Identification of a Binding Site for Ganglioside on the Receptor Binding Domain of Tetanus Toxin†

Heather A. Louch,‡ Ellen S. Buczko,§ Mary A. Woody,‡ Richard M. Venable,§ and Willie F. Vann*,‡

Laboratory of Bacterial Toxins and Laboratory of Biophysics, Division of Bacterial, Parasitic, and Allergenic Products, Office of Vaccine Research and Review, Center for Biologics Evaluation and Research, Federal Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20892

Received April 18, 2002; Revised Manuscript Received September 9, 2002

ABSTRACT: The carboxyl-terminal region of the tetanus toxin heavy chain (H_C fragment) binds to di- and trisialylgangliosides on neuronal cell membranes. To determine which amino acids in tetanus toxin are involved in ganglioside binding, homology modeling was performed using recently resolved X-ray crystallographic structures of the tetanus toxin H_C fragment. On the basis of these analyses, two regions in tetanus toxin that are structurally homologous with the binding domains of other sialic acid and galactose-binding proteins were targeted for mutagenesis. Specific amino acids within these regions were altered using site-directed mutagenesis. The amino acid residue tryptophan 1288 was found to be critical for binding of the H_C fragment to ganglioside GT1b. Docking of GD1b within this region of the toxin suggested that histidine 1270 and aspartate 1221 were within hydrogen bonding distance of the ganglioside. These two residues were mutagenized and found also to be important for the binding of the tetanus toxin H_C fragment to ganglioside GT1b. In addition, the H_C fragments mutagenized at these residues have reduced levels of binding to neurites of differentiated PC-12 cells. These studies indicate that the amino acids tryptophan 1288, histidine 1270, and aspartate 1221 are components of the GT1b binding site on the tetanus toxin H_C fragment.

The tetanus and botulinum neurotoxins are closely related clostridial toxins with similar structure and function. Both tetanus and botulinum toxin are synthesized as 150 kDa polypeptides. Cleavage of the toxin at a surface-exposed loop results in an active two-chain molecule, composed of a 50 kDa light (L) chain and a 100 kDa heavy (H) chain linked by a single disulfide bond. The heavy chain has two regions, each of which is involved in a distinct function. The toxin is first bound to presynaptic nerve terminal membranes within the muscle, and it is the carboxyl-terminal domain of the heavy chain, the H_C fragment,[1] which is involved in this initial binding to neuronal cells. The toxin is then internalized and undergoes retrograde axonal transport to the spinal cord. The amino-terminal domain of the heavy chain (H_N) possesses the ability to translocate the L-chain into the target cell. The toxin inserts itself into the membrane lipid bilayer, and the L-chain is translocated across the membrane. The disulfide bond is then reduced, and the L-chain is free in the cytosol to selectively cleave proteins of the neuroexocytosis machinery. Tetanus toxin blocks inhibitory neurotransmitted impulses in the central nervous system, leading to spastic paralysis. The seven types of botulinum toxin (A−G) target peripheral sensory neurons, resulting in a flaccid paralysis (*1*).

Clostridial neurotoxins are structurally very similar, and their sequences are ∼65% homologous and 35% identical (*2*). Tetanus toxin and botulinum toxins A, B, and E are all synthesized with an amino-terminal proline, not the methionine that the DNA sequence predicts (*3*−*5*). Hence, the numbering of these amino acid sequences should begin with proline. Crystal structures for the receptor-binding domain of tetanus toxin (*6*), the botulinum type A holotoxin (*7*), and the botulinum type B holotoxin (*8*) have recently been reported. The receptor-binding domains for each of these clostridial neurotoxins (tetanus toxin and botulinum toxins A and B) were found to be structurally similar, with the same structural elements: a carboxyl-terminal β-trefoil subdomain and an amino-terminal lectin-like jellyroll subdomain. The tetanus toxin H_C fragment can be expressed independently of the holotoxin. Likewise, the nontoxic toxin H_C fragment displays the binding activity of the intact toxin molecule, and it can undergo retrograde transport (*9*, *10*).

Polysialylgangliosides GD1b and GT1b located on neuronal membranes have long been implicated as receptors for tetanus and botulinum toxins. The addition of ganglioside GD1b and GT1b to chromaffin cells results in tetanus toxin sensitivity (*11*), and treatment of spinal cord cultures with a

† This project was supported in part by an appointment to the Research Participation Program at the Center for Biologics Evaluation and Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

* To whom correspondence should be addressed: Laboratory of Bacterial Toxins, Division of Bacterial, Parasitic, and Allergenic Products, OVRR, CBER, FDA, 8800 Rockville Pike, Bethesda, MD 20892. Phone: (301) 496-2008. Fax: (301) 402-2776. E-mail: wvann@helix.nih.gov.

‡ Laboratory of Bacterial Toxins.

§ Laboratory of Biophysics.

[1] Abbreviations: H_C fragment, carboxyl-terminal heavy chain fragment of tetanus toxin; PC-12, rat pheochromocytoma cells; L-chain, amino-terminal catalytic domain of tetanus toxin; IPTG, isopropyl thiogalactoside; CD, circular dichroism analysis; NGF, nerve growth factor; VDW, van der Waals; GM1, $II^3NeuAcGgOse_4Cer$; GD1b, $II^3(NeuAc)_2GgOse_4Cer$; GT1b, $IV^3NeuAcII(NeuAc)_2GgOse_4Cer$.

10.1021/bi020291j
Published on Web 10/26/2002

ganglioside synthesis inhibitor eliminates binding of tetanus toxin at neuronal surfaces, abolishing tetanus toxin activity (*12*). Evidence has also been presented for the involvement of a 15 kDa glycoprotein receptor in toxin binding (*13*).

While it is known that the H_C fragment region of tetanus toxin and the botulinum toxins contains the receptor-binding domain, little is known about the actual binding site or sites. There have been studies suggesting specific regions of these toxins that may be involved in binding to gangliosides. In one study (*10*), a ganglioside-based radioiodinated photoaffinity ligand was found to bind near tetanus toxin amino acid residue H1292. On the basis of the linker arm structure, any amino acid residue within 20 Å of H1292 could be part of a ganglioside-binding domain. As might be expected, when H1292 was mutagenized, a reduced level of binding to ganglioside GT1b was observed (*14*). Cocrystal structures of clostridial toxins with di- and trisaccharide components of the ganglioside have also provided evidence for a GT1b-binding site in this same region of the toxin (*8*, *15*). In a study with botulinum toxin type A, quenching of tryptophan fluorescence was observed in the presence of ganglioside (*16*). On the basis of the solvent accessibility of the tryptophan residues, amino acid W1265 of botulinum toxin type A was predicted to be part of a ganglioside-binding domain (*2*). The homologous residue in tetanus toxin is W1288, which is in the immediate vicinity of H1292. Tyrosine 1289 of tetanus toxin has recently been reported to be a very important amino acid in binding to both GT1b gangliosides and synaptosomal membranes (*17*). Collectively, these findings suggest that the area around tetanus toxin H1292 is part of a ganglioside-binding pocket. It is also likely that there may be other ganglioside-binding sites on the tetanus toxin H_C fragment.

