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Protein Toxins that Utilize Gangliosides as Host Receptors

Madison Zuverink and Joseph T. Barbieri

Medical College of Wisconsin, 8701 Watertown Plank Road, BSB-255, Milwaukee, WI, 53226, USA

Abstract

Subsets of protein toxins utilize gangliosides as host receptors. Gangliosides are preferred receptors due to their extracellular localization on the eukaryotic cell and due to their essential nature in host physiology. Glycosphingolipids, including gangliosides, are mediators of signal transduction within and between eukaryotic cells. Protein toxins possess **AB** structure-function organization, where the **A** domain encodes a catalytic function for the posttranslational modification of a host macromolecule, including proteins and nucleic acids, and a **B** domain, which encodes host receptor recognition, including proteins and glycosphingolipids, alone or in combination. Protein toxins use similar strategies to bind glycans by pockets and loops, generally employing hydrogen bonding and aromatic stacking to stabilize interactions with sugars. In some cases, glycan binding facilitates uptake, while in other cases, cross-linking or a second receptor is necessary to stimulate entry. The affinity that protein toxins have for host glycans is necessary for tissue targeting, but not always sufficient to cause disease. In addition to affinity for binding the glycan, the lipid moiety also plays an important role in productive uptake and tissue tropism. Upon endocytosis, the protein toxin must escape to another intracellular compartment or into cytosol to modify a host substrate, modulating host signaling, often resulting in cytotoxic or apoptotic events in the cell, and a unique morbidity for the organism. The study of protein toxins that utilize gangliosides as host receptors has illuminated numerous eukaryotic cellular processes, identified the basis for developing interventions to prevent disease through vaccines and control bacterial diseases through therapies. In addition, subsets of these protein toxins have been utilized as therapeutic agents to treat numerous human afflictions.

Protein toxin AB structure-function organization

AB protein toxins are classified by domain organization (Figure 1). **A** is the catalytic domain, which posttranslationally modifies a host protein(s), changing host physiology and often causing death or intoxication¹. **B** is the binding domain, which binds a host receptor(s) and may also contain a translocation domain to facilitate delivery of **A** into the host cytosol¹. Inactivation of either the **A** or **B** domains results in a nontoxic protein. For single-chain **AB** toxins, **A** and **B** domains are synthesized within a single protein, while for **AB5** toxins, **A** and **B** domains are synthesized independently and assemble into an oligomer composed of one molecule of **A** bound to a **B** pentamer. The **A** domains of **AB5** toxins comprise an **A1** catalytic domain and an **A2** linker domain. The alpha-helical **A2** linker domain inserts into the **B** pentamer through non-covalent interactions to link the **A1** subdomain with the **B** pentamer to produce the **AB5** toxin².

Single-chain **AB** toxins often contain an interchain disulfide bond, while **AB5** toxins often contain an intrachain disulfide bond between the **A1**- and **A2**- subdomains. In each case, reduction is usually required during the delivery of the **A** domain into the host cell cytosol and expression of cellular toxicity^{3–7}. **AB** toxins are typically synthesized in an inactive form and require proteolytic processing to be converted into an active form. Single-chain **AB** toxins, such as the clostridial botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT), cleave a trypsin-sensitive site located between **A** and **B** retaining the interchain disulfide⁸. **AB** toxins, such as diphtheria toxin and anthrax toxin, are also proteolyzed by a host protease, furin, for interdomain cleavage or **B** subunit assembly, respectively^{9,10}. Most **AB5** toxins also contain a protease sensitive site, which is proteolyzed by a bacterial- or host- protease^{10,11}. The four **AB5** family members; cholera toxin (Ctx), Shiga toxin (Stx), pertussis toxin (Ptx), and subtilase cytotoxin (SubAB), are proteolyzed within the **A** domain, between the **A1** catalytic and **A2** linker-subdomains, which are linked by a disulfide bond^{12–14}.

Protein toxin host receptors

AB toxins exploit host receptors to coordinate host cell binding and delivery of **A** into the host cell cytosol. Members of the BoNT family have dual host receptors, often a glycolipid (ganglioside) and a protein receptor, while TeNT utilizes two gangliosides as receptors^{15–20}. Ctx binds a single class of glycolipid (GM1a) while other members of the Ctx family, the heat labile *E. coli* enterotoxins (LTx's) LT-I and LT-IIa-c, bind multiple gangliosides species with varied affinities^{21,22}. Other toxins, such as ricin and Ptx are promiscuous, and bind specific carbohydrate moieties on glycolipids and glycoproteins^{23,24}.

The specificity and affinity of protein toxins for host receptors is often assessed by *in vitro* biochemical and cell biological techniques, including solid-phase assays, surface plasmon resonance, receptor loading, and receptor depletion. Additional *in vivo* gene knockout studies confirm receptor specificity and tissue tropism. Ultimately, protein toxin action requires a receptor, a productive pathway to traffic the **A** domain to the target substrate, and the presence of the target substrate. This review focuses on protein toxins that utilize a subset of glycosphingolipids, gangliosides, and related carbohydrate moieties as host cell receptors.

