Identification of a Ganglioside Recognition Domain of Tetanus Toxin Using a Novel Ganglioside Photoaffinity Ligand*

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Robert E. Shapiro‡, Chelsea D. Specht§, Brian E. Collins¶, Amina S. Woods¶, Robert J. Cotter¶, and Ronald L. Schnaar†**

From the Departments of ‡Neurology, ¶Pharmacology and Molecular Sciences, and **Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Tetanus toxin entry into vertebrate motorneurons may involve binding of neuronal surface gangliosides containing the “1b” substructure (a NeuAc2,8NeuAc group on an internal galactose residue). The domains of tetanus toxin involved in ganglioside binding are known to reside within the carboxyl-terminal half of the toxin’s heavy chain (“C fragment”). We developed a novel photoaffinity reagent based upon the structure of the 1b ganglioside GD1b (125I-azido-GD1b) to define the ganglioside-binding domains of tetanus toxin. Using this ligand, we observed radiolabeling of tetanus toxin C fragment which could be specifically inhibited by a ganglioside of the 1b series (G1b1b), but not by a non-1b series ganglioside (G3M). When tetanus toxin C fragment was proteolyzed with c teamwork, before or after reaction with 125I-azido-GD1b, a radiolabeled band was observed by SDS-polyacrylamide gel electrophoresis autoradiography, which was selectively inhibited by GT1b. Protein sequencing of proteolyzed tetanus toxin C fragment co-migrating with that band revealed the carboxyl-terminal 34 amino acid residues of tetanus toxin. Matrix-assisted laser desorption/ionization mass spectrometry of a photoaffinity labeled synthetic polypeptide representing the 34-amino acid domain revealed modification at a single residue (His1293). We propose that this domain of tetanus toxin is sufficient for ganglioside binding.

The major Clostridial neurotoxins, botulinum and tetanus toxins, are a family of homologous proteins with selective toxicity for vertebrate motorneurons (1). Their neurotropism is a long studied phenomenon (for an excellent historical review and primary historical references, see Niemann (1)). These toxins are believed to gain neural entry by recognition of specific binding sites on motorneuronal axonal processes, followed by endocytosis, intracellular transport, and targeting of the toxins to sites of action. The motorneuron membrane structures responsible for reception of Clostridial toxins have not been definitively established. However, a wealth of data implicates cell surface glycolipids as toxin-binding sites (2). Early studies demonstrated that a crude preparation of mixed brain gangliosides, glycosphingolipids containing anionic sialic acid (NeuAc) carbohydrate residues, could “fix” tetanus toxin in vitro (3, 4). Subsequently, purified gangliosides containing the “1b” substructure (a NeuAc2,8NeuAc group on an internal galactose residue) were shown to directly support toxin binding, and to inhibit toxin binding to brain membranes (5–7). Ganglioside GT1b has so far demonstrated the highest affinity for tetanus toxin (5, 7) and most of the botulinum toxin serotypes (8–10), although ganglioside species with higher affinities may exist.

Considerable progress has been made in defining the toxin peptide sequences necessary and sufficient for both cell membrane and ganglioside binding. The high degree of amino acid sequence homology among the 7 cloned Clostridial toxins (11–17), combined with the conservation of ganglioside binding among these proteins (9), suggests that a conserved amino acid motif may define a common carbohydrate recognition site responsible for toxin-ganglioside binding. For three toxins (tetanus toxin (18) and botulinum toxin serotypes A (19, 20) and E (21)), ganglioside binding is supported by the isolated carboxy-terminal half (>50 kDa) of the respective heavy chains. This region of tetanus toxin is commonly termed the “C fragment” and roughly comprises amino acids 864–1315 (1).

Recently, in a highly informative study, Halpern and Loftus (22) synthesized various peptide fragments near the carboxyl terminus of tetanus toxin and measured their binding to immobilized GT1b ganglioside and neurons at physiological pH and ionic strength. Their data indicated that ganglioside binding is mediated by the carboxyl-terminal portion of the tetanus toxin C fragment, although contributions of protein secondary and tertiary structure to binding (e.g. interactions of non-contiguous polypeptide sequences) may be significant.