In this study, we report the use of molecular modeling and site-directed mutagenesis of target amino acid residues in defining the ganglioside-binding domain(s) of tetanus toxin based on homology to known carbohydrate-binding proteins. Two regions of the toxin were selected for site-directed mutagenesis, and surface plasmon resonance was utilized to screen each mutant protein for the ability to bind to a ganglioside GT1b receptor.

EXPERIMENTAL PROCEDURES

Construction of pWL400 and pWL411. Oligonucleotide primers 5′-GCGGGATCCTCTAAAAATCTGGATTGT-TGGGTT-3′ and 5′-GCGCTGCAGCTGTTTAATCATTTG-TCCATCCTTC-3′ (1 μM each) were used to amplify the tetanus toxin H_C fragment gene from pSS1261 (1 ng of template) (*9*) using the Ready-to-go PCR bead system (Amersham Biosciences, Piscataway, NJ) and the following program (45 s at 94 °C; 25 cycles of 45 s at 92 °C, 1 min at 50 °C, and 2 min at 72 °C; and a final extension for 10 min at 72 °C). The product of the PCR was cloned using the pBAD TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). The recombinant plasmid was digested with *Bam*HI and *Pst*I, and the digestion products were separated by agarose gel electrophoresis. The band containing tetanus toxin H_C fragment DNA was excised and purified (GenecleanII Kit, Bio101, Vista, CA). The expression vector pMALcRI (New England Biolabs, Inc., Beverly, MA) was also digested with *Bam*HI and *Pst*I, electrophoresed on an agarose gel, and purified in the same manner. An aliquot of the ligation reaction mixture between pMALcRI and the tetanus toxin H_C fragment gene was transformed into *Escherichia coli* DH5α. Recombinant clones were selected on LB medium supplemented with ampicillin (100 μg/mL). Clones were screened by restriction digestion with *Pst*I and *Bam*HI. Confirmed recombinant clones were assessed for the ability to synthesize a maltose binding protein−H_C fragment fusion protein. To construct pWL411, PCR amplification performed as described above was used to transfer the wild-type H_C fragment gene from pWL400 into pTrcHis (Invitrogen). The wild-type H_C fragment from pWL411 was sequenced in both strands by ACGT, Inc. (Northbrook, IL), and compared to that (entry X06214) in GenBank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD). The pWL411 sequence contained two nucleotide changes that result in conservative amino acid changes.

Expression of Amino-Terminal His-Tagged Tetanus Toxin H_C Fragment Proteins. Overnight cultures of *E. coli* TOP10 carrying plasmid pWL411 or other pTrcHis-derived vectors were diluted (3:100) into fresh, prewarmed (37 °C) LB broth (0.2−3.0 L) containing ampicillin (100 μg/mL) and glucose (2 g/L). Cultures were grown to an OD_{600} of ∼0.6 prior to IPTG (1 mM) induction (2 h at 37 °C). Cultures were harvested at 6500*g* for 15 min, and cell pellets were resuspended in 50 mM Tris (pH 8). Cells were lysed in a French pressure cell, and broken cells were removed by centrifugation (10 min at 13000*g*); then the supernatant was centrifuged for 1 h at 100000*g*. For large cultures (2.0−3.0 L), supernatants were applied directly to a 17−22 mL Ni−NTA agarose (Qiagen, Valencia, CA) column and incubated with the resin overnight. Columns were washed with 10 volumes of 50 mM Tris (pH 8) and eluted with 4 volumes of 50 mM Tris (pH 8.0), 0.2 M NaCl, and 0.25 M imidazole. For small cultures (0.2 L), supernatants were tumbled overnight with 3 mL of Ni−NTA agarose resin. The mixture was then poured into a column; the column was washed and the protein eluted. All steps were performed on ice or in a cold room (8 °C). Initial attempts to express mutant H_C fragments as amino-terminal maltose binding protein fusions were abandoned in favor of the His-tagged proteins because of low yields. Expression of His-tagged H_C fragments yielded ample amounts of the protein after purification.

SDS−Page and Western Blotting. Samples were suspended in a 2× SDS loading buffer, boiled, and then loaded and electrophoresed on a polyacrylamide gel (8 to 12%). The Kaleidoscope protein standard (Bio-Rad Laboratories, Hercules, CA) was included on each gel. Either gels were stained [0.1% Coomassie Blue R250 dissolved in an acetic acid/methanol/water mixture (1:4:5)] to visualize protein bands, or proteins from the gel were transferred onto nitrocellulose membranes for Western blotting. Following the transfer for Western blotting, the membranes were blocked (1 h at 25 °C) with a 5% BSA/1× phosphate-buffered saline (PBS) mixture; after blocking buffer had been decanted, the primary antibody diluted in a 0.1% Tween/5% BSA/1× PBS mixture was added to the membrane (1 h at 25 °C). The primary antibody was removed, and the membrane was washed three times for 10 min with a 0.1% Tween/1× PBS mixture. The secondary antibody (an alkaline phosphatase conjugate), diluted in a 0.1% Tween/5% BSA/1× PBS mixture, was

added to the membrane (1 h at 25 °C). The membrane was washed again as described above, and then the substrate (NBT/BCIP tablets, Roche Diagnostics, Indianapolis, IN) was added to the membrane. The colorimetric reaction was allowed to proceed for up to 1 h, but typically was complete in less than 10 min. When the desired color intensity was achieved, the reaction was stopped with 0.5 M EDTA and the membrane rinsed in water. To visualize His-tagged H_C fragments by Western blotting, anti-Xpress antibody (Invitrogen) was utilized as the primary antibody, and goat anti-mouse alkaline phosphatase conjugate antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used as the secondary antibody.

Primary and Secondary Homology Studies. Sequence alignments of the template proteins against the tetanus toxin H_C fragment were performed with Clustal in PC Gene (Intelligenetics, Mountain View, CA). Secondary structural homology studies were performed in Quanta 98 (Accelrys, San Diego, CA) on a Silicon Graphics platform with CHARMM for energy calculations. These studies used the following carbohydrate-binding templates: the influenza virus type A−Neu5Ac complex (PDB entry 2QWB) (*18*) and the plant toxin ricin B chain−galactose complex (PDB entry 2AAI) (*19*), which contains a β-trefoil motif similar to tetanus toxin, and two Gal binding domains centered at amino acids W37 and Y248. These proteins were chosen because of structural homology identified using the SCOP (structural classification of proteins) index of "Folds" and "Superfamilies" (SCOP database 1.55, scop@mrc-lmb.cam.ac.uk) (*20*). The modeling method used least-squares distance algorithms for superposition, where a sphere of a defined radius (6 Å) was centered on the binding carbohydrate of the superimposed template. Translation of the H_C fragment revealed the homologous residues within 6 Å of the template carbohydrate-binding domain. Additional studies were based on the three-dimensional geometry of key residues in known carbohydrate-binding domains. Distance geometry methods, including superposition in Quanta, were performed using a monomeric unit of the trimer sialoadhesin in complex with sialyllactose (PDB entry 1QFO) (*21*).

