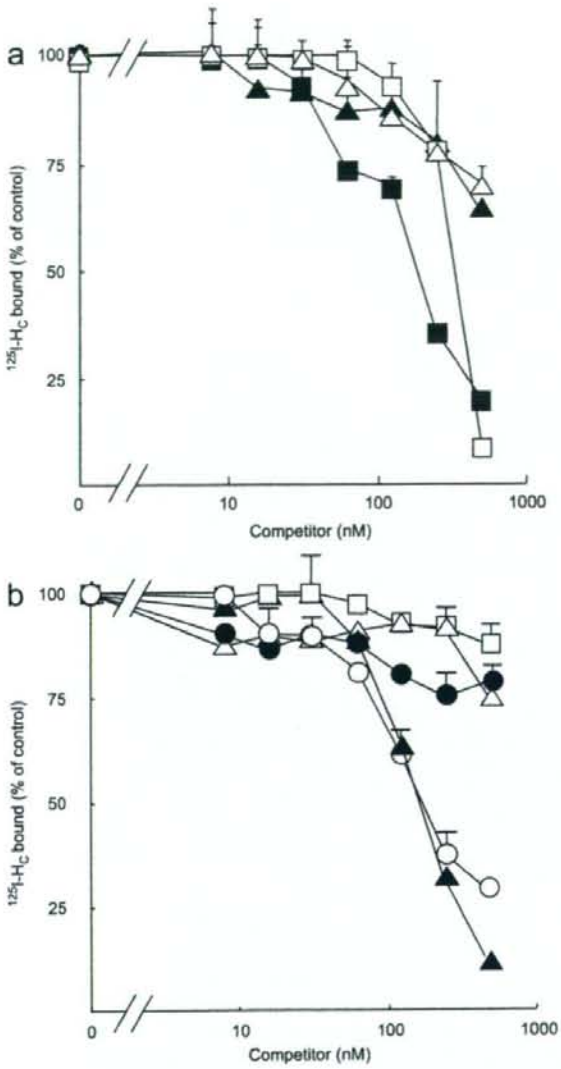

Fig. 3. Competition binding assays of ^{125}I-labeled H_C to ganglioside GT1b with unlabeled representative H_C mutants. ^{125}I-labeled H_C (0.5 nM) was added to ganglioside GT1b immobilized on microtiter plate (50 ng NeuAc/well) in presence of increasing concentrations of unlabeled H_C mutants. Each point represents the mean of three determinations. The bar shows standard error. (a) Loop mutants: K1187A (open triangle); K1187E (closed triangle); E1190A (open square); E1190K (closed square). (b) Mutants of W1261 surrounding residues: H_{CC} (open circle); H1240A (closed circle); K1260A (open triangle); L1263A (closed triangle); W1261A (open square).

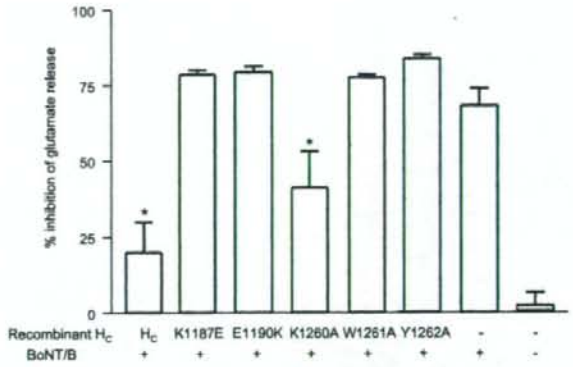

Fig. 4. Effects of BoNT/B with competitor on the depolarization-evoked glutamate release from cultured cerebellar cells. Inhibition of potassium-stimulated glutamate release from rat cerebellar granule cells was determined in the presence of BoNT/B and competing H_C mutants. One nanomolar BoNT/B was added to rat cerebellar granule cells with 500 nM H_C mutants. Each point represents the mean of three determinations. The bar shows standard error. (* = $P<0.05$ as compared to BoNT/B treated cells).

conserved in the ganglioside pockets of TeNT, BoNT/A and BoNT/B, which similarly interact with common sugar structures of disaccharide portions of the GT1b. The hydroxyl group of Y1262 interacts with the protruding glyceryl side groups of GT1b [11]. In addition, the corresponding residue of Y1289/TeNT was shown to play a key role in the functional binding of TeNT to spinal cord neurons leading to the inhibition of neurotransmitter release [12,13]. In this study, alanine mutants of S1259, W1261 and Y1262 showed a significantly decreased binding affinity to the Stg2/GT1b complex as well as to the ganglioside alone. H1240A significantly decreased the binding affinity to GT1b and moderately to the Stg2/GT1b complex. This is consistent with a previous study [18] in which H1240A resulted in the significant loss of binding to a synaptosome and moderate loss of binding to GT1b. It was confirmed that H1240, S1259, W1261 and Y1262 have a critical role in the binding to ganglioside and to the Stg2/GT1b complex. Besides the four residues, we found that some residues contributed to the ganglioside recognition. Mutation of K1260 to alanine and glutamic acid decreased the binding affinity to GT1b but not to the Stg2/GT1b complex, indicating that K1260 contributed to the ganglioside recognition. Mutation of K1260 to arginine did not alter the binding affinity to GT1b, suggesting that a positive charge at this site may be important to bind to GT1b. Three mutants of K1186A, E1189A and K1191A decreased the binding to GT1b but not to the Stg2/GT1b complex. Thus, the residues contributing to the ganglioside recognition were widely distributed.

K1187E, E1190K, W1261A and Y1262A did not affect the Okra/NT action on the glutamate release from rat cerebellar granule cells. These findings are comparable to the results that the mutants showed decreased binding activity to the Stg2/GT1b complex, which suggests that these residues are functionally related with the receptor recognition. In contrast, K1260A, which only inhibited the binding to the ganglioside, was hardly inhibited. This result suggests that ganglioside binding is scarcely associated with the toxic action on the neural cells, while the binding to the

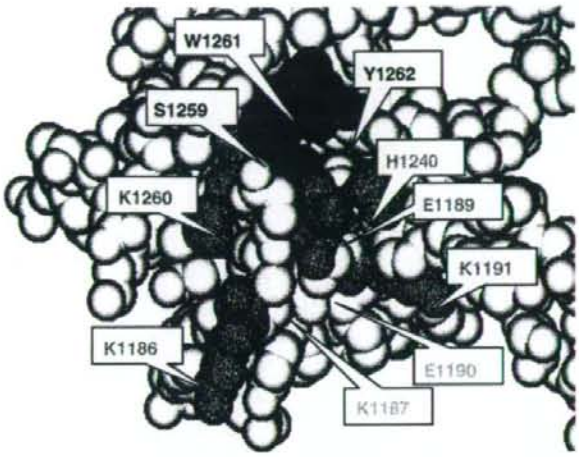

Fig. 5. Three-dimensional structure of β-trefoil subdomain of H_{CC}. Red residues are Stg2/GT1b and ganglioside GT1b binding site, green residues are ganglioside GT1b binding sites and yellow residues may be determinants of the different binding affinities between Okra/NT and 111/NT.