To extend this analysis, we developed a novel ganglioside-based photoaffinity ligand to identify polypeptide domains of tetanus toxin involved in binding to 1b series gangliosides. A radiiodinated aryl azide derivative of GD1b ganglioside (125I-azido-GD1b) was synthesized, incubated with tetanus toxin C fragment, and then the reaction was photolyzed, thereby covalently fixing the ligand at the toxin’s presumptive ganglioside-binding site(s). The photoaffinity-labeled tetanus toxin C fragment was then enzymatically proteolyzed and the resultant radiolabeled peptides purified and sequenced. An advantage of azido-G1b1b photoaffinity labeling is that ganglioside binding may be performed prior to toxin fragmentation, while the protein is in its native conformation. Using this technique we identified the 34-amino acid peptide at the carboxyl terminus of tetanus toxin as sufficient for ganglioside binding, and demonstrate specific photoaffinity labeling at His1293.

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‡ To whom correspondence should be addressed: Dept. of Neurology, College of Medicine, Given Building B202, University of Vermont, Burlington, VT 05405. Tel.: 802-456-1480. Fax: 802-456-5844. E-mail: robert.shapiro@vtmednet.org.

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1 Ganglioside nomenclature is according to Svennerholm (35).
Ganglioside Photoaffinity Labeling of Tetanus Toxin

G_{D_{18}} Oxidation—G_{D_{18}} (10 mg, Matreya) was dissolved in 9 ml of ice-cold aqueous 50 mM sodium phosphate, 150 mM NaCl, pH 7.4, and chilled on ice. Ice-cold aqueous sodium periodate (20 mM, 1 ml) was added to the mixture and the reaction incubated on ice for 90 min. The selectively oxidized product was purified by reverse phase chromatography as described previously (23). Briefly, methanol (7.82 ml) and chloroform (0.36 ml) were added, and the resulting single-phase solution loaded onto pre-washed Sep-Pak C_{18} cartridges (Waters, 4 in series) which were then washed with 15 ml of chloroform/methanol/water (2:63:35; 7.5 ml) of methanol/water (1:1), and 7.5 ml of methanol/water (4:1) prior to elution of the product with 15 ml of methanol. Elution was monitored by TLC (24) developed in isopropyl alcohol/0.25% aqueous KCl (3:1), and stained for sialic acids using an acid/resorcinol reagent. Nearly all of the product eluted in the methanol fraction, and migrated slower than G_{D_{18}}.

Reductive Amination—The methanol fractions were pooled and evaporated under nitrogen. Aqueous 1 M ammonium acetate, pH 6.5 (5 ml), was added, the residue dissolved, and 140 mM aqueous recrystallized sodium cyanoborohydride (0.5 ml) added. The reaction tube was flushed with nitrogen, sealed, and stirred at 42 °C for 24 h. After the incubation, methanol (4.3 ml) and chloroform (0.2 ml) were added and the product was purified by reverse phase chromatography as described above. TLC analysis, using isopropyl alcohol/aqueous 1 M ammonium acetate (3:1) as developing solvent, revealed a less mobile product which stained positively for the presence of both sialic acid (resorcinol reagent) and primary amine (fluorescamine reagent).

Azido-G_{D_{18}}—Aryl azide reactions were performed in foil-wrapped tubes and/or away from direct sunlight to minimize photolysis. A portion (83%) of the amine product was evaporated under nitrogen in a 1.5-ml microcentrifuge tube, and 1 ml of 10 mM sulfosuccinimidyl 2-p-azidosalicylamidoethyl-1,3'-dithiopropionate (Pierce), 10 mM triethylamine in dimethyl sulfoxide/ethanol (1:1) was added. After 36 h with end-over-end mixing at ambient temperature in the dark, methanol (3.8 ml), chloroform (0.2 ml), and water (5.5 ml) were added and the product purified by reverse phase chromatography as above. TLC analysis, using isopropyl alcohol/aqueous 1 M ammonium acetate (3:1) or chloroform/methanol/aqueous 1 M ammonium acetate (60:35:5) as developing solvents, revealed a more mobile product which adsorbed UV light (on fluorescence-impregnated TLC plates) and stained for the presence of sialic acid (resorcinol reagent).

Fractions from the reverse phase column containing the desired product were evaporated under nitrogen, then under vacuum. The residue was dissolved in 600 μl of running solvent (isopropyl alcohol/aqueous 1 M ammonium acetate 9:2), loaded onto a silicic acid column. Fractions containing product (as monitored by TLC) were combined, evaporated to dryness, and re-chromatographed on the same column. Fractions containing product were combined, evaporated, and subjected to reverse phase chromatography as described above. Product was stored in methanol at −20 °C.