For docking within the region 1 domain, GD1b and/or its oligosaccharide components were built in CHARMM (*22*) starting from the GM1 structure of cholera toxin (*23*) or the lactose conformer of tetanus toxin H_C fragment (1DLL). This was energy-minimized with the steepest descents method (*24*). Docking within the region 1 domain (W1288 and H1292) was performed using a grid-walking method (*25*), with modifications. A rigid ligand was translated and rotated within a 17−21 $Å^3$ volume of the tetanus toxin H_C fragment. Candidate amino acids were evaluated by determining the nonbonded energy between the ganglioside and the protein residue within an interaction radius of 4 Å and by considering the number of ligand−protein contacts. Ligand−protein docking positions with minimal electrostatic energy were further evaluated in an effort to identify likely amino acid candidates for mutagenesis.

Site-Directed Mutagenesis. Specific amino acid changes and deletions were introduced into the tetanus toxin H_C fragment using the Stratagene QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and pairs of mutagenic oligonucleotide primers. Initial H_C fragment mutations were introduced into the pWL400 vector and mutant proteins expressed as maltose binding protein−H_C fragment fusions. However, problems were encountered in purifying these fusion proteins, necessitating the transfer of the mutant genes to a different host vector. Thus, the mutant genes were amplified using PCR and cloned into pTrcHis (Invitrogen), enabling mutant proteins to be expressed as His-tagged H_C fragments. Subsequent mutations were all introduced into pWL411. Each mutated plasmid was sequenced in the mutagenesis region to confirm the successful introduction of the desired mutation.

Liposome Preparation. Dimyristoyl-L-α-phosphatidylcholine (C14:0) (DMPC) (Sigma Chemical Co., St. Louis, MO) was dissolved in chloroform and methanol (2:1) at a concentration of 10−20 mg/mL (w/v). For preparation of ganglioside-containing liposomes, 5 mol % of ganglioside GT1b (Sigma Chemical Co.) or ganglioside GM1 (Calbiochem-Novabiochem Corp., San Diego, CA) dissolved in chloroform and methanol (2:1) was added to the DMPC solution. Samples were dried under nitrogen in glass vials and lyophilized for 1 h, and the lipid film was rehydrated at a concentration of 0.5 mM in aqueous solution at ≥27 °C. Over the course of 1 h, the lipid suspension was periodically removed from the 27 °C bath and vortexed. Finally, the sample was sonicated in a bath sonicator at 27 °C for 2 min.

Surface Plasmon Resonance Binding Studies. The Biacore 3000 instrument (Biacore, Piscataway, NJ) was used to assess the binding of the tetanus toxin H_C fragment to different liposomal membrane surfaces attached to the surface of the L1 sensor chip (Biacore). Three of the cells of this sensor chip were coated with different liposome preparations (DMPC alone, DMPC with ganglioside GM1, and DMPC with ganglioside GT1b), and the fourth cell on the chip surface was used as a control surface by leaving it free of liposomes. Liposome coating was established by injections across the chip surface in the following order: 20 mM CHAPS (0.1 mL, flow rate of 10 μL/min), liposome solution (30 μL, flow rate of 2 μL/min), 20 mM NaOH (20 μL, flow rate of 100 μL/min), BSA (0.1 mg/mL, 25 μL, flow rate of 5 μL/min), and 10 mM glycine (pH 2.0) (40 μL, flow rate of 20 μL/min). After successful lipid coating had been carried out, measured by a change in the overall response (RU), binding of the tetanus toxin H_C fragment to these different membrane surfaces was examined. As expected, all tetanus toxin H_C fragments that were analyzed in these studies (wild type and mutants) were unable to bind to the DMPC alone or GM1-containing DMPC liposomal surfaces.

Initial binding studies performed with the H_C fragment were carried out using a variety of buffers (30 mM MES at pH 5.5, 6.0, and 6.5, 30 mM MOPS at pH 6.5, 7.0, and 7.5, 30 mM HEPES at pH 7.5 and 8.0, and 30 mM BICINE at pH 8.0, 8.5, and 9.0) containing 100 mM NaCl. All buffers were filtered prior to use. The tetanus toxin H_C fragment was dialyzed into the appropriate experimental buffer prior to binding analysis. These initial binding studies revealed that the 30 mM MES buffer (pH 6.0) containing 100 mM NaCl was optimal for studying H_C fragment binding, and thus, this buffer system was utilized in all subsequent experiments. H_C fragment injections (0.1 mL) were performed at a flow rate of 50 μL/min.

Circular Dichroism. Circular dichroism (CD) measurements were taken at 298 K using a Jasco 710 spectropolarimeter with a 0.1 mm path length cylindrical cuvette.

Samples were measured at wavelengths between 190 and 280 nm with a 0.1 nm step resolution, a measurement speed of 2 nm/min, a 1 s response time, and a 2 nm bandwidth. CD measurements were carried out on protein samples ranging in concentration from 48 to 120.6 μM, all prepared in 10 mM Tris buffer. For all samples, eight individual spectra were averaged, and a spectrum of 10 mM Tris buffer was subtracted to obtain the final protein spectra.

Maintenance of Rat Pheochromocytoma Cells. Rat pheochromocytoma (PC-12) cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT) containing 4.00 mM L-glutamine, 4.5 mg/mL glucose, 10 mM HEPES, 5% fetal bovine serum, 5% equine serum, and 100 μg/mL penicillin−streptomycin at 37 °C in a 5% CO_2 humidified incubator. Cultures were split at 90−95% confluence, which generally occurred during the tenth day of growth. Culture medium was replaced every 3−5 days. Cells ready for passage were dislodged by striking the culture flask. Cells were then suspended in fresh culture medium, and new 75 cm^2 flasks were seeded at a 1:8 dilution.

Immunofluorescent Detection of the Recombinant Tetanus Toxin H_C Fragment Bound to Differentiated PC-12 Cells. PC-12 cells were grown on glass coverslips pretreated for 1 h at room temperature with 5 μg/cm^2 mouse collagen IV (BD Labware, Bedford, MA) diluted in 0.05 M HCl containing 5 μg/mL poly-L-lysine. Coverslips were washed with sterile deionized water and PBS and then seeded with 50 μL of a cell suspension at passage (5000 to 1×10^4 cells). Cultures were maintained as described above. To induce neurite outgrowth and expression of tetanus toxin H_C fragment receptors, culture medium was supplemented with 100 ng/mL mouse nerve growth factor 2.5S (NGF) (Roche Diagnostics). Supplemented medium was replaced every 2 days.

