Molecular characterization of binding subcomponents of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes

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*Clostridium botulinum* type C 16S progenitor toxin consists of a neurotoxin (NTX), a non-toxic non-HA (NTNH), and a haemagglutinin (HA). The HA acts as an adhesin, allowing the 16S toxin to bind to intestinal epithelial cells and erythrocytes. In type C, these bindings are dependent on sialic acid. The HA consists of four distinct subcomponents designated HA1, HA2, HA3a, and HA3b. To identify the binding subcomponent(s) of HA of type C 16S toxin, all of the HA-subcomponents and some of their precursor forms were produced as recombinant proteins fused to glutathione S-transferase (GST). These proteins were evaluated for their capacity to adhere to intestinal epithelial cells of guinea pig and human erythrocytes. GST-HA1, GST-HA3b and GST-HA3 (a precursor form of HA3a and HA3b) bound intestinal epithelial cells and erythrocytes, whereas GST alone, GST-HA2 and GST-HA3a did not. GST-HA3b and GST-HA3 showed neuraminidase-sensitive binding to the intestinal epithelial cells and erythrocytes, whereas GST-HA1 showed neuraminidase-insensitive binding. TLC binding assay revealed that GST-HA3b and GST-HA3 recognized sialosylparagloboside (SPG) and GM3 in the ganglioside fraction of the erythrocytes, like native type C 16S toxin [Inoue, K. et al. (1999). *Microbiology* 145, 2533–2542]. On the other hand, GST-HA1 recognized paragloboside (PG; an asialo- derivative of SPG) in addition to SPG and GM3. Deletion mutant analyses of GST-HA3b showed that the C-terminal region of HA3b is important for its binding activity. Based on these data, it is concluded that the HA component contains two distinct carbohydrate-binding subcomponents, HA1 and HA3b, which recognize carbohydrates in different specificities.

INTRODUCTION

Seven antigenically distinct types (types A to G) of botulinum neurotoxin are produced by the Gram-positive, spore-forming bacterium *Clostridium botulinum*. The neurotoxin (NTX, ~150 kDa) is in the form of a stable complex due to its association with nontoxic proteins. The complex, designated a progenitor toxin, is found in three

Abbreviations: GSL, glycosphingolipid; GST, glutathione S-transferase; HA, haemagglutinin; LacCer, lactosylceramide; NTNH, non-toxic non-haemagglutinin; NTX, neurotoxin; PG, paragloboside; SPG, sialosylparagloboside.
forms; 12S toxin (~300 kDa), 16S toxin (~500 kDa) and 19S toxin (~900 kDa) (Sakaguchi et al., 1984). Type A progenitor toxin has three forms, 19S, 16S and 12S. Type B, C and D toxins have two forms, 16S and 12S. Type E and F toxins have only a single form, 12S, as does type G toxin, 16S. By comparative genetic and biochemical analyses of these progenitor toxins, precise subunit structures of the toxins were elucidated (for reviews, see Minton, 1995; Oguma et al., 1995, 1997, 1999). The 12S toxin consists of an NTX and a nontoxic component having no haemagglutinin (HA) activity, designated non-toxic non-HA (NTNH). The 16S toxin consists of an NTX, an NTNH and an HA. The HA of types A–D consists of four different subcomponents: HA3b, HA1, HA3a and HA2 with the nomenclature introduced in this paper. They correspond respectively to HA-53 (53 kDa), HA-33 (33 kDa), HA-22–23 (22–23 kDa) and HA-17 (17 kDa) in type C (Fujinaga et al., 1994) and type D (Ohyama et al., 1995), HA-52, HA-35, HA-19–20 and HA-15 in type A (Inoue et al., 1996), and HA-51, HA-34, HA-19–23 and HA-18 in type B (Arimitsu et al., 2003). The type A 19S toxin consists of the same protein components as the 16S toxin; the 19S toxin is presumed to be a dimer of the 16S toxin conjugated through HA1 molecules (Inoue et al., 1996). NTX, NTNH and HA are associated non-covalently in the 16S and 19S toxins. Four different subcomponents of the HA are also associated non-covalently. In alkaline conditions (> pH 7.2), the 16S and 19S toxins are dissociated into two molecules: an NTX and a nontoxic component (a complex of NTNH and HA).