Stg2/GT1b complex is functionally related to the toxic action.

These results provide new evidence suggesting that the residues contributing to the binding to the Stg2/GT1b complex in H_C/B were different from those responsible for binding to GT1b. A summary of the results is illustrated in Fig. 5. The results suggest that various residues of H_C in a broad region interact with GT1b in the absence of Stg2, while the contribution of individual residues binding to the Stg2/GT1b complex is limited.

4. Material and methods

4.1. Construction of recombinant genes

DNA fragments encoding H_C (amino acids 854–1291) of Okra/NT and 111/NT were subcloned into pQE30 (Qiagen, Valencia, CA, USA) to express recombinant H_C with His-tag as previously reported [17]. The plasmids were termed pQE30/OkraH_C and pQE30/111H_C, respectively. For H_C mutants, a *Bgl* II site was newly created in pQE30/OkraH_C by mutagenesis of codon 1139 (TTA to CTA) without altering the amino acid residues using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Following the digestion with *Bgl* II and *Hind* III, DNA fragments encoding the C-terminal region of H_C (amino acids 1139–1291) were purified and ligated into the *Bgl* II- and *Hind* III-digested pUC18 vector (Takara, Tokyo, Japan) in which a *Bgl* II site was newly created by PCR (pUC18-*Bgl* II) to yield a plasmid for mutation (pUC18/$H_{C1139-1291}$). Mutagenesis of pUC18/$H_{C1139-1291}$ was performed using the QuickChange site-directed mutagenesis kit. Pairs of complementary oligonucleotides were used to construct mutant H_C molecules as shown in Table 1. Nucleotide sequences of the mutants were verified by DNA sequencing. After digestion of the mutated plasmid with *Bgl* II and *Hind* III, the fragments were ligated into *Bgl* II- and *Hind* III-digested pQE30/Okra H_C. The recombinant plasmids were introduced into *Escherichia coli* M15. Expression of recombinant H_C was performed according to the pQE system manual. Expressed recombinant H_C's were purified by Ni-NTA column as described previously [17].

4.2. Binding assay

The recombinant N-terminal domain of Stg2 (amino acids 1–87) was expressed in *E. coli* DH5α as a fusion protein with maltose binding protein, and purified on an amylase resin as described previously [16]. *C. botulinum* type B strain Okra was used for purification of BoNT/B as described [19]. The purified BoNT/B and recombinant H_C were labeled with $Na^{125}I$ (PerkinElmer, Boston, MA, USA) by the chloramines T method as described previously [20]. The specific activities of BoNT/B and H_C were 137 and 396 kBq/mg proteins, respectively. The residual toxicity of BoNT/B was higher than 80% compared to the unlabeled one. The purified recombinant Stg2 was incorporated into phosphatidylcholine lipid vesicles together with ganglioside GT1b (Wako, Osaka, Japan) by the acetone precipitation method [21]. The binding of ^{125}I-labeled BoNT/B or ^{125}I-labeled H_C to the reconstituted lipid vesicles was measured by filtration assay as described previously [21] in the presence or absence of competitors. The ^{125}I-labeled H_C bound to the Stg2/GT1b lipid vesicles at the same extent with ^{125}I-labeled BoNT/B.

GT1b was dissolved to 1 μg/ml in methanol and applied to wells of microtiter plates (50 ng N-acetyl-neuraminic acid/well). After evaporation for dryness, blocking solution of 3 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES)–NaOH buffer, (pH 7.0) containing 2.5 mM KCl, 2 mM $MgCl_2$ and 2 mM $CaCl_2$–0.5% bovine serum albumin (HEPES–BSA) was added, and the plates were incubated for 1 h at 37 °C. After washing six times in HBS (3 mM HEPES–NaOH buffer, pH 7.0, containing 0.12 M NaCl, 2.5 mM KCl, 2 mM $MgCl_2$ and 2 mM $CaCl_2$), plates were incubated for 1 h at 37 °C with ^{125}I-labeled H_C (1 nM) and various concentrations of unlabeled H_C. The wells were then washed twice with HEPES–BSA buffer, and the radioactivity of bound H_C was liberated with 0.1 N NaOH and determined by a γ-counter. The concentration giving 50% inhibition (IC_{50}) was calculated using PRISM software (GraphPad, San Diego, CA, USA).

4.3. Measurement of glutamate release from rat cerebellar granule cells

Rat cerebellar granule cells were prepared and maintained as described previously [22]. For competition

experiments, cultures at 7 days were washed with a minimum essential medium (MEM) containing 2% B-27 supplement (Invitrogen, Carlsbad, CA, USA), 25 mM KCl, 50 U/ml penicillin and 100 μg/ml streptomycin (HK-MEM/B-27) and incubated with 1 nM BoNT/B with or without recombinant H_C for 30 min at 37 °C in HK-MEM/B-27. The toxin mixture was removed and the cells were washed twice with HK-MEM/B-27 and incubated for 18 h at 37 °C. The cerebellar granule cells were washed four times with prewarmed low-K^+ solution [128 mM NaCl, 1.9 mM KCl, 1.2 mM KH2PO4, 2.5 mM CaCl2, 1.2 mM MgSO4, 11 mM glucose, 10 mM HEPES–NaOH (pH 7.4)] and then basal release was determined by a 2 min incubation with low-K^+ solution. Cells were depolarized with high-K^+ solution [80 mM NaCl, 50 mM KCl, 1.2 mM KH2PO4, 2.5 mM CaCl2, 1.2 mM MgSO4, 11 mM glucose, 10 mM HEPES–NaOH (pH 7.4)] for 2 min. Glutamate content in the medium was determined by reverse-phase HPLC using precolumn derivation with *o*-phthalaldehyde and fluorescence detection [23].

4.4. CD spectroscopy

Circular diachronic spectra were recorded in the wavelength range 185–260 nm using J-720w spectropolarimeter (JASCO, Tokyo, Japan) in a quartz cell with path length of 0.1 cm and maintained at 25 °C, at a speed of 50 nm/min and response time of 2 s. The detection was performed in 50 mM phosphate buffer (pH 6.8) at a protein concentration of 2 μM. Ten reproducible scans were collected for each sample. The buffer alone was used as a control blank in three experiments. The ellipticities were calculated using J700 software (JASCO, Tokyo, Japan).

4.5. Other methods

Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard [24]. The ganglioside content was determined by the Svennerholm method using sialic acid (NeuAc) as a standard [25].