The cells were used for experiments after stimulation with NGF for 10−14 days, when neurite extension was maximal. The coverslip cultures were fixed with 4% paraformaldehyde in PBS for 10 min, washed three times for 5 min with PBS, and then incubated with horse serum diluent for 30 min. Recombinant H_C fragments were added to cells at final concentrations of 4 μg/mL in PBS containing 5% horse serum. Cultures were washed with PBS to remove the nonspecifically bound H_C fragment. Binding was detected by incubating the cells with a 1:1000 dilution of goat anti-tetanus toxoid serum for 1 h, followed by a 30 min incubation with a 1:100 dilution of rabbit anti-goat IgG conjugated with FITC. After the final wash, stained cultures were treated with mounting medium and applied to slides. Cultures were examined by phase-contrast and fluorescence microscopy within 24 h on Nikon fluorescence microscope equipped with Spot RT Imaging Equipment and software (Diagnostic Instruments, Sterling Heights, MI).

The fluorescence images were analyzed using a Photometric cooled CCD (model CH250). A background area of 289 pixels selected from regions with minimum fluorescence was measured for fluorescence intensity. Next the fluorescence intensity and area of each cell were measured excluding the nucleus. Since the areas of the cells varied, the sum of the fluorescence intensities was normalized to an area of 2000 pixels for both the background and the cell fluorescence prior to subtraction of the background intensity.

RESULTS

Identification of Potential Ganglioside-Binding Domains and Candidate Amino Acids for Mutagenesis through Molecular Modeling. Our approach to identifying the amino acid residues within the tetanus toxin H_C fragment that are important for ganglioside binding has been to use both the available data in the literature and computational methods based on the three-dimensional structure of the protein to identify amino acid targets for site-directed mutagenesis. Our first objective was to identify regions of the protein that would likely contain the oligosaccharide-binding site and test these regions for their importance in binding. Using available data in the literature, one likely binding domain was defined by mapping a 20 Å radius around tetanus toxin histidine 1292. The photoaffinity labeling experiments of Shapiro et al. (*10*) showed that a modified ganglioside GD1b was cross-linked to H1292 of tetanus toxin. Mapping of a 20 Å radius around H1292, which directly reflects the size of the photoaffinity ligand linker arm, defines an area on the toxin near H1292 containing amino acids that are potentially involved in ganglioside binding.

Within this radius is contained tryptophan 1288, which has been implicated in ganglioside binding on the basis of fluorescence binding studies with botulinum type A toxin. The importance of aromatic residues, particularly tryptophan residues, has been established for a number of carbohydrate-binding domains (*26*). A potential sialic acid binding region was identified on the basis of the three-dimensional geometry of the sialic acid binding proteins sialoadhesin (*21*) and influenza neuraminidase A (*18*). A triad of three residues (an aromatic residue, a basic residue, and a H-bonding residue) common to the Neu5Ac binding site in sialoadhesin and influenza neuraminidase was identified as a possible "marker" for a binding domain. Although other residues were also implicated in carbohydrate binding (*21*), the triad was used to indicate a possible sialic acid-binding domain. A similar domain was identified involving amino acids W1288, R1222, and S1263 on the tetanus toxin H_C fragment. This area of the H_C fragment was designated as "region 1" (Figure 1), and the residues in this triad were selected as targets for mutagenesis.

Several computational approaches were used to evaluate other possible ganglioside GD1b and GT1b binding domain(s) on the tetanus toxin H_C fragment. These included primary sequence alignments, secondary structural alignments, and homology modeling with known protein- and carbohydrate-binding domains from several candidate proteins. The proteins that were chosen as templates for secondary structural analysis were proteins with structural folds similar to that of the tetanus toxin heavy chain that also bind Neu5Ac or Gal.

A secondary structural superposition in Quanta of the entire H_C fragment (amino acids 862−1314) or of the β-trefoil domain (amino acids 1126−1314) of the carboxyl subdomain in the H_C fragment was performed against the Neu5Ac-binding and Gal-binding templates, respectively. To identify common protein partner residues in the homology model, the carbohydrate-binding domains were translated to the tetanus toxin homologue. Tetanus toxin residues superimposed on the Gal-binding domain of ricin are Y1201 and the N1202−N1204 region. The H_C fragment residues that

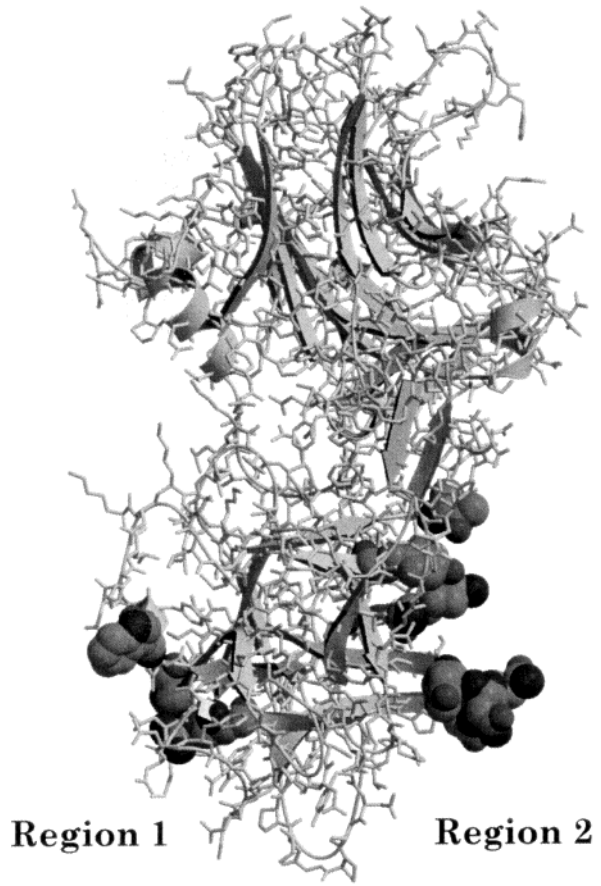

FIGURE 1: Mutagenesis regions 1 and 2 on the tetanus toxin H_C fragment. Two distinct subdomains are apparent on the tetanus toxin H_C fragment. In this depiction, the amino-terminal subdomain, with a distinct lectin-like jellyroll appearance, is located at the top of the molecule. Located at the bottom portion of the H_C fragment is the carboxy-terminal β-trefoil subdomain. The region 1 and 2 mutations all fall within the carboxyl-terminal subdomain. Mutagenized residues in each region are indicated as space-filled molecules.

are homologous and/or superimposable on the influenza virus neuraminidase Neu5Ac-binding domain are Y1128, K1173, and T1307. These seven amino acids are located on the same face of the protein. Moreover, the putative Gal and Neu5Ac binding domains are proximal, indicating a ganglioside-binding domain within this region, designated as "region 2" (Figure 1). The seven residues in region 2 were targeted for mutagenesis.