Protein toxin entry into cells

Protein toxins enter cells by multiple pathways. Most **AB** toxins bind a cell surface host receptor and enter via receptor-mediated endocytosis. Early endosomal entry involves clathrin-dependent and clathrin-independent pathways such as bulk uptake, caveolae, and micropinocytosis. **AB5** toxins Ctx, LTx, Stx, and SubAB are associated with both clathrin-dependent and clathrin-independent pathways depending on the host cell type^{25–28}. Following uptake, the catalytic domain escapes the early endosome upon endosome acidification or retrograde traffics to the endoplasmic reticulum (ER) where the **A** domain enters the cytosol to modify a cytosolic host substrate, leading to host cell intoxication or apoptosis²⁹ (Figure 2).

Other **AB** toxins enter a retrograde endosomal pathway and traffic to the Golgi and the ER. For **AB5** toxin family members, Ctx/LTx, Stx, Ptx, and SubAB, the **B** pentamer encodes a binding domain, but lacks a translocation domain^{2,29–32}. Once these **AB5** toxins retrograde traffic to the ER, the **A** domain dissociates from the **B** pentamer and interacts with a chaperone such as protein disulfide isomerase (PDI) to reduce the intrachain disulfide^{33–37}. Following dissociation and release, the **A1** subdomain usurps the Unfolded Protein Response (UPR) pathway to escape through the Sec61 translocon³⁸. Upon retrograde translocation into the cytosol, via the Sec61 retrieval system, the **A1** subdomain binds cytosolic chaperones, including Arf^{39,40}, and refolds before trafficking to ADP-ribosylate the stimulatory heterotrimeric G-protein alpha subunit ($G\alpha_s$). ADP-ribosylation locks $G\alpha_s$ in the GTP bound form to constitutively activate host adenylate cyclase and stimulate cAMP, uncoupling host signaling⁴⁰. An exception within the **AB5** family is SubAB which remains in the ER lumen to modify a chaperone protein involved in UPR^{41,42}. The Ctx family members contain a KDEL, RDEL, or HDEL sequence at the C terminus of the **A** domain, which has been associated with retention in the ER by the KDEL receptor. The **B** pentamer also independently retrograde traffics to the ER, which indicates the KDEL sequence(s) optimize retrograde trafficking, but are not necessary nor sufficient to deliver **AB5** toxins to the ER^{43,44}.

Glycolipids

Eukaryotic cells are enveloped by a carbohydrate-rich coating, the glycocalyx. The glycocalyx contains glycoproteins and glycosphingolipids anchored in the outer leaflet of the plasma membrane⁴⁵. Glycans face the exterior milieu and mediate cell-cell interactions via binding complementary molecules on other membranes or regulating activity by forming a buffer zone between host cell and extracellular microbes. Pathogens utilize a capsular glycocalyx coating to avoid host detection and immune cell assault and have evolved to utilize the host cell glycocalyx as receptors, by binding these molecules with adhesins, hemagglutinins, or by releasing protein toxins⁴⁶.

Although glycosphingolipids refer to sphingosine-based lipids containing glycosidic bonds to simple carbohydrate molecules, the lipids are further classified into series based on a core structure⁴⁷. The lipid portion of the molecule, ceramide, contains a sphingoid base connected to a fatty acid through an amide bond⁴⁸. The ceramide portion of the ganglioside can be composed of sphingonine, sphingosine, or phytosphingosine and fatty acids can vary in chain length from ~16->24 carbons, which is associated with differences in trafficking^{48,49}. Glycosphingolipids are abundantly found in the grey matter of the brain, with 10–12% of brain sphingolipids being composed of gangliosides⁵⁰. Ganglio-series gangliosides have a common core of Gal β 1–3GalNAc β 1–4Gal β 1–4Glc β Cer, while another common glycolipid species, the globosides, have Gal α 1–4Gal β 1–4Glc β Cer (Gb3) as a core⁴⁵ (Figure 3).