Acknowledgment

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Identification of the receptor-binding sites in the carboxyl-terminal half of the heavy chain of botulinum neurotoxin types C and D

Kentaro Tsukamoto[a], Yuiko Kozai[b], Hideshi Ihara[c], Tomoko Kohda[b], Masafumi Mukamoto[b], Takao Tsuji[a], Shunji Kozaki[b,*]

[a]*Department of Microbiology, Fujita Health University School of Medicine, Toyoake, Aichi 470 1192, Japan*
[b]*Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599 8531, Japan*
[c]*Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599 8531, Japan*

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Abstract

Botulinum neurotoxin (BoNT) binds to presynaptic neuronal cells and blocks neurotransmitter release. The carboxyl-terminal half of the heavy chain (H_C) of the neurotoxin recognizes its specific receptor on the plasma membrane. We have previously demonstrated that BoNT/C binds to gangliosides GD1b and GT1b under physiological conditions, while BoNT/D interacts with phosphatidylethanolamine (PE). Here we report that the recognition sites for gangliosides and PE are present in the carboxyl-terminal domain of H_C. Chimeric mutants and site-directed mutants of BoNT/C-H_C and BoNT/D-H_C were generated and their binding activities evaluated. The chimeric H_C that consisted of the amino-terminal half of BoNT/D-H_C and the carboxyl-terminal half of BoNT/C-H_C possessed activity similar to the authentic BoNT/C-H_C, suggesting that the carboxyl-terminal region of H_C is involved in the receptor recognition of BoNT/C. Moreover, analysis using site-directed mutants indicated that the peptide motif $W^{1257}Y\cdots G^{1270}\cdots H^{1282}$ plays an important role in the interaction between BoNT/C and gangliosides. In contrast, we revealed that two lysine residues of BoNT/D-H_C are involved in the formation of the critical binding site for receptor binding.

1. Introduction

Seven distinct types of botulinum neurotoxins (BoNTs/A–G) produced by *Clostridium botulinum* are the causative agents of botulism [1]. BoNTs/A, B, E, and F are the most potent in causing human intoxication, and BoNTs/C and D are responsible for avian and animal botulism [2,3]. Some type C strains produce unique BoNT called C/D mosaic toxin (BoNT/CD) that comprises two thirds of BoNT/C and one third of BoNT/D corresponding to the H_C portion [4,5]. These neurotoxins as well as the other clostridial toxin, tetanus neurotoxin (TeNT), are synthesized as a single polypeptide chain molecule with a molecular mass of about 150 kDa, and thereafter nicked by proteases into heavy (100 kDa) and light (50 kDa) chains linked together by a disulfide bond. The light chain is a zinc endopeptidase that cleaves the presynaptic soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins [6–9]. Assembly of SNARE proteins is an essential step in neuroexocytosis. The correlation between SNARE protein cleavage by BoNTs and the blockade of neurotransmission is well documented [10–12]. The heavy chain mediates binding to neuronal receptors, leading to internalization of the light chain and its translocation into the cytoplasm of nerve terminals [13,14].

The specific binding of neurotoxin to the peripheral neuromuscular junction is considered to be the first step in intoxication. It is well known that the adhesion between neurotoxins and nerve endings involves gangliosides [15–18], and the carboxyl-terminal half domain of the heavy chain (H_C) binds to the gangliosides. The domain is

Abbreviations: BoNT, botulinum neurotoxin; H_C, carboxyl-terminal domain of the heavy chain; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; PE, phosphatidylethanolamine.

*Corresponding author. Tel.: +81 72 254 9504; fax: +81 72 254 9499.
E-mail address: kozaki@center.osakafu-u.ac.jp (S. Kozaki).

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composed of two subdomains; an amino-terminal jelly roll (H_{CN}) and a carboxyl-terminal β-trefoil (H_{CC}). The crystallographic data on BoNTs/A and B revealed that H_{CC} subdomains structurally resemble the lactose-binding pocket of TeNT [19–23], but the H_{CC} amino acid sequence is poorly conserved among BoNTs. Hence, BoNTs are considered to recognize type-specific receptors other than gangliosides. Synaptotagmin I and II, two homologous synaptic vesicle proteins, have been indicated to function in the entry of BoNT/B into neuronal cells [24–26]. Meanwhile, Synaptic vesicle protein SV2 acts as the protein receptor for BoNT/A [27,28]. We previously reported that the receptor for BoNTs/C and D might not include a protein component, as opposed to BoNTs A and B. In BoNT/C, unlike other types of BoNTs, gangliosides are predicted to play a significant role in the binding to neuronal cells, because a deficiency of gangliosides produced little sensitivity to BoNT/C *in vivo* [29]. On the other hand, BoNT/D exhibited binding activity to phosphatidylethanolamine (PE). These findings suggest that the binding sites in BoNTs/C and D molecules are possibly maintained in different amino acid residues compared with TeNT and BoNTs/A and B. To clarify this diversity, the present study is an attempt to identify the crucial region in BoNTs/C and D for binding to the receptor by using chimeric and site-directed H_C mutants.

2. Materials and methods

2.1. Preparation of recombinant H_C

Wild-type and mutant H_C proteins (type C: H_C/C, type D: H_C/D_1 and H_C/D_2) were expressed and purified as described previously [29]. H_C/C was originated from

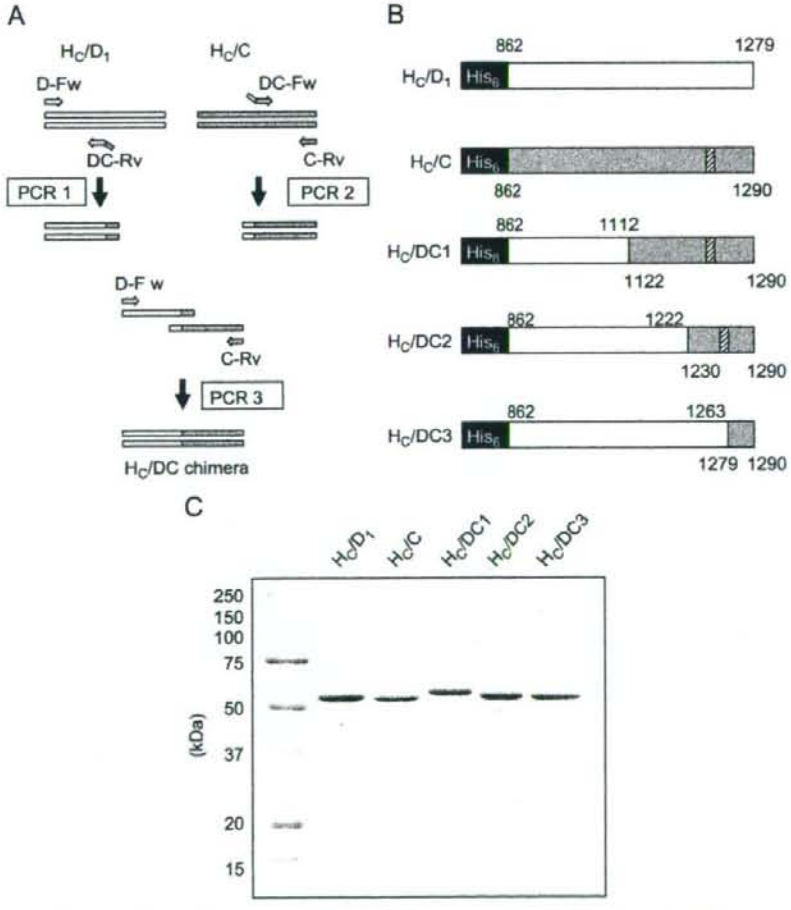

Fig. 1. Preparation of chimeric H_C mutants: (A) schematic overview of overlap extension PCR. Two separate DNA fragments were amplified in PCR reactions 1 and 2. The primers DC-Rv and DC-Fw introduce overlapping regions. These overlap regions hybridize in PCR reaction 3. Thus, using the primers D-Fw and C-Rv the full-length chimeric H_C fragment was finally amplified; (B) schematic representation of the recombinant wild-type H_C and the chimeric H_C. The squares filled by oblique lines indicate the ganglioside-binding motif. The numbers correspond to the position of the amino acids; and (C) SDS-PAGE profile of the recombinant H_C. The sample (1 μg/lane) was applied to a 10% polyacrylamide gel.