The final product migrated as a single spot (resorcinol positive, UV absorbent) by TLC in two solvent systems (isopropyl alcohol/aqueous 1 M ammonium acetate (3:1) or chloroform/methanol/aqueous 1 M ammonium acetate (60:35:5)). For quantitation, an aliquot was hydrolyzed (100 mM HCl, 250 mM NaCl, 80 °C, 2 h) and the released N-acetylneuraminic acid was analyzed using Dionex anion exchange high performance liquid chromatography with pulsed amperometric detection (25), revealing a final yield of 1.0 mg (8.8% overall). The azido-G_{D_{18}} product was further characterized by fast atom bombardment mass spectrometry (Fig. 2). Two prominent molecular ions were detected, corresponding to the two sphingosine lengths (C20 and C18) on bovine brain gangliosides. Ions corresponding to fragmentation at the disulfide bond, and the loss of the derivatized sialic acid were also prominent, demonstrating selective modification of the terminal sialic acid residue.

Radioiodination—An aliquot of azido-G_{D_{18}} (10 nmol) was evaporated under nitrogen in a 1.5 ml microcentrifuge tube. The residue was dissolved in 100 μl of methanol, then 400 μl of 0.1 M sodium phosphate (pH 7.2) and 1 ml of IODO-BEADS (Pierce) were added. After 5 min at ambient temperature, 1 MCl of sodium iodide (carrier free) was added. After 30 min, the reaction solution was added to a glass culture tube containing 4.2 ml of methanol, 5.1 ml of water, and 200 μl of chloroform. The resulting solution was loaded on a Sep-Pak C_{18} cartridge, which was then washed with 8 ml of chloroform/methanol/water (2:43:55), 4 ml of methanol/water (1:1), and 4 ml of methanol/water (4:1). The initial

FIG. 1. Synthesis of azido-G_{D_{18}}. The terminal sialic acid of G_{D_{18}} is shown, where “R” designates the α2,3-linkage to the rest of the ganglioside. G_{D_{18}} was treated with periodic acid under conditions which selectively oxidize the glycerol side chain of the terminal sialic acid (Reaction 1), resulting in a unique aldehyde at carbon 7. Reductive amination with ammonium acetate and sodium cyanoborohydride (Reaction 2) generated a unique primary amine at carbon 7. The 7-amine derivative was subsequently treated with a commercial photoaffinity intermediate sulfosucinimidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (Reaction 3) to generate the desired product, which was amenable to radiiodination vicinal to the hydroxyl group on the phenyl ring.

MATERIALS AND METHODS

Scheme for 125I-Azido-G_{D_{18}} Synthesis—A G_{D_{18}} ganglioside photoaffinity ligand was designed (Fig. 1) to minimize disturbance of the ganglioside substrutures most responsible for Clostridial toxin binding, yet place a photactivatable aryl azide in close proximity to the toxin binding determinant. The glycerol side chains (carbons 7–9) of terminal sialic acids on 1b gangliosides are not essential for Clostridial toxin binding, yet are positioned near the carboxyl groups of the sialic acids, which are necessary for such binding (19). Accordingly, an azido-G_{D_{18}} ligand was synthesized by linking a commercially available aryl azide photoreagent to carbon 7 of the terminal sialic acid of G_{D_{18}}. The aryl azide can be readily radioiodinated, and is spaced from the ganglioside by a short disulfide-containing linker arm, making it cleavable. This design provides for limited mobility to the aryl azide with respect to the ganglioside determinants necessary for toxin binding, as well as a simple means of cleaving the ligand, following photolysis, to yield photoaffinity labeled toxin free of the ganglioside structure.

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The sample in methanol was mixed with an equal volume of monooctylglycerol (MTG), applied to the tube tip of a Kratos Concept 3 mass spectrometer, and subjected to analysis in the negative mode. Areas of the spectrum corresponding to the molecular ions (representing the two sphingosine chain lengths in bovine brain gangliosides), as well as prominent fragment ions (as indicated on the structural diagram) are presented.