While gangliosides are not required for cell survival, gangliosides are necessary for survival in whole-organisms⁵¹. The hydrophilic, negatively charged headgroups of gangliosides spread on the cell surface and exist in varied conformations at each branch points. Gangliosides are negatively charged due to decoration with 9-carbon sugars, sialic acids, off

the core sugars⁵⁰. In humans, sialic acids are composed of N-acetylneuraminic acid (Neu5Ac) while other mammals also synthesize N-glycolylneuraminic acid (Neu5Gc)⁵². In addition to variation in ceramide composition, the number of sialic acid groups connected to the core sugars also vary. Gangliosides contain 75% of the brain's sialic acid, primarily GM1a, GD1a, GD1b, and GT1b (Figure 3)⁵³. Gangliosides like other glycosphingolipids are restricted to the outer leaflet of the cell membrane, with the ceramide and first core sugar glucose engaged in this lipid layer⁵¹. Gangliosides are primarily found in the extracellular plasma membrane, but are also detected in intracellular organelles. The Golgi is responsible for the diversity of cell surface glycans⁵⁴. Neither gangliosides nor globosides are uniformly distributed in the plasma membrane, but cluster in lateral microdomains called detergent-insoluble glycosphingolipid-enriched domains (DIGs) which self-associate due to biophysical properties of long, unsaturated carbon chains⁵⁵. DIGs are known as lipid rafts in living cells and concentrate glycosylphosphatidylinositol-(GPI) anchored proteins, cholesterol, and gangliosides⁵⁶. Lipid rafts are often coupled to signaling complexes, which contain receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and downstream partners such as mitogen-activated protein (MAP) kinases and heterotrimeric G-proteins (GαPy). The binding of protein toxins to glycosphingolipids is prerequisite, but coupling of the lipid species to signaling domains for productive uptake affects tissue susceptibility.

Complex gangliosides, GD1a and GT1b, are ligands for myelin-associated glycoprotein (MAG), promoting axon stability and neurite inhibition⁵⁷. Altered surface expression or storage of gangliosides is associated with abnormal cell-cell contact inhibition and various diseases⁵⁸. Mice lacking some complex gangliosides, GM2 or GD2 synthase knockouts, develop hearing loss and abnormal motor pathology with age⁵⁹. Double knockouts in these aforementioned glycan synthase systems (*St3gal5* or *St8Sia1/B4galnt1*) die shortly after birth; therefore, the necessity for ganglioside synthesis is commonly exploited by pathogens and toxins that bind carbohydrates^{60–62}.

Protein Toxins utilize Gangliosides as Host Receptors

Clostridial Neurotoxins of *Clostridium botulinum* and *C. tetani* The clostridial neurotoxins (CNTs) of *Clostridium botulinum* and *tetani* target motor neuron presynaptic termini in the periphery and inhibitory interneurons in the central nervous system, respectively⁸. CNTs comprise seven serologically distinct BoNTs (A-G) and TeNT⁶³. CNTs are synthesized as single-chain **AB** toxins cleaved into a di-chain; an **A** catalytic light chain (LC) connected to a **B** heavy chain (HC) containing an N-terminal translocation domain and a C-terminal receptor binding domain⁶⁴. The LCs of CNTs are zinc-metalloproteases, which cleave neuron-specific isoforms of SNARE proteins involved in vesicle fusion at the plasma membrane^{65,66}. Following cleavage, the tertiary complex cannot form between vesicle and plasma membrane-associated SNARES, and fusion of neurotransmitter-containing vesicles to the presynaptic membrane is inhibited, resulting in paralysis^{67,68}. Toxin trafficking determines the type of paralysis, as both BoNT/B and TeNT cleave the host SNARE at the same scissile bond^{66,69}. Flaccid paralysis is characterized by inhibition of acetylcholine release at the neuromuscular junction, with the motorneuron not signaling the downstream muscle to contract⁷⁰. For TeNT, signaling is uncoupled between an interneuron and

motorneuron when release of inhibitory glycine into the synaptic cleft is inhibited. This results in spastic paralysis as the motor neuron is persistently releasing acetylcholine, causing muscle contraction⁷⁰. CNTs bind complex gangliosides, which are enriched in the outer leaflet of neurons⁷¹.

TeNT, when isolated from brain and cultured neuronal cell membranes, was found bound to a glycolipid, later identified as GT1b^{72,73}. TeNT has two ganglioside binding pockets in the receptor binding domain⁷⁴. Toxin binding two gangliosides is necessary and sufficient for entry into neuronal and non-neuronal cells²⁰. Although protein receptors for the toxin have been proposed, none are necessary for intoxication^{75–77}. TeNT has been observed to colocalize with signaling endosome proteins such as the growth factor receptors, which like gangliosides, are enriched on lipid rafts and DIGs⁷⁸. Recent studies indicate that the receptor binding domain pivots with respect to the N-terminal domains of full-length TeNT in response to a change in pH⁷⁹. This may influence the orientation of the translocation domain with respect to channel formation, as BoNT-receptor interactions regulate channel formation^{80,81}. Botulinum neurotoxins, excluding BoNT/C, bind a ganglioside and a protein receptor sequentially; upon depolarization of the neuronal membrane, fusion of synaptic vesicle exposes the luminal domains of a synaptic vesicle protein receptors⁸². Interactions with both the ganglioside and SV2 appear to be sequential for entry to neurons⁸³. The overall structure of BoNT/C aligns well with other BoNTs serotypes, except at the C terminus where there are two unique ganglioside binding pockets, which appear sufficient for the entry of BoNT/C into neurons⁸⁴, precluding the use of a host protein receptor.