The NTX blocks, specifically and with high potency, the release of acetylcholine from the peripheral nerve terminus. This poisoning mechanism was characterized as the cleavage of the proteins involved in the fundamental process of exocytosis (reviewed by Jahn & Niemann, 1994; Montecucco & Schiavo, 1994; Schiavo et al., 2000). Human and animal food-borne botulism are caused by ingestion of food or feed containing the progenitor toxin. The orally ingested progenitor toxin is absorbed from the upper small intestine into the lymphatic system (Sakaguchi et al., 1984), then enters the bloodstream, and reaches peripheral nerves, where neurological dysfunction is elicited.

It has been reported that the progenitor toxin possesses greater potential for oral toxicity than does NTX alone, since the former is more resistant to low pH and proteases in the digestive tract than the latter (Sakaguchi et al., 1984). Thus the nontoxic components are presumed to have the role of protecting the neurotoxin against acidity and proteases in the digestive tract. In addition, we showed that HA functions as an adhesin in the attachment of 16S and 19S toxins to the microvilli of the upper small intestine. (Fujinaga et al., 1997, 2000). In type A, HA1 and HA3b are the binding subcomponents; HA1 recognizes galactose like HA-positive progenitor toxin (16S and 19S toxins), whereas HA3b recognizes sialic acid (Fujinaga et al., 2000). On the other hand, type C 16S toxin recognizes sialic acid (Fujinaga et al., 1997, Inoue et al., 1999). In this study, we produced all of the type C HA-subcomponents, including some of their precursor forms, as proteins fused with glutathione S-transferase (GST), to identify and characterize the type C HA-subcomponents exhibiting binding activity toward intestinal epithelial cells and erythrocytes.

METHODS

Construction of expression vectors. Purified c-st phage DNA (Fujinaga et al., 1994) was used as a template for amplification by PCR as described previously (Fujinaga et al., 1995). Primer sets (Table 1) were designed from the DNA sequences of type C HA genes (Tsuzuki et al., 1990; Fujinaga et al., 1994; Hauser et al., 1994), and used in PCR to amplify the region encoding HA2, HA3a, HA3am, HA1, HA3b and HA3, and deletion mutants of HA3b. For the amplification of genes encoding HA2, HA3a, and deletion mutants of HA3b, one cycle consisted of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. For the amplification of genes encoding HA3b, HA3, and deletion mutants of HA3b, one cycle consisted of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C. The amplified products were digested with BamHI and Sall, purified, and then ligated to the pGEX-5×3 expression vector. For each of these constructs, the nucleotide sequence of the fused gene was verified to ascertain that no modifications had been introduced during the PCR and cloning procedure.

Expression and purification of GST-HA-subcomponent fusion proteins. These recombinant plasmids were introduced into Escherichia coli DH5α, and GST-HA-subcomponent fusion proteins were expressed and affinity-purified with glutathione Sepharose 4B as described previously (Fujinaga et al., 2000). The yields of the fusion proteins were about 0.5–1 mg for GST-HA2, about 13 mg for GST-HA3am, about 10 mg for GST-HA3am, about 6 mg for GST-HA1, about 40 mg for GST-HA3b, and about 12 mg for GST-HA3, when prepared from 2 litres of broth cultures. The yield of GST-HA2 was extremely low compared with that of the other fusion proteins.

GST-free HA-subcomponents were prepared by Factor Xa protease treatment of the GST-fusion proteins as described previously (Fujinaga et al., 2000).