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typical type C toxin (strain CB-19). H_C/D_1 was derived from BoNT/CD (strain 003-9). H_C/D_2 was derived from typical type D toxin (strain 1873). H_C/D_1 showed a binding activity higher than H_C/D_2 [29]. Overlap extension PCR for the construction of genes encoding chimeric polypeptides was carried out as described originally [30]. In two separate PCR amplifications (the products of PCR 1 and 2), double-stranded fragments were generated and subsequently fused in PCR 3 (Fig. 1A). PCR 1 used a common 5′-far primer (D-Fw) and the unique reverse overlap primer (DC-Rv) that carries information for the construction of the intended chimera (Table 1). In PCR 2, a 3′-far primer (C-Rv) from the stop codon of the BoNT/C gene and a primer that was the complement of the overlap primer used in PCR 1 (DC-Fw) were used to generate the carboxyl-terminal fragment. After the two PCR products were mixed, denatured, and cooled, the complementary overlaps annealed, and the junction was then extended by template-dependent DNA polymeryzation. Finally, the chimeric construct was amplified by using far primers (D-Fw and C-Rv), and the amplified fragment was cloned into vector pET-30a (Novagen, Darmstadt, Germany). H_C/DC1 was composed of the amino acid residues 862-1112 of BoNT/CD and 1122–1290 of BoNT/C. H_C/DC2 comprised the residues 862-1222 of BoNT/CD and residues 1230–1290 of BoNT/C. The two chimeric mutants contain the ganglioside-binding motif (Fig. 1B). H_C/DC3 consisted of residues 862–1263 of BoNT/CD and residues 1279–1290 of BoNT/C. H_C/DC3 possessed the G1270-containing ganglioside-binding motif, but not motifs W1257 and Y1258. The chimeric mutants that consisted of H_C/D_1 and H_C/D_2 (H_C/DD1-H_C/DD4) were constructed using suitable primers in a similar manner (Fig. 2A). These chimeric H_Cs were composed of C-terminal H_C/D_1 (high affinity binding) and N-terminal H_C/D_2 (low affinity binding). Site-directed mutations were introduced into the H_C/C, H_C/D_1, and H_C/D_2 genes in pET-30a by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as instructed by the manufacturer. Briefly, suitable primers were designed for the desired mutation, and a thermocycling reaction permitted *in vitro* synthesis of the plasmid DNA with *PfuUltra*™ high-fidelity DNA polymerase (Stratagene). Parental methylated template DNA was digested by DpnI, and the resultant mutated plasmid mixture was used to transform *Escherichia coli* JM109 cells. All mutants were sequenced and expressed in *E. coli* BL21 CodonPlus (DE3)-RIL (Stratagene) in a similar manner to the wild-type H_C.

2.2. Binding of H_C to synaptosomes

Wild-type recombinant H_C proteins were radioiodinated with $Na^{125}I$ (Perkin Elmer Japan, Yokohama, Japan) using the chloramine-T method as described previously [31]. The specific activities of the H_C proteins ranged from 13 to 17 mCi/mg protein (26–34 MBq/nmol). Synaptosomes were prepared from rat brain [32] and suspended in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffered saline (HBS) [3 mM HEPES–NaOH buffer (pH 7.0) containing 150 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$]. The binding of ^{125}I-H_C to the synaptosomes was measured by a filtration assay in the presence or absence of unlabeled H_C or cinnamycin (Ro 09-198) (Sigma, Tokyo, Japan) [33].

Table 1
Specific primers used for preparation of chimera H_C by overlap extension PCR

Primer	Sequence[b]
D-Fw	5′-CATGCCATGGCTGAATATTTCAATAGTATTAATGATTCA-3′[c]
C-Rv[a]	5′-CCCAAGCTTTTATTCACTTACAGGTACAAAACC-3′[d]
D-Rv	5′-CCCAAGCTTTTACTCTACCCATCCTGGATC-3′[d]
DC1-Fw	5′-TAATTATAATTATATAGATAGG/TATATGTATGCGAACTCACG-3′
DC1-Rv[a]	5′-CGTGAGTTCGCATACATATA/CCTATCTATATAATTATAATTA-3′
DC2-Fw	5′-GTATCTCAAAATAAATATTGTAGTCAA/ATATTTAAATCAAATTTTAATGGAGAAA-3′
DC2-Rv[a]	5′-TTTCTCCATTAAAATTTGATTTAAATAT/TTGACTACAATATTTATTTTGAGATAC-3′
DC3-Fw	5′-CTAATTATGAGACAAAACTATTATCA/ACATCAACTCATTGGGGTT-3′
DC3-Rv[a]	5′-AACCCCAATGAGTTGATGT/TGATAATAGTTTTGTCTCATAATTAG-3′
DD1-Fw	5′-ATGGAGATAATATAATTCTTCAT/TCTAAAGATTTAACTAATTCTCATA-3′
DD1-Rv[a]	5′-TATGAGAATTAGTTAAATCTTTAGA/ATGAAGAATTATATTATCTCCAT-3′
DD2-Fw	5′-GATCTAAATTATATACTGGAAATCCTATT/ACTATTAAATCAGCAGCTAATAAGA-3′
DD2-Rv[a]	5′-TCTTATTAGCTGCTGATTTAATAGT/AATAGGATTTCCAGTATATAATTTAGATC-3′
DD3-Fw	5′-GATACAGAATATTATATTATTAATGATAATTATATAGAT/AGGTATATAGCACCTAAAAATAATATACT-3′
DD3-Rv[a]	5′-AGTATATTATTTTTAGGTGCTATATACCT/ATCTATATAATTATCATTAATAATATAATATTCTGTATC-3′
DD4-Fw	5′-TAGTGTTAGTTTTTGGATTAAGATA/TCTAAAGATTTAACTAATTCTCATA-3′
DD4-Rv[a]	5′-TATGAGAATTAGTTAAATCTTTAGA/TATCTTAATCCAAAAACTAACACTA-3′

[a]Oligonucleotide positioned in the complementary strand.
[b]Slashes in parentheses indicate the position of overlap junctions.
[c]The underline indicates Nco I site.
[d]The underline indicates Hind III site.