Cholera toxin family of AB5 toxins (Ctx/LTx) of *V. cholerae* and enterotoxigenic *Escherichia coli* The cholera toxin family is comprised of Ctx produced by *Vibrio cholerae* and the related heat-labile enterotoxins (LTx), LT-I, LT-IIa, LT-IIb, and LTIIc, produced by enterotoxigenic *Escherichia coli* (ETEC)². *V. cholerae* and ETEC are often found in water and food contaminated with fecal matter. Ctx/LTx target intestinal epithelial cells to cause diarrheal disease, with LTx family members generally eliciting pathology of shorter duration and milder illness than Ctx⁸⁵. Ctx and LTx cause distention in the rabbit ileal loop model, previously a gold standard for diagnosing this disease⁸⁶. Cholera outbreaks are endemic in many developing countries, while LT-related illnesses are associated with travelers' diarrhea in developing countries and more recently, livestock disease in developed countries⁸⁶.

Ctx and LT-I invade the intestinal epithelia by binding with high affinity to the ganglioside GM1a and retrograde traffic to the ER^{21,87}. Ctx/LTx **A1** catalytic domain is an ADP-ribosyltransferase that ADP-ribosylates Arg177 of the stimulatory α -subunit of the heterotrimeric G-protein, G_{α_s} , following translocation into the cytosol^{88–91}. Addition of the negatively charged ADP-ribosyl group constitutively activates G_{α_s} by preventing hydrolysis of GTP. G_{α_s} -GTP binds to host adenylate cyclase, which increases cyclic AMP (cAMP) production by the cyclase and modulates ion channels to release ions into the extracellular space⁹². Persistent chloride ion release and efflux of water out of the intestinal epithelial cells and into the intestine results diarrhea, electrolyte imbalances, and severe dehydration⁹³.

Ctx- and LT-I- **B** subunits share ~ 80% identity and both bind GM1a; however, LT-I also binds LPS and A/B glycolipid antigens on human erythrocytes^{21,94–97}. The **B** subunits are

less conserved between Ctx and LT-II family members with ~ 14% shared identity. LT-IIa predominantly binds GD1b, GD1a and GT1b, and to a lesser extent GM1a and other gangliosides, while LT-IIb predominantly binds only GD1a and GD1b^{22,98}. The newest member LT-IIc has been observed to bind GM1 species with long acyl chains⁹⁹. Ctx/LTx bind up to 5 glycans subunits; however, for Ctx, binding of one GM1a molecule is sufficient, though less efficient for retrograde trafficking and cell intoxication⁴³.

Stx of *Shigella dysenteriae* and Stx1/2 of Shiga toxin-producing *E. coli* Stx, produced by *Shigella dysenteriae*, was identified by Kiyoshi Shiga as the causative agent of dysentery¹⁰⁰. Almost a century later, the toxins now renamed as Shiga-like toxins 1 and 2 (Stx1 and Stx2), were identified in Shiga toxin-producing *E. coli* (STEC). Stx and Stx1 differ by a single amino acid, while Stx1 and Stx2 share ~55% identity³⁰. Both *E. coli* Stx1 and Stx2 produce immunologically distinct subtypes. Stx-producing bacteria cause the infection *Shigella*, which is contracted by ingestion of contaminated foods¹⁰¹.

Stxs are **AB5** toxins, the **A** subunit contains N-glycosidase activity responsible for removal of an adenine residue from 28S rRNA of the 60S ribosome subunit¹⁰². Modification of the rRNA inhibits protein synthesis as the ribosome no longer interacts with elongation factor 1^{103,104}. Stalled synthesis induces ribotoxin stress and the UPR, resulting in apoptosis¹⁰⁵. Stx translocates through intestinal epithelial cells to target the endothelial cell layer, and in serious cases intoxicates the kidneys¹⁰¹. Following modification of the rRNA substrate, the localized infection presents as hemorrhagic diarrhea and if Stx reaches the kidneys, hemolytic uremic syndrome (HUS) occurs as a result of low platelet count, erythrocyte lysis, and kidney failure¹⁰⁰. The five identical **B** pentamers of Stx bind the globoside Gb3, Stx1 and Stx2 weakly bind Gb4 (GalNAc β 1–3Gal α 1–4Gal β 1–4Glc β Cer), while subtype Stx2e preferentially binds globoside Gb4¹⁰⁶. Cells and mice lacking Gb3 are insensitive to Stx, while mice with Fabry's disease containing excess Gb3 throughout tissues also possess increased resistance to Stx¹⁰⁷. Gb3, like other glycosphingolipids, clusters in DIGs or lipid rafts in cells¹⁰⁸. The presence of cholesterol in microdomains enhances binding and entry of the Stx and Stx1, as pre-treatment with cholesterol-binding agents such as methylcyclodextrin (M β CD) reduces Golgi-localization and cytotoxicity¹⁰⁹. Stx2 is associated with lipid rafts *in vitro* and clinically associated with development of HUS; Stx2 does not interact with the host *trans*-Golgi cycling resident protein, GPP130^{106,110,111}

Shiga toxins are endocytosed by clathrin-dependent and clathrin-independent pathways, depending on the cell type^{32,112}. In HeLa cells, clathrin-independent tubular invaginations contribute to sorting of Stx **B** subunits, which bind GPP130 to facilitate retrograde traffic^{113,114}.