Purification of 16S toxin. The type C 16S toxin was purified from the culture fluid of C. botulinum type C strain Stockholm (C-ST) according to the procedure employed for purifying type A toxins (Inoue et al., 1996). Briefly, the toxin in the culture fluid was precipitated by 50% saturation of ammonium sulfate. The precipitate was dialysed against 50 mM sodium acetate buffer (pH 4.2), and then applied on to an SP-Toyopearl 650M (Tosoh) column. The 16S toxin separated by this column chromatographic procedure was further purified by gel filtration on a Sephacryl S-300 (Amershams) column.

SDS-PAGE and immunoblot analysis. SDS-PAGE was performed by the method of Laemmli (1970), using a 4% stacking gel and a 12.5% separating gel under reducing conditions. Proteins were stained with Coomassie brilliant blue R-250 (Merck). For immunoblot analysis, proteins were electrotransferred and reacted with 104-fold-diluted rabbit polyclonal anti-type C 16S toxin serum (Oguma et al., 1980), and then reactive bands were visualized using the ECL detection system (Amershams).

Binding assay with intestinal tissue sections. The binding of the 16S toxins and recombinant proteins to paraformaldehyde-fixed sections of guinea pig upper small intestine was assayed as described previously (Fujinaga et al., 2000).
Table 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Sequence (5’–3’)</th>
<th>Product</th>
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<tr>
<td>HA2F</td>
<td>TGAatggtcctcataagtgaagaacccc</td>
<td>HA2 (from Ser-2 to Leu-146)</td>
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<tr>
<td>HA2R</td>
<td>TCTtgctgctaatagatgtgcatc</td>
<td>HA3a (from Glu-7 to Lys-192)</td>
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<tr>
<td>HA3a-F</td>
<td>TATggtcctcataagtgaagaacccc</td>
<td>HA3a (from Met-1 to Lys-192)</td>
</tr>
<tr>
<td>HA3a-R</td>
<td>ATtgctgctaatagatgtgcatc</td>
<td>HA3a (from Ser-2 to Ile-286)</td>
</tr>
<tr>
<td>HA3b-F</td>
<td>GITAAAGggtatcccaattcaataataat</td>
<td>HA3b (from Asn-193 to Ser-623)</td>
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<tr>
<td>HA3R</td>
<td>TTATgctgctaatagatgtgcatc</td>
<td>HA3 (from Met-1 to Ser-623)</td>
</tr>
<tr>
<td>HA3F</td>
<td>GGggtatcccaattcaataataat</td>
<td>HA3bN119 (from Lys-312 to Ser-623)</td>
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<tr>
<td>HA3R</td>
<td>TTATgctgctaatagatgtgcatc</td>
<td>HA3bN151 (from Ser-344 to Ser-623)</td>
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<td>HA3N297F</td>
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<td>HA3bN297 (from Ser-490 to Ser-623)</td>
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<td>HA3bN363 (from Thr-556 to Ser-623)</td>
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<td>HA3C25R</td>
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<td>HA3bC13 (from Asn-193 to Leu-610)</td>
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<td>HA3C13R</td>
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Binding assay with erythrocytes. The binding of the 16S toxins and recombinant proteins to human erythrocytes (type O) was assayed as described previously (Hoschützky et al., 1989; Fujinaga et al., 2000).

Neuraminidase treatment of erythrocytes. Human type O erythrocytes (10% suspension in PBS) were treated with *Actinobacillus urefaciens* neuraminidase (a highly purified preparation containing no protease, N-acetylneuraminic acid aldolase or glycosidase; Nacalai Tesque) at a final concentration of 0.01 unit ml⁻¹, at 37°C for 1 h. The erythrocytes were then washed twice with PBS and suspended in PBS (50% neuraminidase-treated erythrocytes).