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2.3. TLC overlay assay

The recombinant H_C proteins were biotinylated with EZ-Link™ Sulfo-NHS-LC-biotin (Pierce, Rockford, IL). The gangliosides GD1b and GT1b (Sigma) (0.25 nmol each) and PE from bovine brain (Sigma) (0.5 nmol) were chromatographed on plastic-coated TLC plates (Macherey-Nagel, Düren, Germany) in chloroform/methanol/water 5:4:1 (v/v). The TLC plates were then dried and blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) containing 1% polyvinylpyrrolidone for 12 h at 4 °C. After blocking, the plates were incubated with 5 nM biotinylated H_C for 2 h at 37 °C in HBS. After washing three times in HBS to remove unbound H_C, the plates were incubated with 1 μg/ml horseradish peroxidase (HRP)-conjugated avidin (Zymed Laboratories Inc., San Francisco, CA). The H_C binding lipids were detected using 3,3′-diaminobenzidine (DAB)-stable substrate solution (Wako, Osaka, Japan).

2.4. Others

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% gels by the method used by Laemmli [34]. Protein concentration was determined by Lowry's method using bovine serum albumin as a standard [35].

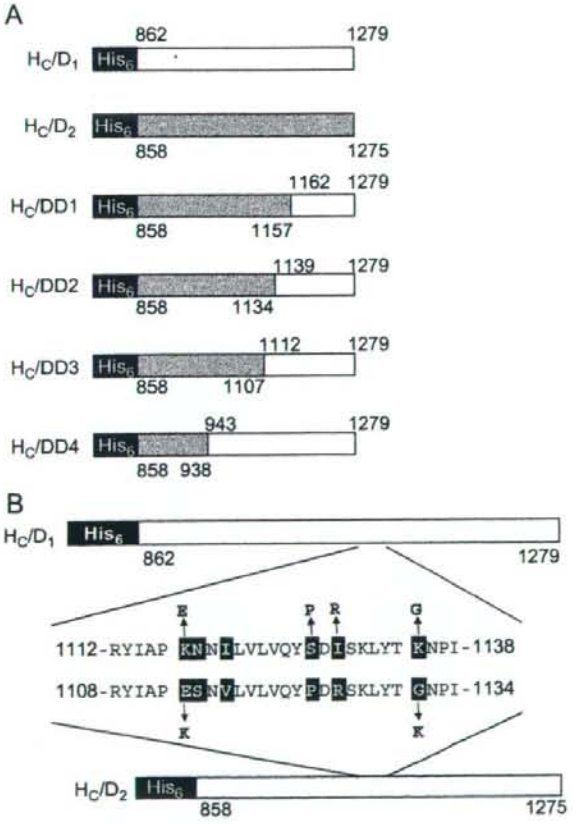

Fig. 2. Preparation of chimeric mutants and site-directed mutants of H_C/D: (A) schematic representation of the recombinant wild-type H_C and chimeric H_C. The numbers correspond to the position of the amino acids and (B) schematic representation of the recombinant site-directed mutants and the amino acid sequence of the region targeted for mutagenesis in this study.

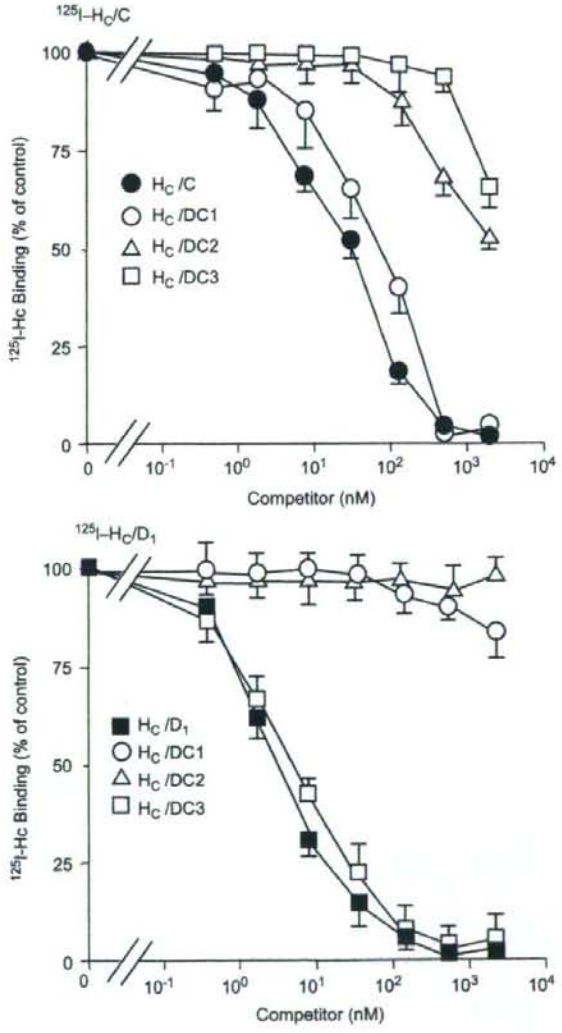

Fig. 3. Competition binding assay of ^{125}I-H_C/C and H_C/D_1 to rat brain synaptosomes with wild type and D/C chimeric H_C. ^{125}I-H_C (0.5 nM) was incubated with synaptosomes (1 μg protein in 0.2 ml) at 37 °C for 60 min in the presence of various concentrations of unlabeled H_C. Each data point represents the mean ± S.D. from three separate experiments each performed in duplicate.

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3. Results

3.1. Expression and binding activities of DC chimeric H_c

We previously constructed H_C genes (H_C/C, H_C/D_1, and H_C/D_2) in pET-30a and obtained recombinant proteins that possessed binding activities similar to that of the neurotoxins [29].

To examine the recognition site for the receptor, we generated three kinds of chimeric H_C (H_C/DC1, H_C/DC2, and H_C/DC3) using overlap extension PCR (Fig. 1B). SDS-PAGE profiles revealed that all recombinant proteins were successfully obtained in a pure state (Fig. 1C).

To evaluate the binding activity of the chimeric H_C, we performed a binding experiment using ^{125}I-wild-type H_C (Fig. 3). H_C/DC1 effectively inhibited the binding of ^{125}I-H_C/C to synaptosomes but not that of ^{125}I-H_C/D_1, whereas H_C/DC3 exhibited an inhibitory effect on the binding of ^{125}I-H_C/D_1. H_C/DC2 did not show any inhibitory effect on the binding of either ^{125}I-H_C/D_1 or ^{125}I-H_C/C. In order to characterize the binding specificity of chimeric H_C, a TLC overlay assay was performed (Fig. 4). H_C/DC1 was found to bind GD1b and GT1b, but not PE. On the other hand, H_C/DC3 retained the PE binding ability. H_C/DC2 hardly associated with any lipid molecules.