Ricin of *Ricinus communis* Ricin (Rtx) is a toxigenic lectin produced by seeds of the castor oil plant *Ricinus communis*. Exposure to Rtx by inhalation or injection is more lethal than digestion, as the toxin is sensitive to bile acids¹¹⁵. The ricin **A** subunit, like Stx, is a type II ribosome-inactivating protein, which inhibits protein synthesis by depurination of the rRNA catalyzed by N-glycosidase activity^{102,116}. Stx- and Rtx- **A** subunit share < 50% identity, but residues involved in adenine abstraction are conserved¹⁰³. Cytotoxic effects of include cell lysis through blebbing and DNA degradation²⁷. The route of exposure determines the

symptoms; intravenous injection in rodents results in hypoglycemia, inflammation, and ketosis, while inhalation results in fluid accumulation in the lungs and eventual respiratory failure¹¹⁷. Like other single-chain **AB** toxins, Rtx **A** subunit catalytic domain connects to the **B** subunit binding domain by a peptide linker. The **B** domain contains two homologous lectin-binding sites specific for galactose^{117–119}. Rtx undergoes post-translational glycosylation on both chains, which remain associated following host cleavage by an interchain disulfide bridge^{4,23}.

Rtx binds glycolipids (including gangliosides) and glycoproteins containing terminal galactose (or N-acetylgalactosamine) in β i-4-linkage. In early endosomal sorting, Rtx enters a recycling pathway and traffics back to the cell surface as well as to lysosomes¹²⁰. Uptake of ricin occurs by two pathways: ricin **B** subunit binds galactose-containing host glycolipid and glycoproteins and conversely, glycosylated residues of ricin toxin are bound by a host mannose receptor, which cycles between *trans*-Golgi and late endosomes^{121,122}. While galactose-binding by the **B** subunit results in robust uptake of Rtx, the latter mannose-dependent uptake by the mannose receptor is responsible for the cytotoxic effects by delivering Rtx to a productive *trans*-Golgi pathway¹²¹.

SubAB of STEC Recently, a new AB5 toxin member, subtilase cytotoxin (SubAB) was found to cause HUS-like illness in STEC strain O113:H21. SubAB binds non-human Neu5Gc as a receptor and is more cytotoxic than Stx in *in vitro* cell culture assays². SubAB is also unique, as the **A** subunit is not required to translocate into the cytosol, since the target substrate is located within the lumen of the ER. The **A** subunit contains subtilase-like serine protease activity and cleaves the ER-resident protein BiP⁴². BiP contributes to the UPR as a chaperone for folding nascent proteins to be degraded by the proteasome¹²³. BiP also forms a seal within the Sec61 pore; proteolysis of BiP destroys the integrity of this barrier leading to ER permeability¹²³. Humans, in contrast to the great apes, have acquired mutations in the biosynthetic pathways to synthesize and convert Neu5Ac into Neu5Gc⁵². With loss of Neu5Gc, Neu5Ac becomes the only sialic acid synthesized. Intriguingly, humans are susceptible to SubAB due to incorporation of dietary Neu5Gc into cellular glycolipids and proteins via meat and dairy consumption¹²⁴.

While SubAB specifically recognizes the sialic acid Neu5Gc, which contains an additional hydroxyl substituent relative to Neu5Ac, many human pathogens have evolved to exploit Neu5Ac as a receptor. For example, the newest member of the enterotoxins, LT-IIc was isolated from avians, some of which produce Neu5Gc, but has similar affinity to both sialic acid species^{99,125}. Incorporation of dietary sialic acids and pathogens that bind multiple sialic acid species has potential to expand the host range for zoonotic diseases.

Ptx of *Bordetella pertussis* Ptx is a major virulence factor produced by *B. pertussis*, which is transmitted by inhalation of aerosolized droplets transmitted early in the infectious state of an infected individual¹²⁶. Although Ptx alone does not cause whooping cough, entry into pulmonary epithelial and endothelial cells leads to inflammation and white blood cell recruitment¹²⁷. Ptx alteration of host signaling also leads to hyperinsulinemia and pulmonary hypertension^{127,128}.