Peptide Sequencing—Transfer of peptides from Tricine SDS-PAGE to polyvinylidene difluoride membranes (Bio-Rad) was performed using a Bio-Rad transblot apparatus. Gels were treated for 30 min in transfer buffer (25 mM Tris, 192 mM glycine), apposed to methanol-treated nitrocellulose membranes, and polypeptides transferred at 90 V for 1 hr. Following transfer, membranes were stained (10% acetic acid, 40% methanol, 0.5% Coomassie Blue), destained (10% acetic acid), and dried. Autoradiography in the gel was analyzed by PhosphorImager analysis (Fuji BAS 1000) or x-ray film autoradiography.

MALDI-MS—The 34-amino acid carboxyl-terminal domain of tetanus toxin was synthesized and purified by the Biosynthesis and Sequencing Facility of the Johns Hopkins School of Medicine. Purified peptide (5 μg, 0.5 nmol) and azido-GD1b (0.5, 0.5, or 1 nmol, not radiolabeled) in a total volume of 100 μl of 25 mM Tris-HCl, pH 6.5, were incubated for 3 h at 4 °C in the dark with gentle agitation. Following incubation, the reactions were individually transferred, using glass micropipettes, to a quartz vial designed to fit directly on top of a Vivitar 352 xenon flash lamp (kindly provided by Dr. Tae Ji, University of Wyoming). Each reaction was exposed to one photoflash. The reactions were then transferred back to glass tubes, lyophilized, and subjected to polyacrylamide gel electrophoresis as described below.

MALDI-MS—The 34-amino acid carboxyl-terminal domain of tetanus toxin was synthesized and purified by the Biosynthesis and Sequencing Facility of the Johns Hopkins School of Medicine. Purified peptide (5 μg, 0.5 nmol) and azido-GD1b (0.5, 0.5, or 1 nmol, not radiolabeled) in a total volume of 100 μl of 25 mM Tris-HCl, pH 6.5, were incubated for 3 h at 4 °C in the dark, then photolyzed as described above. Dithiothreitol (10 μl, 50 mM) was added and reactions incubated in the dark for 60 min at ambient temperature. Each reaction was diluted to 1 ml with acetonitrile/water/trifluoroacetic acid (15:85:0.1), and loaded onto prewashed reverse phase cartridges (C18 Sep-Pak, Waters). The cartridges were washed with 2 ml each of the loading solvent and acetonitrile/water/trifluoroacetic acid (30:70:0.1), then peptide
eluted with acetonitrile/water/trifluoracetic acid (70:30:0.1). The eluate was evaporated under vacuum, redissolved in 10 μl of acetonitrile/ water (7:3) and subjected to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS (27)). Spectra were acquired by drying 0.3 μl of the sample, 0.3 μl of saturated ammonium sulfate, and 0.3 μl of matrix (saturated α-cyano-4-hydroxycinnamic acid, Aldrich Chemical Co.) on the probe. Samples were analyzed in linear positive mode on a Kratos Kompact MALDI 4 (Kratos Analytical, Manchester, UK) equipped with a 337-nm nitrogen laser and 20 kV extraction voltage.

RESULTS

Incubation of tetanus toxin C fragment with 125'I-azido-GD1b followed by photoflash activation, produced radiolabeling of a polypeptide band which co-migrated with the tetanus toxin C fragment (Fig. 3A). When tetanus toxin C fragment was preincubated with ganglioside GT1b prior to reaction with 125'I-azido-GD1b, radiolabeling was inhibited (Fig. 3B). Half-maximal inhibition of 125'I-azido-GD1b labeling of tetanus toxin C fragment occurred at ~1 μM GT1b. Preincubation of tetanus toxin C fragment with ganglioside GM3 to a concentration of 50 μM was without significant effect on subsequent radiolabeling (Fig. 3B), whereas preincubation with ganglioside GM1 resulted in half-maximal inhibition at approximately 10 μM (data not shown), consistent with previously published inhibition studies (5).