Amino Acid Residue W1288 in Region 1 Is Important for Binding of the Tetanus Toxin H_C Fragment to Ganglioside GT1b. Amino acid residues R1222, S1263, and W1288 (region 1) and Y1128, K1173, Y1201, T1307, and N1202−N1204 (region 2) were targeted for mutagenesis on the basis of the modeling studies described above. Seven mutants with a single alanine substitution and a triple mutant deletion construct, ΔN1202−1204, were each expressed with an amino-terminal His tag.

Binding of mutant His-tagged H_C fragments to GT1b-containing liposomal membranes was compared to binding of the wild-type His-tagged H_C fragment. All H_C fragments with an amino acid mutation in region 2 of the protein and two of the three mutant H_C fragments with an amino acid substitution in region 1 were found to bind to GT1b-containing liposomes in a manner similar to that of the wild-type toxin fragment. Binding of the H_C fragment to ganglioside GT1b was abolished when tryptophan 1288 from H_C fragment region 1 was changed to an alanine (Figure 2). Circular dichroism (CD) analysis of both the wild-type His-tagged H_C fragment and the WA1288 mutant protein indicated that the structures of the two proteins were similar, and thus, a gross structural alteration probably does not account for the observed differences in binding (results not shown). The W1288 residue was mutated to a phenylalanine, and this WF1288 mutant was found to bind to GT1b liposomes at levels that are around 50% of that of the wild-type H_C fragment (results not shown). CD analysis of WF1288 was consistent with the findings for the WA1288 mutant protein (results not shown).

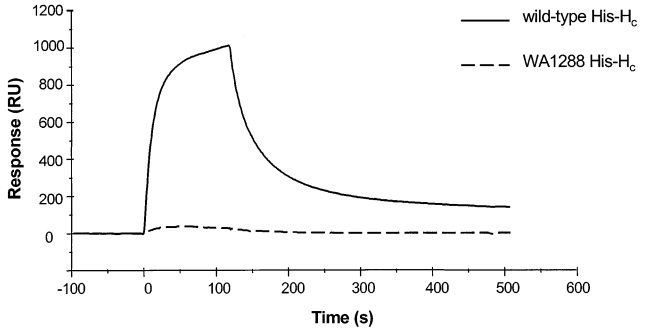

FIGURE 2: Binding of wild-type and mutant WA1288 tetanus toxin H_C fragments to GT1b gangliosides was examined using surface plasmon resonance. H_C fragment preparations (1 μM) were individually injected across the surface of an L1 sensor chip (Biacore) coated with ganglioside-containing liposomal membranes. The level of binding of the H_C fragments was measured as an increase in the response units (RU) over the baseline level.

Amino Acids H1270 and D1221 Are Also Important for Binding to GT1b. Tetanus toxin H_C fragment residues W1288 (this report), Y1289 (*17*), and H1292 (*14*) are all involved in binding of the toxin to ganglioside GT1b. Conversely, it has been found that amino acids R1222 and S1263 (this report) and K1296, K1294, and N1291 (*17*) are not involved in binding to GT1b. In light of these observations, we have focused our studies on the amino acids located in tetanus toxin H_C fragment region 1; specifically, we have focused on the area of the cleft where H1292, W1288, and Y1289 are located (Figure 3).

To identify low-energy binding positions for GD1b or GT1b, the lactose component of the oligosaccharide was built in CHARMM with coordinates extracted from the reported tetanus toxin H_C fragment−lactose crystal complex (*15*). The lactose was docked over a 20 Å^3 volume within region 1. The lactose was used as a disaccharide probe for the identification of possible lactose and/or Gal-β1−3-GalNAc binding domains. The justification for this comes from their elegant work showing that the galactose in lactose binds to the putative Gal-β1−3-GalNAc binding domain in region 1, and thus has served as a pyranose probe in identifying the binding position of another disaccharide component of the ganglioside. Using the coordinates of the lactose in this complex as a starting point, the lactose was translated over 20 Å in the *X*, *Y*, and *Z* directions. From this, a van der Waals (VDW) interaction energy was calculated using the CHARMM potential function (*22*) between the receptor and all protein atoms within a 4 Å radius of the receptor at each translational increment. At the original crystal binding

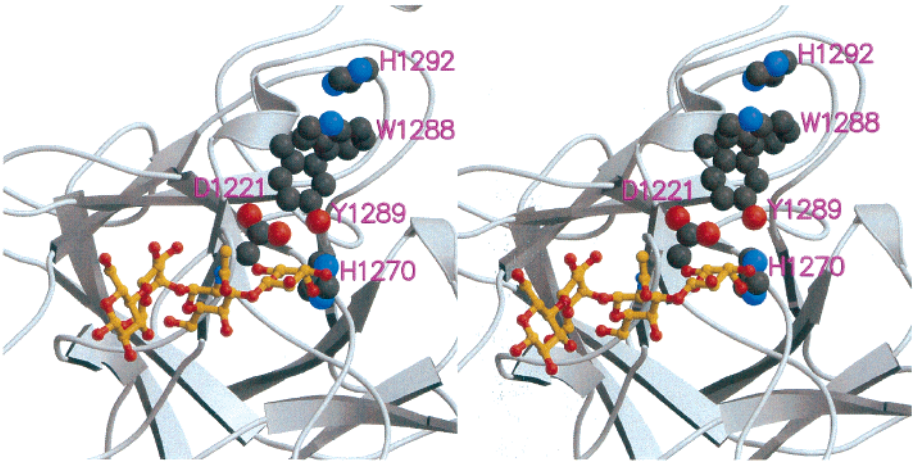

FIGURE 3: Enlarged stereoview of tetanus toxin H_C fragment region 1 with the GM1 tetrasaccharide docked. The figure shows the docking position of the GM1 tetrasaccharide (Gal-β1−3-GalNAc-β1−4-Gal-β1−4-Glc) receptor (ball and stick) on the tetanus toxin H_C fragment. Mutated residues Y1289 (*17*), H1292 (*14*), and W1288 (from this study) are indicated. Other residues suggested to be involved in binding on the basis of the calculated putative H-bond distances to the docked tetrasaccharide are highlighted. The calculated putative H-bond distances are as follows: 3.5 Å for the D1221 OD1−Gal O6 bond and 2.83 Å for the H1270 ND1−Gal O4 bond. The depicted orientation of the H_C fragment reveals a cavity for the terminal Neu5Ac extension of GT1b below W1288; however, in the absence of the terminal sialic acid (as in GD1b), the docking position of the terminal Gal markedly resembles the Gal/ricin binding domain.

position (0,0,0), the VDW energy of lactose was 1.64 kcal/mol. This VDW energy was reduced to −3.16 kcal/mol when lactose was translated 2 Å units in the X^+ direction (toward the surface) and 3 Å toward the bottom of the cavity to the relative coordinates of (2,−3,−5). This localized the tetrasaccharide (Gal-GalNAc-lactose) binding domain to the low-energy regions near the bottom of the region 1 cavity.