Ptx is an **AB5** family member. Like Ctx, the **A** subunit known as S1, ADP-ribosylates the inhibitory heterotrimeric G-protein (G_{α_i}) at a C-terminal cysteine, causing dysregulation of cAMP signaling and ion efflux¹²⁹. The **B** pentamer is comprised of four heterologous subunits known as S2, S3, S4, and S5, in a 1:1:2:1 ratio, respectively¹³⁰. Heterogeneity of the **B** pentamer subunits, including the two carbohydrate binding subunits, S2 and S3, allows for Ptx to recognize multiple glycans with varied affinities^{131,132}. As a result, Ptx associates with glycolipids and glycoproteins such as the serum protein fetuin, which is decorated with N-linked glycans²⁴. **B** pentamer binding to host receptors alone can induce T cell proliferation and agglutinate erythrocytes¹³³. S2 and S3 are 77% identical and each contain a sialic acid (Sia) binding site and an N-terminal carbohydrate binding site¹³⁰. In a glycan array screen, Ptx bound $\alpha 2-6$ linked Sia in N-glycans, $\alpha 2-3$ linked Sia in O-glycans, and complex gangliosides^{134,135}. To discriminate glycans bound by the N-terminal region, dimers lacking the Sia domain were assembled with S4 for stability and bound asialo N-linked glycans¹³⁴.

Although not unique to Ptx, characterization of glycans bound by the **B** subunits of toxins varies depending on the format of the assay. For example, Ptx binds gangliosides, such as GD1a on liposomes, but unlike Ctx, does not detectably bind lysate-derived gangliosides separated by thin layer chromatography (TLC)^{135,136}. Additional considerations for interpretation of toxin affinity for glycan substrates are the orientation of the glycan, flat or curved surfaces, as well as the linker composition, ceramide, or acyl species of the glycolipids.

Alpha-toxin of *C. perfringens* *C. perfringens* produces many virulence factors including alpha-toxin, a virulence factor for pathogenesis. Alpha-toxin is the causative agent of gas gangrene, which is characterized by edema, myonecrosis, and gas. Inflammation, resulting from alpha-toxin action leads to septic shock and organ failure^{137,138}. Alpha-toxin is also associated with Crohn's Disease, where the toxin targets intestinal epithelial cells and resident macrophages¹³⁹.

Alpha-toxin is a single-chain **AB** toxin, which contains an **A** subunit with zinc-metalloprotease-dependent phospholipase C catalytic activity, which hydrolyzes phospholipids into diacylglycerol (DAG) and ceramide. The **A** subunit is connected to a binding domain that contains both hemolytic activity and a C2-like polycystin-1, lipoyxygenase, alpha-toxin (PLAT) domain¹³⁷. In the presence of calcium, the **B** domain binds and partially inserts into the phospholipid membrane¹⁴⁰. There is also a ganglioside binding site, specific for GM1a, located on a loop between the **A** and **B** subunits¹⁴¹. The generation of DAG, a common eukaryotic second messenger, results in uncoupled signaling and release of cytokines¹⁴².

Molecular interactions of Protein toxins with sugars

The **AB5** family members Ctx and SubAB contain five sugar binding sites, one per **B** subunit, while Ptx contains up to two functional binding sites on each of the S2 and S3 subunits, and Stx contains up to 3 binding sites per subunit^{106,130,143,144}. Each **AB5** family member **B** subunit contains a homologous fold at their C terminus comprised of five or six

antiparallel beta strands, which form a barrel topped by a short alpha helix (Figure 4, **panel A**) with and without sugar. This oligonucleotide and oligosaccharide binding domain is known as Oligomer Binding Fold (OBF)¹⁴⁵. Ptx S2 and S3 subunits contain an additional N-terminal domain with homology towards C-type eukaryotic lectins such as rat mannose binding protein¹³⁴.

Cholera toxin family of AB5 toxins (Ctx/LTx) of *V. cholerae* Ctx and LT utilize a pocket formed by loops to bind GM1a; in Ctx these loops are found between $\beta 1$ to $\alpha 1$, $\beta 4$ to $\alpha 2$, $\beta 5$ to $\beta 6$ on one monomer and the loop between $\beta 2$ to $\beta 3$ on the other monomer¹⁴⁴. The pocket is oriented towards the glycolipid receptor at the membrane. For Ctx, the terminal sugars of GM1a, galactose and sialic acid, predominantly interact with the B subunit^{2,146}. The galactose forms hydrogen bonds via oxygen with the nitrogen atoms in the B subunit residues Asn90 and Lys91¹⁴³. A hydrophobic interaction between galactose and the indole ring of Trp88 is also observed (Figure 4, **panel B**). The sialic acid of GM1a forms hydrogen bonds with the carbon backbone of residues Glu11 and His13, and a hydrophobic interaction with Tyr12⁹⁴. Gly33 lies in a loop between $\beta 2$ and $\beta 3$ of the adjacent monomer and forms the pocket edge with one hydrogen bond to galactose^{94,146}. In addition to GM1a, LT binds asialo-GM1 and GD1b, derivatives that lack and gain an additional sialic acid, respectively; the substitution of His13 for Arg13 in LT is suspected to accommodate this promiscuity¹⁴⁷. LPS binding and erythrocyte group A or B antigens by LT have been mapped to residues not involved in binding GM1a¹⁴⁸.