Preparation of glycosphingolipid (GSL) fractions from erythrocyte membranes. Packed erythrocytes (human, type A) were lysed in 10 vols 0.5% acetic acid, and then the stroma was collected and washed five times with water by centrifugation at 2600 g for 20 min. Lipids were extracted sequentially from the stroma with 20 vols each of chloroform/methanol (successively 2:1, 1:1, and 1:2, v/v), and chloroform/methanol/water (30:60:8, by vol.) at room temperature overnight. The lipid extracts were combined and evaporated to dryness in vacuo, then the residue was dissolved in chloroform/methanol (1:1, v/v). The redissolved extracts were subjected to mild alkaline hydrolysis by addition of 1 M sodium methylate (Wako Pure Chemical) at room temperature for 3 h. The solution was neutralized with acetic acid, and then dialysed against distilled water, and evaporated to dryness in vacuo. Standard GSLs were collected and dried against distilled water, and evaporated to dryness in vacuo. Standard GSLs were purified from human erythrocytes (Hakomori & Siddiqui, 1974; Ledeen et al., 1982). Paragloboside (PG) was isolated from bovine erythrocytes (Uemura et al., 1978). The structures of these GSLs are shown in Table 2.

TLC binding assay. The binding assay for GSLs on TLC plates was performed according to the method of Magnani et al. (1982) with slight modifications. GSLs were spotted on a high-performance TLC plate (Silica Gel 60 F-254, Merck), and developed with chloroform/methanol/water (60:40:9, by vol.). The dried plate was soaked for 1 min in a 0.02% solution of polyisobutylmethacrylate (Tokyo Kasei Kogyo) dissolved in hexane, allowed to air-dry, and then blocked by incubation in PBS containing 1% BSA (Wako Pure Chemical), 1% polyvinylpyrrolidone (Wako Pure Chemical) and 0.02% NaN₃ (Wako Pure Chemical) (blocking buffer) at 37°C for 30 min. It was then rinsed five times with PBS containing 0.1% Tween 20 (Wako Pure Chemical) (washing buffer) and incubated with GST-HA-subcomponent fusion protein (200 μg ml⁻¹) at room temperature overnight. The plate was then washed five times with washing buffer and soaked at 37°C for 30 min in blocking buffer. After another wash with the washing buffer, the plate was reincubated with 10⁻³-fold-diluted goat anti-GST antiserum (Amersham) at 37°C for 1 h. After that, the plate was washed five times with washing buffer and incubated with 2 x 10⁻³-fold-diluted horse–donkey-peroxidase-conjugated rabbit anti-goat antiserum (DAKO) at 37°C for 1 h. As a final step, it was washed five times with washing buffer, and the activity of the peroxidase was detected with the ECL system (Amersham).
Table 2. Structures of GSLs used in this study

<table>
<thead>
<tr>
<th>GSL</th>
<th>Chemical structure</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>GM3</td>
<td>NeuAc2-3Galβ1-4Glcβ1-1Cer*</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>SPG</td>
<td>NeuAc2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>LacCer</td>
<td>Galβ1-4Glcβ1-1Cer</td>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>Paragloboside</td>
<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer</td>
<td>Bovine erythrocytes</td>
</tr>
</tbody>
</table>

*Cer, ceramide.

Determination of protein concentration. Protein concentration was determined using the Lowry method with BSA as a standard.

RESULTS

Expression of GST-HA-subcomponent fusion proteins

All of the HA-subcomponents present in a type C strain Stockholm 16S toxin and some of their precursor forms were produced as polypeptides fused to Schistosoma japonicum GST (Fig. 1). GST-HA1, GST-HA2, GST-HA3α7 (an N-terminal processed form of HA3αm, starting from 7Glu) and GST-HA3b have the same N-terminals as the corresponding mature HA-subcomponents of the native 16S toxin (Fujinaga et al., 1994). HA3 (a precursor of HA3a and HA3b), which is found in some type C 16S toxins (Kouguchi et al., 2001, 2002), and HA3αm (an N-terminal unprocessed form of HA3α7) were also expressed as GST-fusion proteins. Fusion proteins of the expected sizes (see Fig. 1b) were visualized by SDS-PAGE (Fig. 1c). Western blot analysis shows that all these recombinant proteins were immunoreactive with anti-type C nontoxic-component serum (Oguma et al., 1980), like the HA-subcomponents of native type C 16S toxin (data not shown). In the case of GST-HA2, a band of about 30 kDa was seen in addition to the GST-HA2 band at 43 kDa. By direct N-terminal sequencing of the 30 kDa band and by Western blotting using anti-type C nontoxic-component serum, this protein was found to be a C-terminal degradation product of GST-HA2 fusion protein.