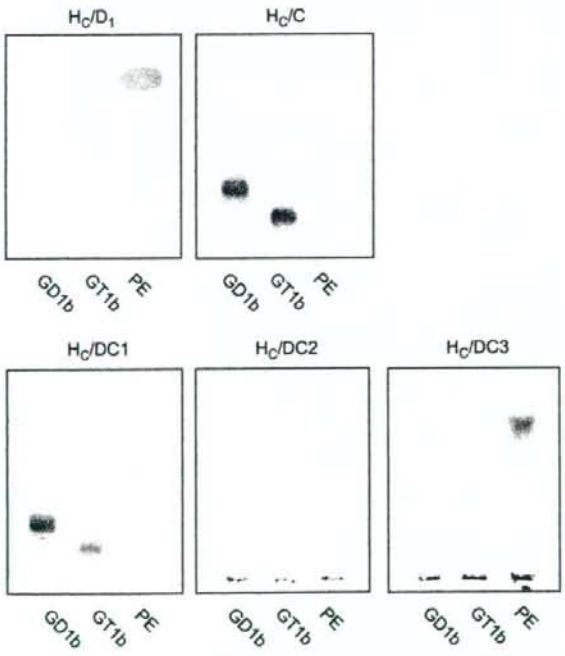

Fig. 4. TLC overlay analysis to detect the direct binding of H_C to lipid receptors. Gangliosides (0.25 nmol) and phosphatidylethanolamine (1 nmol) were chromatographed on plastic-coated TLC plates. After blocking, the plates were incubated with 5 nM biotinylated H_C at 37 °C for 2 h in HBS, followed by treatment with 1 μg/ml HRP-avidin. The H_C binding lipids were detected using DAB substrate solution.

3.2. Determination of the critical amino acid residues involved in ganglioside-binding

Since previous chimeric mutagenesis studies had implicated the region between Y1122 and S1278 of BoNT/C as being important for ganglioside-binding, eight single point mutations of H_C/C were generated using the QuickChange kit. All mutations were confirmed by nucleotide sequencing of both strands of the mutated H_C/C. The previous experiments with TeNT found that the lactose binding site is characterized by the presence of the peptide motif H···SXWY···G [36]. The motif is conserved among most BoNTs (Fig. 5). Especially, W1283 in BoNT/C is common residues among all clostridial neurotoxins, but residue H1282, which has a positive charge, specifically exists immediately prior to W1283 in only BoNT/C. We thus constructed mutants with single amino acid changes in H_C/C, as the following W1257A, W1257F, Y1258A, G1270A, H1282A, H1282E, W1283A, and W1283F (Fig. 6), and determined their binding activities with rat brain synaptosomes. All the mutants except for W1283A and W1283F had strong decreases in binding activity against synaptosomes (Fig. 7).

3.3. Binding activity of H_c/D mutants

We showed that H_C/D interacts with PE in the TLC overlay assay, but it was still unclear whether H_C/D could bind to PE on synaptosomes. Cinnamycin (Ro 09-0198), which is a tetracyclic peptide antibiotic, is known to associate with PE [37,38]. To investigate which molecule was recognized by H_C/D on the membrane, the inhibitory effects of cinnamycin on the binding of ^{125}I-H_C/D to rat brain synaptosomes were examined. The binding of H_C/D_1 and H_C/D_2 was inhibited in the presence of cinnamycin in a dose dependent manner, but the binding of H_C/C was not inhibited (Fig. 8).

To explore the critical region for the binding of H_C/D, four kinds of chimeric H_C (H_C/DD1, H_C/DD2, H_C/DD3, and H_C/DD4) were prepared (Fig. 2A). The binding of ^{125}I-labeled H_C/D_1 was competed by unlabeled wild-type H_C/D_1 and H_C/D_2 in a concentration dependent manner, whose IC_{50} (50% inhibition concentration) values were 0.4 and 152 nM, respectively (Table 2). H_C/DD1 and H_C/DD2 inhibited the binding of ^{125}I-labeled H_C at the same level as H_C/D_2 (IC_{50} = 178 and 165 nM, respectively), whereas H_C/DD3 and H_C/DD4 were found to possess high affinity binding activity (IC_{50} = 0.7 and 0.4 nM, respectively), whose values were equivalent to that of H_C/D_1. Accordingly, residues 1112–1139 of BoNT/CD are assumed to take part in the formation of the high affinity binding site. In this region, there are six amino acid residue differences between H_C/D_1 and H_C/D_2. To identify the critical residues that participate in the binding, point mutations of H_C/D_1

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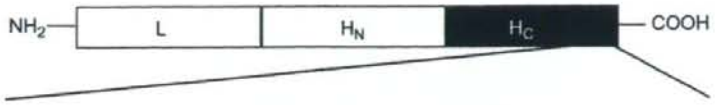


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A     1221 VMKSKNDQGITNKCKMNL-QDNNGNDIGFIGFHQFNN---------I 1257
B     1209 IKEYDEQP-TYSCQLLFKKDEESTDEIGLIGIHRFYE-SGIVFEEYK 1253
C     1217 IFQIQPMNNTYYYASQIFKSNFNGENISGICSIGTY----------- 1249
D/C   1207 SFIIQPCNTFDYYASQLFLSSNATTNRLGILSIGSYS---------- 1243
C/D   1208 -FSIKNIVSQNKYCSQIF-SSFMKNTMLLADIYKPW---------- 1241
D     1204 -FSIKNIVSKNKYCSQIF-SSFRENTMLLADIYKPW---------- 1237
E     1188 VMNS-----VGNNCTMNF-KNNNGNNIGLLGFKA---------- 1215
F     1214 VMDS-----IGNNCTMNF-QNNNGGNIGLLGFHS---------- 1241
G     1217 KKYYEKT--TYNCQILCEKD---TKTFGLFGIGKFVKDYGYVWDTYD 1259
TeNT  1241 AVKLRDLK--TYSVQLKLY-DDKNASLGLVGTH---NGQIGNDPNR  1280