SubAB of STEC Unlike Ctx and Stx, SubAB binds the non-human derived sialic Neu5Gc in a shallow binding pocket located on outer face of the B pentamer⁴¹. The SubAB pocket resembles the sialic acid pocket of Ptx B subunits S2, S3, and S5, where S2 and S3 bind sialic acid Neu5Ac in a similar orientation, but unique interactions are involved in binding the different sialic species⁴¹. The chemical structure of Neu5Gc differs from human-derived Neu5Ac by a single hydroxyl group⁵². However, the presence of this hydroxyl group allows SubAB pocket residues Tyr78 and Met10 to form hydrogen bonds with sialic acid, where Neu5Gc stacks with Phe11 of SubAB, forming a van der Waals interaction (Figure 4, **panel C**)^{2,41}. The affinity of SubAB for Neu5Gc is unique and specific; serum from chimpanzees containing glycolipids and proteins decorated with Neu5Gc, but not human serum that predominantly contains Neu5Ac, can compete with SubAB binding of immobilized the Neu5Gc glycan¹²⁴.

Ptx of *Bordetella pertussis* Ptx binds glycoproteins through B subunits, S2 and S3, which each contain two unique carbohydrate domains. Analysis of a crystal structure incubated with glycolipids identified electron density for Neu5Aca2–6Gal, but not galactose within the binding pocket^{130,131} (Figure 4, **panel D**). Sialic acid forms hydrogen bonds with Ser104, and hydrophobic interactions with Tyr102 and Tyr103, on the sides of the pentamer^{130,131}. It was previously observed that deletion of Tyr102 and Tyr103 in S2 and S3, or substitution of Ser104 with a charged group reduced biological activity of Ptx¹⁴⁹.

Stx of *Shigella dysenteriae* Stx1 binds the Gb3 analogue in up to three binding sites per B subunit. Gb3 binding pockets are located near the bottom of the B pentamer to allow Stx1 to bind the membrane face¹⁵⁰. Two binding sites are formed between subunits, while one is

formed near the membrane. The first site is a pocket formed on a single monomer by loops $\beta 2$ – $\beta 3$, $\beta 5$ – $\beta 6$, and strands $\beta 3$ and $\beta 4$; hydrogen bonding is observed between both galactose sugars and the **B** subunit residues Thr21, Glu28, Gly60, and Asp17¹⁵⁰. Additionally, hydrophobic interactions, including a ring stacking interaction with Phe30 are observed with Gb3^{106,150}. The second Gb3 binding site is located between monomers and is comprised of loops $\beta 2$ – $\beta 3$ from site one, $\beta 4$ – α , and $\beta 5$ – $\beta 6$ (Figure 4, **panel E**)^{106,109}. This site was originally predicted based on known crystal structures of other **AB5** family members and appears to be highly occupied. The binding pocket contacts Gb3 through hydrogen bonding the two galactose sugars with the Asp16, Asn32, Arg33, Asn55, and Phe63 while many hydrophobic interactions are observed between the galactose sugars, glucose interacts with Asn55¹⁵⁰. At binding site 3, Gb3 is bound within the pentamer cleft by $\beta 4$ – α of the second site, and $\beta 2$ – $\beta 3$ loop at the first site. Asp18, Trp34 and Asn35 hydrogen bond with the first galactose while the residue Trp34 located within and on an adjacent monomer stack with both the first and second galactose moiety^{106,150}.

Rtx of *Ricinus communis* The Rtx crystal structure exhibits two non-cooperative lectin binding domains containing a p-trefoil fold separated by ~ 3.5 nm¹¹⁹. Rtx, binds glycolipids or glycoproteins, and the distance between lectin binding domains allows the **B** subunit to sterically accommodate host receptors¹⁵¹. The two domains are composed of three lobes referred to as 1(α , β , and γ) and 2(α , β , and γ), with 1 α and 2 γ confirmed to bind galactose^{23,118,152,153}. Each lobe contains a tripeptide kink and an aromatic residue; the motif Gln,X,Trp¹¹⁸. For ricin, mutagenesis, docking analysis, and comparison to other lectin-binding proteins predicts two interactions upon receptor binding: ring stacking of the galactose moiety against an aromatic residue and hydrogen bonding between pocket residues and oxygen groups on the sugar¹⁵¹.