To identify specific protein residues of the tetanus toxin H_C fragment interacting with the ganglioside, it was necessary to build and dock the tetrasaccharide Gal-β1−3-GalNAc-β1−4-Gal-β1−4-Glc. With the coordinates of the tetrasaccharide in cholera toxin GM1 as a starting point, the carbohydrate was walked across region 1 over a 17 $Å^3$ volume, resulting in approximately 5000 docking positions. Favorable amino acid candidates both had more than 40 protein atoms within a 4 Å interaction sphere of the carbohydrate and an electrostatic energy of less than 100 kcal/mol. Modeling of GD1b in region 1, using an energy-minimized structure based on the GM1 conformation of cholera toxin, revealed the minimum electrostatic energy docking orientation (−67 kcal/mol) with the C6OH group of the terminal galactose proximal to tetanus toxin residues H1270 and D1221, possibly also interacting with the C3OH, C4OH, and O5 atoms (Figure 3). On the basis of these studies, H1270 and D1221 were targeted for mutation.

Site-directed mutagenesis was used to introduce alanine substitutions at D1221 and H1270 in the tetanus toxin H_C fragment. Mutant H_C fragment proteins DA1221 and HA1270 were found to have reduced levels of binding to GT1b liposomal membranes when compared to the wild-type protein using surface plasmon resonance (Figure 4). Neither did the mutant H_C fragment bind to any of the other surfaces on the Biacore L1 sensor chip surface. CD analysis confirmed that the structures of each of these mutant proteins are similar to the wild-type protein (results not shown). While the WA1288 mutation rendered the toxin fragment unable to bind to ganglioside GT1b, the DA1221 and HA1270 mutants retained a binding ability at ∼50% of the wild-type levels (Figure 4).

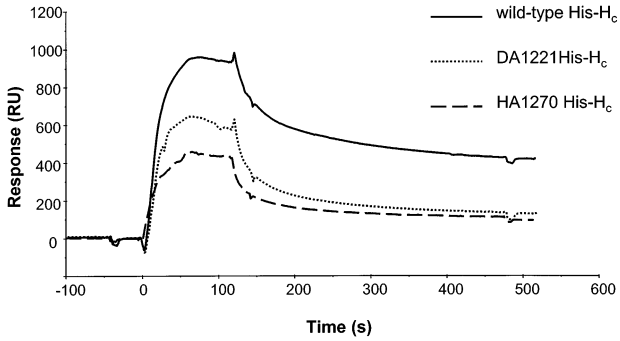

FIGURE 4: Binding of wild-type and mutant DA1221 and HA1270 tetanus toxin H_C fragments to ganglioside GT1b was examined using surface plasmon resonance. H_C fragment preparations (1 μM) were individually injected across the surface of an L1 sensor chip (Biacore) coated with ganglioside-containing liposomal membranes. The level of binding of the H_C fragments was measured as an increase in the response units (RU) over the baseline level.

Mutant H_C Fragments Bind Very Weakly to PC-12 Cells. Differentiated PC-12 cells are commonly used as a model system for measuring the effect of clostridial neurotoxins on neuronal cells. Because mutations in residues W1288, D1221, and H1270 affect the binding of the tetanus toxin H_C fragment to ganglioside in surface plasmon resonance experiments, we examined the interaction of mutant H_C fragments with PC-12 cells. PC-12 cells stimulated to differentiate with nerve growth factor (NGF) exhibited bright fluorescence when treated with the wild-type H_C fragment and anti-tetanus antibody prior to staining with FITC-labeled anti-immunoglobulin (Figure 5). In a similar experiment, mutant proteins DA1221 and HA1270 exhibited a marked decrease in fluorescence to near background. This qualitative result with mutant proteins DA1221 and HA1270 was confirmed by quantitation of the relative fluorescence in the micrographs (data not shown). These results suggest that D1221 and H1270 are important for binding of the tetanus toxin H_C fragment to differentiated nerve cells.

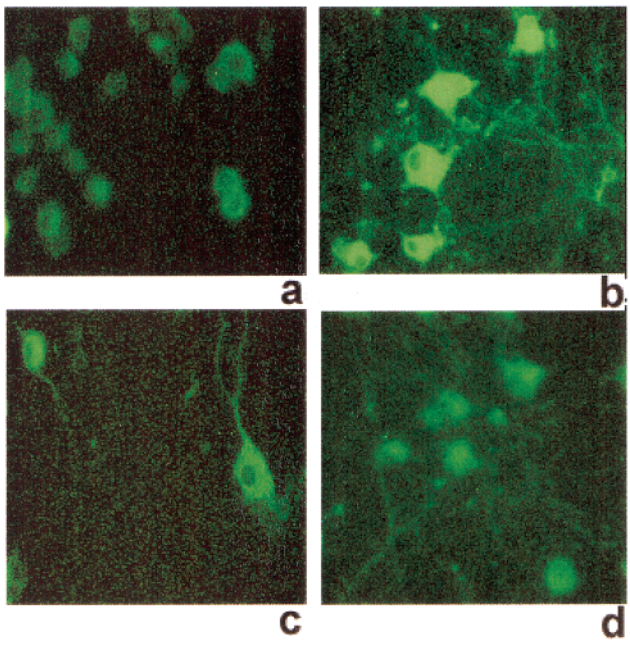

FIGURE 5: Binding of tetanus toxin H_C fragments to PC-12 cells. (a) Tetanus toxin H_C fragment binding to cells that were not stimulated with NGF, (b) tetanus toxin H_C fragment binding to cells that were stimulated with NGF, (c) mutant tetanus toxin H_C fragment DA1221 binding to cells that were stimulated with NGF, and (d) mutant tetanus toxin H_C fragment HA1270 binding to cells that were stimulated with NGF.

DISCUSSION

In this study, amino acid residues within the tetanus toxin H_C fragment were targeted for site-directed mutagenesis using molecular modeling; each mutant toxin protein was then examined for the ability to bind to ganglioside receptors. Using this approach, we have identified three residues (D1221, H1270, and W1288) in a single region of the protein, which we propose to be a binding site for the oligosaccharide portion of the ganglioside receptor. Other researchers have taken a slightly different approach to identifying amino acid residues possibly involved in binding of clostridial toxins to gangliosides. Two reports (*8*, *15*) describe the cocrystallization of clostridial toxin H_C fragments with mono-, di-, or trisaccharide components of gangliosides. Emsley et al. (*15*) determined crystal structures for the native tetanus toxin H_C fragment and the H_C fragment soaked in solutions of lactose, galactose, *N*-acetylgalactosamine, and sialic acid. Similarly, Swaminathan and Eswaramoorthy (*8*) crystallized botulinum toxin type B in complex with sialyllactose, and then determined which amino acid residues from the toxin were involved in interacting with this trisaccharide.