Binding properties of recombinant HA-subcomponent proteins to guinea pig intestinal epithelial cells

The six fusion proteins were evaluated for their capacity to adhere to intestinal epithelial cells of guinea pigs. GST-HA1, GST-HA3b and GST-3 bound to these cells (Fig. 2), whereas GST alone, GST-HA2, GST-HA3α7 and HA3αm did not. The relative intensity of the binding is shown in Table 3. The binding activity of GST-HA1 and GST-HA3 was very strong. GST-HA3b showed weak binding activity compared to GST-HA3. The same results were obtained using GST-free forms of these recombinant proteins (data not shown).

Neuraminidase treatment of the tissue sections completely abolished the binding of native type C 16S toxin, GST-3b and GST-3, but did not abolish the binding of GST-HA1 (Table 3).

Binding properties of recombinant HA-subcomponent proteins to erythrocytes

The binding of the recombinant HA-subcomponents to erythrocytes was analysed. As shown in Fig. 3, GST-HA1, GST-HA3b and GST-HA3 showed strong binding activity, the same as the native type C 16S toxin. GST alone, GST-HA2, GST-HA3α7 and GST-HA3αm showed no binding activity. The same results were obtained using GST-free forms of these recombinant proteins (data not shown).

Neuraminidase treatment of erythrocytes completely abolished the binding of GST-HA3b, GST-HA3 and native type C 16S toxin, but did not affect the activity of GST-HA1. In order to study the cellular receptor(s) for GST-HA1 on the neuraminidase-treated erythrocytes, a binding inhibition test with various saccharides was carried out (Fig. 4a). The binding of GST-HA1 to neuraminidase-treated erythrocytes was markedly inhibited by 100 mM of galactose (Gal), N-acetylgalactosamine (GalNAc) and lactose (Lac). These inhibitory effects were concentration-dependent, with half-maximal inhibition at about 10 mM (Fig. 4b). However, the binding was not inhibited by 100 mM of glucose (Glc), mannose (Man), N-acetylglucosamine (GlcNAc) or NeuAc (Fig. 4a).

Reactivity of GST-HA1, GST-HA3b and GST-HA3 with GSLs in erythrocytes

To identify the carbohydrate epitopes recognized by HA1 and HA3b, the ganglioside fraction and neutral GSL fraction obtained from human erythrocytes were separated by TLC, and the binding of GST-HA1, GST-HA3b and GST-HA3 to them was tested (Fig. 5, Table 3). GST-HA1 bound strongly to GM3 and SPG, the major gangliosides of human erythrocytes, but not to the neutral GSLs. It also reacted strongly with paragloboside (PG; an asialo-derivative of GM3), but not with LacCer (an asialo-derivative of GM3). GST-HA3b and GST-HA3 also bound to GM3 and SPG, but their binding activity was weak compared with that of GST-HA1. Neither GST-HA3b nor GST-HA3 reacted with LacCer, PG or the neutral GSL fraction from human erythrocytes. In addition, GST alone did not show a positive reaction with any GSLs tested, even though 2 mg ml⁻¹ was employed.
Deletion mutant analysis of HA3b

To define the region within HA3b that is required for cell binding, derivatives containing amino-terminal and carboxy-terminal deletions were expressed as GST-fusion proteins (Fig. 6a). These derivatives were stably expressed and purified (data not shown). As shown in Fig. 6(b), deletion at the amino-terminus reduced the binding activity, and GST-HA3bN151, missing 151 amino acids of the amino-terminus of HA3b, was the smallest protein that bound erythrocytes. On the other hand, the deletion of only 13 amino acids from the carboxy-terminus abolished all binding activity.