Tetanus toxin C fragment (1 μg/reaction) was preincubated (without inhibitor or with 10 μM of either GT1b or GM3) and proteolyzed with clostripain, and the polypeptides subjected to Tricine SDS-PAGE (Fig. 4A). Alternately, tetanus toxin C fragment was first proteolyzed with clostripain, then the mixture of fragments preincubated with inhibitors (as indicated) and photoaffinity labeled with 125'I-azido-GD1b (Fig. 4B). Autoradiographic analysis of these reactions after Tricine SDS-PAGE demonstrated only one band for which radiolabeling was selectively inhibited by GT1b, regardless of whether the tetanus toxin C fragment was proteolyzed before or after reaction with 125'I-azido-GD1b. This band co-migrated with a polypeptide fragment detected by Coomasie Blue staining of unlabeled proteolyzed tetanus toxin C fragment electrophoresed in an adjacent lane (not shown). In the sample which was first photoaffinity labeled, then proteolyzed, this appeared to be the only radiolabeled polypeptide fragment, in that other radiolabeled bands were lipid-related (they appeared in control reactions in which tetanus toxin C fragment was excluded, data not shown). These results suggest that a peptide fragment of tetanus toxin binds to 125'I-azido-GD1b in a manner that is selectively inhibitable by Lb series gangliosides (GT1b). The observation that a comparable pattern of labeling was observed whether the 125'I-azido-GD1b reaction was performed before or after proteolysis indicates that the labeled peptide is sufficient to mediate ganglioside binding (see below). Furthermore, the observation that quantitatively greater label-
ing of comparable amounts of tetanus toxin C fragment occurred if the $^{125}$I-azido-$G_{D1b}$ reaction followed proteolysis (Table I) suggests that other domains of the polypeptide may attenuate ganglioside binding.

In a separate experiment, clostripain proteolysis of unlabeled tetanus toxin C fragment was repeated using 20 µg of unlabeled tetanus toxin C fragment per reaction. $^{125}$I-azido-$G_{D1b}$-labeled polypeptide and clostripain-proteolyzed unlabeled tetanus toxin C fragments were resolved in adjacent lanes on the same Tricine SDS-PAGE gel. Following electrophoresis, the gel was sliced in half. The side containing radio-labeled tetanus fragments was analyzed by autoradiography, whereas the side containing the lanes of unlabeled tetanus fragments was subjected to electrotransfer to a polyvinylidene difluoride membrane. Based upon the mobility of the $G_{D1b}$-inhibitable radiolabeled band, a rectangle of polyvinylidene difluoride membrane was excised from the lane containing proteolyzed unlabeled tetanus toxin C fragment and subjected to microsequencing. The results indicated a peptide with amino-terminal sequence DILIASNWYFNHLKDKILG (where X represents no determination). This sequence corresponds to the amino acids following the clostripain cleavage site closest to the carboxyl terminus of tetanus toxin (Fig. 5). Therefore the clostripain cleavage product of tetanus toxin C fragment which co-migrates with the $^{125}$I-azido-$G_{D1b}$ labeled fragment, representing the carboxyl-terminal 34 amino acids of tetanus toxin, is shown. Six homologous botulinum toxin sequences are included for comparison, in rank order (top to bottom) of their reported abilities to be inactivated by ganglioside $G_{T1b}$ (9). Sequence similarity (indicated by shading) is based on BOXSHADE (http://ubiec3.unlch/software/BOX_form.html). Numbers relate to the amino acid sequence of intact tetanus toxin (11).

Fig. 6. MALDI-MS of the synthetic polypeptide corresponding to the carboxyl-terminal 34 amino acids of tetanus toxin, and its photoaffinity labeled derivative. Spectra for the underivatized 34-mer polypeptide (spectrum 1) and products resulting from incubation of the polypeptide with equimolar (spectrum 2) and 2-fold molar excess (spectrum 3) of azido-$G_{D1b}$ were obtained as described under “Materials and Methods.” The spectrum of the underivatized polypeptide revealed masses consistent with the molecular ion (M + H, 4091.5) and the molecular ion less a hydroxyl (4074.5). A peak corresponding to the polypeptide derivatized with a single 2-p-aminosalicylamidoethanethiol group was detected in spectra 2 and 3. The additional mass/charge 4132.0 peak in spectrum 3 is a doubly charged species apparent only in reactions having $\geq$2-fold molar excess of the photoaffinity ligand (data not shown), and is considered an artifact.