A     1258 AKLVASNWYNRQIERSSRTL--GCSWEFIPVDD-GWGERPL       1295
B     1254 DYFCISKWYLKEVKRKPYNLKLGCNWQFIPKDE-GWTE          1290
C     1250 RFRLGGDWYRHNYLVPTVKQ--GNYASLLESTSTHWGFVPVSE     1290
D/C   1244 FKLGDDYWFNHEYLIPVIKI--EHYASLLESTSTHWVFVPASE     1284
C/D   1242 RFSFENAY------TPVAVTN--YETKLL-STSSFWKFISRDPGWVE 1279
D     1238 RFSFKNAY------TPVAVTN--YETKLL-STSSFWKFISRDPGWVE 1275
E     1216 DTVVASIWYYTHMRDH--TNSNGCFWNFI-SEEHGWQEK        1251
F     1242 NNLVASSWYYNNIRKN--TSSNGCFWSFI-SKEHGWQEN        1277
G     1260 NYFCISCWYLRRISENINNLRLGCNWQFIPVDE-GWTE          1296
TeNT  1281 DILIASNWYFNHL----KDKILGCDWYFVPTDE-GWTND        1314
                 **          *          **
```

Fig. 5. Amino acid sequence alignment of the carboxyl-terminal region of TeNT and BoNT (A-G). The amino acid residues forming the ganglioside-binding pocket in TeNT and BoNTs/A and B are presented as white letters on a black background. Positions of amino acids of BoNT/C selected for mutational analyses are highlighted by asterisks below the sequence of TeNT.

and H_C/D_2 were generated (Fig. 2B). Consequently, S1127P and I1129R retained the inhibitory effect on the binding of ^{125}I-H_C/D_1 to synaptosomes ($IC_{50} = 0.5$ and 0.3 nM, respectively), but K1117E and K1135G were shown to reduce the binding affinity ($IC_{50} = 173$ and 1.2 nM, respectively) (Table 2). These results indicate that two lysine residues are involved in the binding, but the K1117 is more effective residue than K1135. On the contrary, the mutations E1113 K and G1131 K in H_C/D_2 were shown to increase the binding affinity ($IC_{50} = 9.1$ and 2.0 nM, respectively). The double mutation of E1113 K and G1131 K was characteristic of the high affinity binding of authentic H_C/D_1 ($IC_{50} = 0.6$ nM).

4. Discussion

Clostridial neurotoxins bind to nerve terminals through the H_C region derived from the heavy chain. The binding sites on TeNT were localized by co-crystallization experiments and biochemical approaches using TeNT mutants [19,20,36]. The lactose binding site is characterized by the presence of a peptide motif H···SXWY···G. A similar motif is conserved among the BoNTs/A, B, E, F, and G proteins, but not BoNTs/C and D. BoNT/C retains a part of the motif, "WY···G"; however, BoNT/D lacks this peptide motif completely [39]. These observations also suggest that BoNTs/C and D share different properties with respect to the interaction with carbohydrate molecules in comparison with other clostridial toxins. To confirm this hypothesis, we have analyzed the binding properties of H_C mutants at the carboxyl-terminal region of BoNTs/C and D. Our present mutagenesis study has now identified several specific amino acid residues that possess a critical role in binding to gangliosides and PE.

First, we focused on the H_{CC}-subdomain and generated chimeric mutants that comprise parts of BoNT/D-H_C and BoNT/C-H_C by overlap extension PCR. The degree of sequence homology between the BoNT/C-H_C and BoNT/D-H_C genes is 64%, and they have very few restriction sites in common that can be used in constructing chimeric mutants. Therefore, overlap extension PCR was useful to construct the chimeric mutants and to analyze the binding region in BoNT. In the binding experiment using brain synaptosomes, a chimeric H_C that included the carboxyl-terminal half of BoNT/C-H_C (H_C/DC1) exhibited the same level of activity as BoNT/C-H_C, while the other chimeric H_C consisting mainly of BoNT/D-H_C and 11 residues of the carboxyl-terminal of BoNT/C-H_C (H_C/DC3) showed a binding activity similar to that of BoNT/D-H_C. Halpern et al. reported that the removal of H_{CN} from the heavy chain of tetanus neurotoxin did not reduce the binding activity, whereas deletion of only 10 residues from the carboxyl-terminal abolished its binding to spinal cord neurons [40]. However, the present data on

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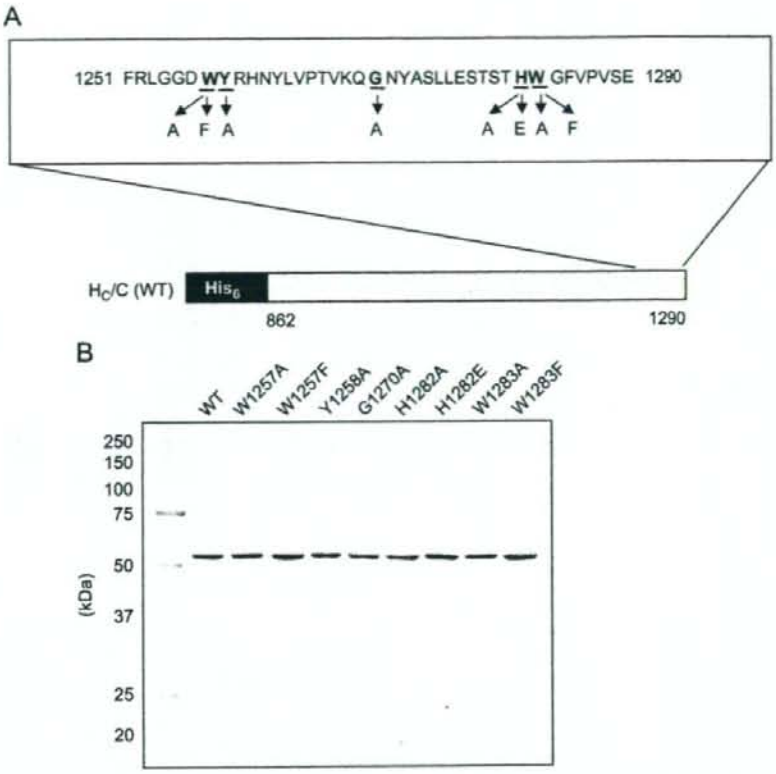

Fig. 6. Preparation of site-directed mutants of H_C/C: (A) schematic representation of the recombinant site-directed mutants and the amino acid sequence of the region targeted for mutagenesis in this study and (B) SDS-PAGE profile of the recombinant H_C. The sample (1 μg/lane) was applied to a 10% polyacrylamide gel.

BoNT/D showed that the carboxyl-terminus 10 residues of BoNT/D were not involved in the binding because H_C/DC3 retained its ability to bind to PE. H_C/DC1 actually bound both gangliosides GD1b and GT1b but not PE, whereas H_C/DC3 interacted only with PE. Although H_C/DC2 contained ganglioside-binding motif, H_C/DC2 was not able to recognize any molecule. This discrepancy is not able to explain clearly. The binding region of H_C/DC2 was probably hooded, owing to the conformational change by chimeric mutation. These results suggest that the binding site for PE formed an inherent structure. Consequently, this study documents the critical regions of the H_{CC}-subdomain of BoNT/C in receptor recognition. In BoNT/C, the recognition site for GD1b and GT1b is located within amino acid residues 1122–1290. In BoNT/D, these results show a loss of inhibition of H_C/DC1 and H_C/DC2 on ^{125}I-H_C/D_1 binding, thus suggesting, according to Fig. 1B, a recognition site located within amino acid residues 1222–1279. Even more, this site can be restricted to 1222–1263, as amino acid residues 1264–1279 of BoNT/CD do not appear to be necessary for the inhibition to occur as they can be replaced by amino acid residues 1279–1290 of BoNT/C.