DISCUSSION

In this study, we have shown that HA1, HA3b and HA3 of type C 16S toxin have the capacity to bind guinea pig intestinal epithelial cells and human erythrocytes. The erythrocyte-binding activity of type C HA1 was demonstrated...
using an HA1 which exists in a free state in type C culture medium (Inoue et al., 1999; Kouguchi et al., 2001), and an HA1 isolated from type C 16S toxin by chromatography in the presence of denaturing agents (Kouguchi et al., 2001). Kouguchi et al. (2001) also demonstrated that HA3 and HA3b isolated from type C 16S toxin by chromatography in the presence of denaturing agents can bind erythrocytes. Our results are consistent with theirs. In the binding assay with guinea pig intestinal tissue sections, GST-HA3 showed stronger binding than GST-HA3b, but these proteins showed similar activity toward erythrocytes. This discrepancy may be explained by the limitation to the quantifiability of our erythrocyte-binding assay: dose curves of the erythrocyte binding obtained using various amounts of native 16S toxin, GST-HA1, GST-HA3 and GST-HA3b were not sigmoid, but steeply sloped (data not shown).

In the TLC binding assay, GST-HA3b and GST-HA3 were reactive to GM3 and SPG, which contain NeuAc\(\alpha\)2-3Gal\(\beta\)1-4Glc\(\beta\)1- and NeuAc\(\alpha\)2-3Gal\(\beta\)1-4Glc\(\beta\)1-Nac\(\beta\)1-3Gal\(\beta\)1-4Glc\(\beta\)1-residues, respectively, but not with the neutral GSL fraction, LacCer (an asialo- derivative of GM3) and PG (an asialo-derivative of SPG). We reported previously that type C 16S toxin exhibits the same ligand specificity toward these GSLs (Inoue et al., 1999). These findings indicate that the epitope recognized by GST-HA3b, GST-HA3 and 16S toxin may be the terminal (at least) disaccharide, NeuAc\(\alpha\)2-3Gal\(\beta\)1- sequence of glycoconjugates, and that NeuAc is crucial for the binding of GST-HA3b, GST-HA3 and 16S toxin to guinea pig intestinal epithelial cells and human erythrocytes. GST-HA1 recognized GM3 and SPG, but not the neutral GSL fraction or LacCer, like GST-HA3b and GST-HA3. This protein, however, could bind to PG. LacCer and PG have a Gal\(\beta\)1-4Glc\(\beta\)1- structure and Gal\(\beta\)1-4Glc\(\beta\)1-Nac\(\beta\)1-3Gal\(\beta\)1-4Glc\(\beta\)1- structure, respectively. These results suggest that GST-HA1 recognizes the terminal (at least) disaccharide (NeuAc\(\alpha\)2-3Gal\(\beta\)1-) structures in GM3 and SPG and the terminal (at least) disaccharide (Gal\(\beta\)1-4Glc\(\beta\)1-Nac\(\beta\)1-) sequence of PG. In the present study, the binding of GST-HA1 to guinea pig intestinal epithelial cells and human erythrocytes was not stopped by neuraminidase treatment. Thus, GST-HA1 recognized the Gal\(\beta\)1-4Glc\(\beta\)1- structure which is the terminal disaccharide sequence of PG (asialo- derivative of SPG) as a receptor. In addition, binding to neuraminidase-treated erythrocytes was inhibited by the addition of Gal, GalNac or Lac, suggesting that the Gal moiety of the Gal\(\beta\)1-4Glc\(\beta\)1-Nac\(\beta\)1- residue is important for the binding of GST-HA1 to the neuraminidase-treated erythrocytes.