Fig. 5. Sequence comparisons of the carboxyl termini of Clostridial toxins. The sequence of the clostripain proteolytic product of tetanus toxin C fragment which co-migrates with the $^{125}$I-azido-$G_{D1b}$ labeled fragment, representing the carboxyl-terminal 34 amino acids of tetanus toxin, is shown. Six homologous botulinum toxin sequences are included for comparison, in rank order (top to bottom) of their reported abilities to be inactivated by ganglioside $G_{T1b}$ (9). Sequence similarity (indicated by shading) is based on BOXSHADE (http://ubiec3.unlch/software/BOX_form.html). Numerals relate to the amino acid sequence of intact tetanus toxin (11).
to the intact tetanus C fragment in supporting ganglioside binding, since there was greater $^{125}$I-azido-G_Dlb labeling if proteolysis was performed prior to photoaffinity labeling. A synthetic polypeptide corresponding to these 34 amino acids was specifically photolabeled by azido-G_Dlb.

These results agree with the findings of Halpern and Loftus (22) who reported structure-activity relationships for the binding of recombinant tetanus toxin fragments to neurons or to gangliosides at physiological pH and ionic strength. They found markedly enhanced binding of peptide 1120–1315 relative to peptide 858–1315, indicating not only that peptide 1120–1315 is sufficient to support both neuron and ganglioside binding, but that the presence of amino acids 858–1119 inhibits such binding. Whereas peptide 858–1315 supported binding to gangliosides at physiological pH and ionic strength. They found that the presence of amino acids 858–1119 inhibits such binding. Moreover, tetanus toxin binding to neural membranes exhibits a pH maximum near the pH of histidine (pH 6, Ref. 6). Enhanced binding. Our results indicate that the critical residues for ganglioside binding are included in the carboxyl-terminal peptide 1282–1315, and photoaffinity labeling occurred predominantly at residue 1293. The structurally unrelated enterotoxin from another Clostridial species (Clostridium perfringens) also encodes its cell binding function in its carboxyl-terminal 30 amino acids (30).

The presence of a 34-amino acid peptide of tetanus toxin capable of independently supporting specific ganglioside binding suggests that there may be an oligosaccharide recognition domain shared among the 7 homologous Clostridial neurotoxins, all of which are known to possess some ganglioside binding activity. Alignment of the carboxyl-terminal sequences of the 6 botulinum toxins which are homologous to tetanus toxin (11–17), in rank order of their reported relative abilities to be inactivated by G_Dlb (9), indicates the conservation of particular residues which may mediate carbohydrate recognition (Fig. 5). These toxins which functionally interact the least with G_Dlb (e.g. botulinum Types C and D) are more divergent from tetanus toxin in their carboxyl-terminal sequence compared with those which are most inhibited by G_Dlb. The site of photoaffinity labeling (His*1293) is near two lysine residues (Lys*1295 and Lys*1297) which bind the sialic acid carboxylate(s). Furthermore, tetanus toxin binding to neural membranes exhibits a pH maximum near the pH of histidine (pH 6, Ref. 6). Enhanced binding. Our results indicate that the critical residues for ganglioside binding are included in the carboxyl-terminal peptide 1282–1315, and photoaffinity labeling occurred predominantly at residue 1293. The structurally unrelated enterotoxin from another Clostridial species (Clostridium perfringens) also encodes its cell binding function in its carboxyl-terminal 30 amino acids (30).
binding at low pH may reflect protonation of His\textsuperscript{1293} near the sialic acid binding pocket. Botulinum toxins B, F, A, and E have one or more cationic amino acids at comparable positions on their carbonyl-terminal structures (Fig. 5).

All of the above toxins are encoded episomally within \textit{Clostridial} species (i.e. in phage or plasmid) (1). This fact raises the possibility that toxin sequences may have originally been found in vertebrate host genes, and then adopted by these bacteria. Consequently, definition of the amino acid sequences necessary for ganglioside recognition in \textit{Clostridial} toxins may aid in identification of vertebrate proteins with similar carbohydrate binding functions.

Tetanus toxin C fragment, while itself nontoxic, retains not only the ganglioside binding activity of native tetanus toxin, but also its selective cytolytic mobility (e.g. motorneuronal retrograde axonal transport and subsequent retrograde transynaptic transfer), albeit with some loss of transport efficiency (18, 31–34). It remains to be determined whether the tetanus toxin peptide comprising amino acids 1282–1315, which our data indicate contains the ganglioside-binding domain of the toxin, also retains this cytolytic mobility. If true, this would suggest a role for gangliosides in intracellular targeting of endocytosed ligands.

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