Second, we have generated eight single point mutants of five amino acid residues to clarify the molecular interactions between the BoNT/C H_C residues of the deduced binding site and gangliosides. In BoNT/C molecules, a part of the ganglioside binding pocket (WY···G), which are conserved in TeNT, BoNT/A, and BoNT/B, is observed. We have carried out substitutions of W1257, Y1258, G1270, H1282, and W1283. H1282 is the amino acid residue that possesses a positive charge and exists only in BoNT/C. W1283 is a common residue conserved in all clostridial neurotoxins. Mutations of W1257, Y1258, and G1270, residues located in positions comparable to the ganglioside-binding pockets of TeNT and BoNTs/A and B, dramatically affected the binding activity. Since the decrease in binding activity was observed in not only W1257A but also W1257F, the indole ring of W1257 is thought to be essential, forming strong interactions with the hydrophobic side of the sugar ring. This finding suggests that W1257, Y1258, and G1270 form a ganglioside-binding pocket in BoNT/C as well as other types of neurotoxins. The influence of the histidine on the functionality of the BoNT/C binding pocket is also important. The glutamate substitution of H1282 is more

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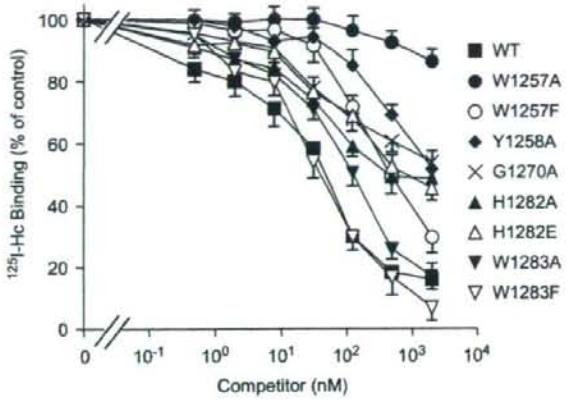

H_C	IC_{50} (nM)
WT	21
W1257A	>1000
W1257F	297
Y1258A	>1000
G1270A	448
H1282A	151
H1282E	267
W1283A	56
W1283F	14

Fig. 7. Competition binding assay of ^{125}I-H_C/C to rat brain synaptosomes with site-directed mutants of H_C/C. ^{125}I-H_C/C (0.5 nM) was incubated with synaptosomes (1 μg protein in 0.2 ml) at 37° C for 60 min in the presence of various concentrations of unlabeled H_C. Each data point represents the mean ± S.D. from three separate experiments each performed in duplicate.

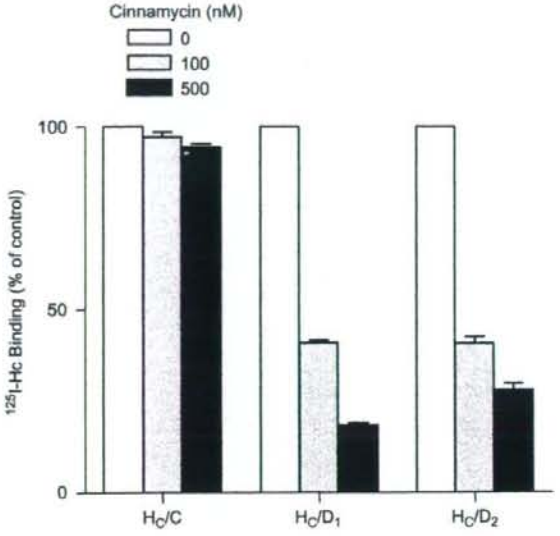

Fig. 8. Effect of cinnamycin on the binding of ^{125}I-H_C to rat brain synaptosomes. ^{125}I-H_C (0.5 nM) was incubated with synaptosomes (1 μg protein in 0.2 ml) at 37 °C for 60 min in the presence of various concentrations (0, 100, and 500 nM) of cinnamycin. Each data point represents the mean ± S.D. from three separate experiments each performed in duplicate.

Table 2
Binding activity of H_C/D mutants

Recombinant H_C	Binding activity[a] (nM)
Wild type	
D_1	0.4
D_2	152
Chimeric mutant	
DD1	178
DD2	165
DD3	0.7
DD4	0.4
Site-directed mutant of H_C/D_1	
K1117E	173
S1127P	0.5
I1129R	0.3
K1135G	1.2
Site-directed mutant of H_C/D_2	
E1113K	9.1
G1131K	2.0
E1113K/G1131K	0.6

[a]Data are the concentrations for 50% binding inhibition by competition binding assays of ^{125}I-labeled H_C/D_1.

effective than the alanine substitution, indicating that the positive charge of H1282 of BoNT/C enhances the binding affinity to gangliosides. Unlike other neurotoxins, BoNT/C is able to bind to gangliosides under physiological ionic strength (0.15 M NaCl) [29]. H1282 is most probably a key amino acid in the high affinity binding of BoNT/C to gangliosides. On the other hand, the mutation of W1283 barely affected the binding, suggesting that W1283 of BoNT/C is not including in the ganglioside-binding pocket.

Third, we focused on the binding site of PE in BoNT/D-H_C. Cinnamycin (Ro 09-0198), which binds specifically to PE [37,38], was used as a competitor of BoNT/D-H_C binding to brain synaptosomes. Interaction of BoNT/D-H_C but not BoNT/C-H_C with synaptosomes was inhibited by the presence of cinnamycin, supporting the notion that BoNT/D-H_C recognized PE on the membranes of brain synaptosomes. For the purpose of identification of the binding region of BoNT/D, we examined the binding activity of four chimeric mutants (H_C/DD1–H_C/DD4) and seven site-directed mutants. As a result, two lysine residues (K1117 and K1135 in H_C/D_1) were the most important amino acid residues in the high affinity binding of BoNT/D to PE. Unlike other types, the C-terminal region of H_C is not probably involved in the recognition of its receptor.

Recently, it was reported that TeNT possesses two binding sites against carbohydrate causing higher affinity to gangliosides than BoNTs/A and B [36,39]. Whether

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BoNT/C contains multiple binding sites is not clear, though the high affinity binding of BoNT/C to gangliosides indicates this possibility. Accordingly, the crystallographic analysis provides useful information to clarify the interaction between BoNT/C and gangliosides. In conclusion, these findings are valuable in that we have elucidated the role of BoNTs/C and D. Although additional studies using other mutants of BoNT/C-H_C and BoNT/D-H_C and three dimensional structural analyses are needed to clarify the form of the binding regions, the present study will be useful in developing effective binding inhibitors or therapeutic agents against neuronal disorders.

Acknowledgments

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Proposed WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins

NOTE: This document has been prepared for the purpose of inviting comments on the proposals contained therein, and for the preparation of the materials to be considered by the Expert Committee on Biological Standardization. Comments proposing modifications for this text MUST be received by 1 October 2008 and should be addressed to the attention of Dr Ana Padilla, World Health Organization, at the following e-mail address padillaa@who.int, with copy to bissumbharb@who.int, or by fax at the number +41 22 791 4889.

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