The binding assays with guinea pig intestinal tissue sections, with human erythrocytes, and with GSLs extracted from erythrocytes showed that GST-HA3 and GST-HA3b have the same ligand specificity as 16S toxin. It is suggested,

| Table 3. Summary of the binding properties of recombinant proteins |
|-------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Intestine | N-treated* | RBC | N-treated* | RBC | Gangliosides | Neutral GSLs | Asialo-gangliosides |
| GST-HA1 | ++ | +/+++ | ++ | ++ | + | ++ | - | - |
| GST-HA2 | - | - | - | - | ND | ND | ND | ND |
| GST-HA3a | - | - | - | - | ND | ND | ND | ND |
| GST-HA3b | + | - | ++ | - | + | + | - | - |
| GST-HA3 | + | - | ++ | - | + | + | - | - |
| Type C 16S toxin | +++ | - | ++ | - | ++ | + | - | - |

ND, Not done.

*N-treated, neuraminidase treated.

ND, Not done.

*N-treated, neuraminidase treated.
therefore, that HA3b in the 16S toxin contributes directly to the binding of these ligands. In contrast, GST-HA1 showed a different ligand specificity to 16S toxin: GST-HA1 recognizes the galactose moiety in addition to the sialic acid moiety. At this time, we have no experimental data to explain why the ligand specificity of HA1 is different from that of 16S toxin. We speculate that the binding of HA1 to the galactose moiety is disrupted by the tertiary structure of the 16S toxin. Sagane et al. (2001) surmised that HA1 in the 16S toxin has an essential role in the binding of 16S toxin to erythrocytes based on the analyses of 16S toxins that have truncated HA1 toxins produced by type D strain 1873 and type C strain Yoichi. In agreement with this idea, our previous data showed that monoclonal antibodies against type C HA1 inhibit the binding of 16S toxin to intestinal epithelial cells and erythrocytes (Mahmut et al., 2002). These studies may indicate that HA1 in 16S toxin also interacts directly with cellular ligands. Further study of the crystal structure and/or reconstitution of 16S toxin containing mutated HA-subcomponents will be required to understand the role of HA1 and HA3b in the binding activity of 16S toxin.

Analyses of deletion mutants of type C HA3b showed that the removal of 13 amino acids from the carboxy-terminus completely abolished the binding activity. This result indicates that the carboxy-terminal region of type C HA3b is important for binding. In the carboxy-terminal region, we found a sequence that has significant similarity with that of the carbohydrate recognition domain of sialic-acid-binding immunoglobulin-like lectins (Siglecs) (Angata & Brinkman-Van der Linden, 2002), as illustrated in Fig. 7. A conserved arginine residue among Siglecs, which is essential for sialic acid binding (Angata & Brinkman-Van der Linden, 2002), also exists in the sequence of HA3b. Presumably, the 13 amino acid deletion at the carboxy-terminus disrupts this domain in type C HA3b and causes the loss of binding activity. Site directed mutagenesis of the conserved arginine residue is in progress to further characterize this domain in HA3b.

**Fig. 4.** Effect of saccharides on the binding of GST-HA1 to neuraminidase-treated erythrocytes. (a) GST-HA1-coated wells were incubated with various saccharides (100 mM) prior to addition of the neuraminidase-treated erythrocytes, and bound erythrocytes were determined as described in Methods. Background binding to BSA was minimal and was subtracted from the absorbance values. Each point is the mean ± SD for triplicate wells. The experiment depicted is representative of three experiments performed. (b) GST-HA1-coated wells were incubated with the indicated saccharides at a given concentration prior to addition of the neuraminidase-treated erythrocytes, and bound erythrocytes were determined as described in Methods. Background binding to BSA was minimal and was subtracted from the absorbance values. Each point is the mean ± SD of triplicate wells. The experiment depicted is representative of two independent experiments.