Emsley et al. (*15*) localized the disaccharide lactose to the area of tetanus toxin that we have designated region 1. Within this region, seven amino acids were reported to be residues that interact with lactose, including N1219, D1221, T1269, S1286, W1288, Y1289, and G1299. In botulinum toxin type B, residues found to form contacts with the sialyllactose trisaccharide included H1240, W1261, Y1262, E1188, and E1189 (*8*). While botulinum toxin type B amino acid residue E1189 does not have a homologous residue in tetanus toxin, the other four amino acids are homologous to tetanus toxin amino acid residues H1270, W1288, Y1289, and D1221, respectively. These four amino acids all fall within our designated region 1 of tetanus toxin. Common residues deduced from both cocrystallization studies include W1288, D1221, and Y1289. The use of a trisaccharide in a soaking experiment would be expected to provide a better approximation of the ganglioside- and oligosaccharide-binding domain than soaking experiments with mono- or disaccharides due to the more realistic representation of the ganglioside oligosaccharide domain. It is interesting to note that our modeling approach implicated tetanus toxin H_C fragment residue H1270, an amino acid that was deduced from the botulinum toxin type B−sialyllactose crystal structure (H1240 in botulinum toxin type B) (*8*), but not reported in the mono- and disaccharide soaking experiments with the tetanus toxin H_C fragment (*15*).

Another recent study (*27*) reports the cocrystallization of the tetanus toxin H_C fragment and an oligosaccharide. This crystal structure implicates toxin amino acids D1221, H1270, W1288, Y1289, H1292, F1217, and T1269 as residues involved in binding to the galactose−*N*-acetylgalactosamine disaccharide of the oligosaccharide. While these results seem to be consistent with our findings, it should be noted that the oligosaccharide used for these crystal structures is a β-anomeric form of ganglioside GT1b, while the natural form of GT1b is an α-form. Thus, these results should be viewed in light of this structural information about the oligosaccharide.

Our first round of modeling and mutagenesis identified two regions of the toxin that might be involved in binding to gangliosides. The targeting of region 1 was supported by several literature reports (*2*, *10*, *16*), while the targeting of region 2 was based mostly on modeling. We did not find any of the amino acids that we targeted in the region 2 area of the toxin to be involved in binding to GT1b. However, a report published by Emsley et al. (*15*) after we had completed our analysis of region 2 suggests that there may be a second binding site in that vicinity of the protein.

In our analysis of region 2, we mutated one residue, T1307, which was previously implicated in binding based on some deletion analyses (*28*). These authors reported that tetanus toxin H_C fragment amino acid residues V1305−E1309 were involved in binding to ganglioside GT1b. In our analysis of the T1307 residue, we found that mutating this residue had no influence on binding to ganglioside GT1b. Similar findings have been reported for amino acid mutations made in this region of the toxin, including T1307, D1308, E1309, ΔG1310−D1314, and ΔV1305−D1314 (*14*, *17*). One residue, E1309, was reported to have had an effect on binding to synaptosomes (*17*), but did not have an effect on binding to ganglioside GT1b (*14*). It is difficult to reconcile the early study (*28*) with the more recent studies (*14*, *17*). However, it can generally be concluded that the region of the toxin encompassing the last 10 amino acids of the H_C fragment is probably not part of a ganglioside-binding domain.

It is also worth noting that in a recent paper (*14*), a deletion analysis approach was used in an attempt to identify amino acids that might be part of a ganglioside-binding domain. Deleted regions included a 12-amino acid stretch, ΔH1270−D1281, and two six-amino acid regions, ΔQ1273−P1278 and ΔD1213−N1218. All three of these mutant H_C fragments were found to have limited ability to bind to ganglioside GT1b. While both of the six-amino acid deletion mutants were unable to undergo retrograde transport, this information

was not determined for the 12-amino acid deletion mutant. Interestingly, this same study analyzed the significance of H1292 in ganglioside binding. A mutation in this residue was found to alter the ability of the protein to bind maximally to ganglioside GT1b; however, the ability to bind to motoneurons and to undergo retrograde transport was retained.

We observed that the binding of the tetanus toxin H_C fragment to gangliosides in vitro is complex when measured by surface plasmon resonance. Surface plasmon resonance studies were initiated on the DA1221, HA1270, and WA1288 mutants and the wild-type H_C fragment in an attempt to obtain binding constants. Theoretically, such data should have been able to be attained from the surface plasmon resonance experiments. However, because of the complex nature of binding seen between the different H_C fragments and GT1b liposomes on the surface of an L1 chip, the binding interaction could not be modeled to obtain binding constants. Thus, surface plasmon resonance was utilized primarily as a qualitative method for screening mutant H_C fragments for binding ability. During the preparation of this paper, Fotinou et al. (*27*) reported the binding of tetanus toxin to two gangliosides using a synthetic β-anomer of the naturally occurring α-form of GT1b.

Finally, mutant H_C fragment binding was examined in PC-12 cells. While one would suppose that tetanus toxin H_C fragment mutants binding weakly to artificial ganglioside GT1b-containing liposomes would also bind weakly to neuronal cells, this is not always the case. Although a report on mutagenesis of H_C fragment residue Y1289 (*17*) indicated a similar reduced level of binding to gangliosides and to synaptosomal membranes for all amino acid changes made, another report (*14*) found quite different results for mutants of H1292. Mutants HA1292 and HS1292 were reported to bind weakly to GT1b, at 12.5 and 43% of wild-type levels, respectively; however, the binding ability of these mutants H_C fragments to rat spinal cord motoneurons was similar to the binding ability of the wild-type H_C fragment. Sinha et al. (*14*) also report that retrograde axonal transport activity was retained for both of these H1292 mutants. The findings for the current study indicate that the binding results from in vitro surface plasmon resonance experiments are consistent with the in vivo neuronal cell binding assay. Binding to neuronal cells by mutant H_C fragments DA1221 and HA1270 is very weak, compared to the binding of the wild-type H_C fragment.

This study presents evidence for the involvement of W1288, D1221, and H1270 in the binding of tetanus toxin to ganglioside GT1b and neuronal cells. Previous mutagenesis studies have implicated H1292 (*14*) and Y1289 (*17*) as residues in tetanus toxin that are important for binding to gangliosides. These five residues all fall within our designated region 1 of the toxin and can be predicted to be a part of a ganglioside-binding pocket (Figure 3).

ACKNOWLEDGMENT

We thank Dr. Ann Dixon of the Center for Biologics Evaluation and Research at the U.S. Food and Drug Administration for performing circular dichroism analyses on mutant DA1221, mutant HA1270, mutant WF1288, and wild-type H_C fragments, Dr. Shousun Szu of the National Institutes for Child Health and Development at the National Institutes of Health for performing circular dichroism analyses on mutant WA1288 and wild-type H_C fragments, and Dr. Richard Pastor of the Center for Biologics Evaluation and Research at the U.S. Food and Drug Administration for helpful comments and discussion during the preparation of this paper.