**Fig. 5.** Profiles of the binding of GST-HA1 to GSLs extracted from erythrocytes and to standard GSLs. (a) The ganglioside fraction from human erythrocytes was assayed for GST-HA1 binding activity with bound GST-HA1 detected by immunostaining as described in Methods (lanes 1–3), or visualized using orcinol reagent (0.2% orcinol in 1 M H₂SO₄) (lane 4). Lanes: 1, standard GM3; 2, standard SPG; 3 and 4, erythrocyte gangliosides. (b) The neutral GSL fraction from human erythrocytes was assayed for GST-HA1 binding activity (lanes 1–3), or visualized using orcinol reagent (lane 4). Lanes: 1, standard LacCer; 2, standard PG; 3 and 4, erythrocyte neutral GSLs. The positions of the standard GM3, SPG, LacCer and PG are shown.
Hazes (1996) and Sagane et al. (2001) reported that the carboxy-terminus of HA1 has a ricin-like lectin domain. Sagane et al. (2001) also demonstrated that the 16S toxin of *C. botulinum* type D strain 1873 and type C strain Yoichi, which has no erythrocyte-binding activity, has 31 amino acids missing from the carboxy-terminus of HA1. These findings indicate that the carboxy-terminus of HA1 has an essential role in binding, presumably via the ricin-like lectin domain.

Maksymowych & Simpson (1998) reported that type A and B botulinum neurotoxins alone bound to human intestinal cell lines and entered the basolateral compartment via transcytosis. In the case of type C, 16S toxin binds strongly to intestinal epithelial cells of guinea pigs, leading to an efficient absorption of the toxin, whereas the neurotoxin alone and 12S hardly bind to the cells, resulting in a much less efficient absorption (Fujinaga et al., 1997). This means that the HA has a crucial role in the intestinal binding and absorption of the neurotoxin and/or neurotoxin complexes at least in type C botulism. It is worth investigating which glycoconjugate(s) are the intestinal receptor(s) for the HA. In the binding assay with guinea pig intestinal tissue sections, protease treatment of the sections greatly reduced the binding of native type C 16S, GST-HA3, GST-HA3b and GST-HA1 (data not shown). This result may indicate that

**Fig. 6.** GST-HA3b variants and analysis of their binding activities. (a) Schematic representation of GST-HA3b variants. (b) Binding of GST-HA3b variants to erythrocytes. Background binding to BSA was minimal and was subtracted from the absorbance values. Each point is the mean±SD for triplicate wells. The experiment depicted is representative of two independent experiments.

**Fig. 7.** Alignment of sugar-binding domains of the sialoadhesin (Siglec) family and C-terminal region of type C HA3b. The sequences aligned to HA3b are as follows: mouse sialoadhesin (GenBank/EMBL accession number S50065), mouse CD22 (NP_033975), human CD22 (X59350), mouse MAG (AH003393), quail SMP (S83711), mouse CD33 (NM_021293), human CD33 (NM_001772). Two or more residues that are identical are boxed in grey. The conserved arginine residues among the sugar-binding domains of the Siglec family which have been shown to interact with sialic acid (May et al., 1998) are indicated by dots.
these proteins recognize glycoproteins rather than glycolipids on the intestinal cells. Further study is needed to identify the intestinal receptor(s) for HA.

In conclusion, our study found that HA of type C 16S toxin has two distinct binding subcomponents, HA1 and HA3b, which recognize carbohydrates with different specificities. The differences in receptor binding between HA1 and HA3b lead us to speculate that a multi-step dependent activation of 16S toxin binding is needed for the toxin to bind and pass through the barrier of intestinal epithelial cells. This study provides fundamentally important information on the interaction of the 16S toxin with intestinal epithelial cells.

ACKNOWLEDGEMENTS

This work was supported in part by a grant (14370093) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant from the Japan Health Sciences Foundation, and a grant from PRESTO, Japan Science and Technology Agency (JST).

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