REFERENCES

1. Herreros, J., Lalli, G., Montecucco, C., and Schiavo, G. (1999) Pathophysiological Properties of Clostridial Neurotoxins, in *The Comprehensive Sourcebook of Bacterial Protein Toxins* (Alouf, J. E., and Freer, J. H., Eds.) 2nd ed., pp 202−228, Academic Press, San Diego.
2. Lacy, D. B., and Stevens, R. C. (1999) Sequence homology and structural analysis of the clostridial neurotoxins, *J. Mol. Biol. 291*, 1091−1104.
3. Eisel, U., Jarausch, W., Goretzki, K., Henschen, A., Engels, J., Weller, U., Hudel, M., Habermann, E., and Niemann, H. (1986) Tetanus toxin: primary structure, expression in *E. coli*, and homology with botulinum toxins, *EMBO J. 5*, 2495−2502.
4. Schmidt, J. J., Sathyamoorthy, V., and DasGupta, B. R. (1984) Partial amino acid sequence of the heavy and light chains of botulinum neurotoxin type A, *Biochem. Biophys. Res. Commun. 119*, 900−904.
5. Schmidt, J. J., Sathyamoorthy, V., and DasGupta, B. R. (1985) Partial amino acid sequences of botulinum neurotoxins types B and E, *Arch. Biochem. Biophys. 238*, 544−548.
6. Umland, T. C., Wingert, L. M., Swaminathan, S., Furey, W. F., Schmidt, J. J., and Sax, M. (1997) Structure of the receptor binding fragment H_C of tetanus neurotoxin, *Nat. Struct. Biol. 4*, 788−792.
7. Lacy, D. B., Tepp, W., Cohen, A. C., DasGupta, B. R., and Stevens, R. C. (1998) Crystal structure of botulinum neurotoxin type A and implications for toxicity, *Nat. Struct. Biol. 5*, 898−902.
8. Swaminathan, S., and Eswaramoorthy, S. (2000) Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B, *Nat. Struct. Biol. 7*, 693−699.
9. Halpern, J. L., Habig, W. H., Neale, E. A., and Stibitz, S. (1990) Cloning and expression of functional fragment C of tetanus toxin, *Infect. Immun. 58*, 1004−1009.
10. Shapiro, R. E., Specht, C. D., Collins, B. E., Woods, A. S., Cotter, R. J., and Schnaar, R. L. (1997) Identification of a ganglioside recognition domain of tetanus toxin using a novel ganglioside photoaffinity ligand, *J. Biol. Chem. 272*, 30380−30386.
11. Marxen, P., Fuhrmann, U., and Bigalke, H. (1989) Gangliosides mediate inhibitory effects of tetanus and botulinum A neurotoxins on exocytosis in chromaffin cells, *Toxicon 27*, 849−859.
12. Williamson, L. C., Bateman, K. E., Clifford, J. C. M., and Neale, E. A. (1999) Neuronal sensitivity to tetanus toxin requires gangliosides, *J. Biol. Chem. 274*, 25173−25180.
13. Herreros, J., Lalli, G., Montecucco, C., and Schiavo, G. (2000) Tetanus toxin fragment C binds to a protein present in neuronal cell lines and motoneurons, *J. Neurochem. 74*, 1941−1950.
14. Sinha, K., Box, M., Lalli, G., Schiavo, G., Schneider, H., Groves, M., Siligardi, G., and Fairweather, N. (2000) Analysis of mutants of tetanus toxin H_c fragment: ganglioside binding, cell binding and retrograde axonal transport properties, *Mol. Microbiol. 37*, 1041−1051.
15. Emsley, P., Fotinou, C., Black, I., Fairweather, N. F., Charles, I. G., Watts, C., Hewitt, E., and Isaacs, N. W. (2000) The structures of the H_C fragment of tetanus toxin with carbohydrate subunit complexes provide insight into ganglioside binding, *J. Biol. Chem. 275*, 8889−8894.
16. Kamata, Y., Yoshimoto, M., and Kozaki, S. (1997) Interaction between botulinum neurotoxin type A and ganglioside: ganglioside inactivates the neurotoxin and quenches its tryptophan fluorescence, *Toxicon 35*, 1337−1340.
17. Sutton, J. M., Chow-Worn, O., Spaven, L., Silman, N. J., Hallis, B., and Shone, C. C. (2001) Tyrosine-1290 of tetanus neurotoxin plays a key role in its binding to gangliosides and functional binding to neurones, *FEBS Lett. 493*, 45−49.
18. Varghese, J. N., Smith, P. W., Sollis, S. L., Blick, T. J., Sahasrabudhe, A., McKimm-Breschkin, J. L., and Colman, P. M. (1998) Drug design against a shifting target: a structural basis

for resistance to inhibitors in a variant of influenza virus neuraminidase, *Structure 6*, 735−746.
19. Rutenber, E., and Robertus, J. D. (1991) Structure of ricin B-chain at 2.5 Å resolution, *Proteins 10*, 260−269.
20. Murzin, A. G., Brenner, S. E., Hubbard, T., and Chothia, C. (1995) SCOP: a structural classification of proteins database for the investigation of sequences and structures, *J. Mol. Biol. 247*, 536−540.
21. May, A. P., Robinson, R. C., Vinson, M., Crocker, P. R., and Jones, E. Y. (1998) Crystal structure of the N-terminal domain of sialoadhesin in complex with 3′ sialyllactose at 1.85 Å resolution, *Mol. Cell 1*, 719−728.
22. Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., and Karplus, M. (1983) CHARMM: a program for macromolecular energy, minimization, and dynamics calculations, *J. Comput. Chem. 4*, 187−217.
23. Merritt, E. A., Sarfaty, S., van den Akker, F., L'Hoir, C., Martial, J. A., and Hol, W. G. J. (1994) Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide, *Protein Sci. 3*, 166−175.
24. Levitt, M., and Lifson, S. (1969) Refinement of protein conformations using a macromolecular energy minimization procedure, *J. Mol. Biol. 46*, 269−279.
25. Gabb, H. A., Jackson, R. M., and Sternberg, M. J. E. (1997) Modelling protein docking using shape complementarity, electrostatics and biochemical information, *J. Mol. Biol. 272*, 106−120.
26. Quiocho, F. A. (1993) Probing the atomic interactions between proteins and carbohydrates, *Biochem. Soc. Trans. 21*, 442−448.
27. Fotinou, C., Emsley, P., Black, I., Ando, H., Ishida, H., Kiso, M., Sinha, K. A., Fairweather, N. F., and Isaacs, N. W. (2001) The crystal structure of tetanus toxin H_C fragment complexed with a synthetic GT1b analogue suggests cross-linking between ganglioside receptors and the toxin, *J. Biol. Chem. 276*, 32274−32281.
28. Halpern, J. L., and Loftus, A. (1993) Characterization of the receptor-binding domain of tetanus toxin, *J. Biol. Chem. 268*, 11188−11192.

BI020291J