Clostridial Neurotoxins of *Clostridium botulinum* and *C. tetani* Alignment of the clostridial toxins alpha-toxin, TeNT, and most BoNTs serotypes, reveals a conserved GM1a binding motif comprised of ~ 30 residues (H...SXWY...G)^{19,141}. TeNT and BoNTs contain this motif within the receptor binding domain, while alpha-toxin contains this sequence on an exposed loop between the A and B subunits^{64,137}. For all toxins, mutagenesis of the aromatic consensus residues tryptophan and tyrosine abrogates binding to the glycolipid receptor.

Tetanus toxin binds the membrane utilizing two pockets. Mutation of Trp1289Ala (W pocket) and Arg1226Leu (R pocket) abrogates ganglioside binding in solid phase and cell binding assays^{20,74}. Mutational analysis of single pockets in TeNT discriminated glycan preference bound by each pocket; the R pocket binding b-series and W pocket binding a-series gangliosides⁷⁴. The crystal structure reveals that the R pocket binds a GT1b analogue in a shallow pocket primarily through the sialic acid moiety. The bound sialic acid forms hydrogen bonds through oxygens to charged and polar residues Asp1147, Asp1214, Asn1216, and Tyr1229⁷⁴. A salt bridge is also formed between the sialic acid and Arg1226^{74,154}. The W pocket, which is also conserved in many BoNTs binds a lactose moiety by stacking with the indole ring of Trp1289 and forming hydrogen bonds between the sugar's hydroxyl group and the charged side chains of His1272, Asp1222 and the amide of Thr1270^{74,154}. The recently solved crystal structure of full length TeNT includes GD1a

within the W pocket which stabilized the crystal⁷⁹. A similar interaction is observed in the W pocket of BoNT/A incubated with a GT1b analogue; analogous hydrogen bonding to His1253, Glu1203, and Ser1264 and hydrophobic interactions with the indole ring of Trp1266¹⁵⁵.

Alpha-toxin does not bind to sialic acids alone or require complex gangliosides, but is specific to GM1a¹⁴¹. Molecular docking simulation predicts that alpha-toxin and GM1a interaction is dependent upon hydrophobic ring stacking and hydrogen bonding of Trp84 in alpha toxin with the sialic acid, while Tyr85 hydrogen bonds with the galactosamine portion of the glycolipid¹⁵⁶. The binding of GM1a is not necessary for endocytosis of alpha-toxin, but is hypothesized to tether the toxin, before binding to phospholipids via C-terminal aromatic residues¹⁴⁰. Binding of alpha toxin to GM1a is necessary to induce signaling through TrkA, a major step in pathogenesis¹⁴².

Protein Toxin-mediated trafficking and signaling

Gangliosides located within lipid rafts are coupled with signaling complexes, which include RTKs and GPCRs that modulate downstream signaling upon ligand binding. Interaction between ganglioside and receptor modulates activity through glycan-glycan or glycan-protein interactions; such interactions include hydrogen bonding that interferes with normal ligand-receptor interactions^{157,158}. For example, how GM3 modulates fibroblast growth factor receptor or epidermal growth factor receptor may be due to reducing receptor-ligand binding affinity or by exclusion of receptors from lipid rafts that leads to dampened signal^{159,160}. Conversely, the ganglioside GM1a stimulates neurotrophin receptors such as TrkA like the endogenous ligand, nerve growth factor (NGF), and leads to receptor activation¹⁶¹. The studies that elucidated gangliosides as ligands for TrkA found brain slices stimulated with high concentrations of whole-ganglioside, but not the components such as sialic acids, glycans, or ceramides, induced autophosphorylation of TrkA leading to downstream signaling through MAP kinase pathway¹⁶¹. In addition to being ligands for growth factor receptors, gangliosides are also ligands for MAG, inhibiting neuriteogenesis and other differentiation processes⁵⁷.

Some protein toxins signal through gangliosides at the membrane to recruit members of endocytic pathways. TeNT receptor binding domain colocalizes with growth factor receptors p75^{NTR} and TrkA in a well-characterized neurotrophic retrograde pathway toward the soma in peripheral motor neurons⁷⁸. TeNT receptor binding domain also stimulates activation of the TrkA receptor in cultured primary neurons¹⁶². Upon addition of TeNT, dose-dependent TrkA autophosphorylation and activation of phospholipase C leads to activation of MAP kinase ERK1/2 and other neuroprotective pathways^{163,164}. TeNT activation of TrkA may mimic the binding of NGF and induce uptake into signaling endosomes toward the retrograde traffic.

Alpha-toxin is associated with lipid rafts. The binding of phospholipids by a C-terminal domain and hydrolysis causes negative membrane curvature and induces endocytosis¹³⁸. Alpha-toxin also binds ganglioside GM1a through a conserved ganglioside motif. This secondary binding of GM1a activates TrkA, causing a phosphorylation cascade of MAP

kinase substrates¹⁴². Unlike TeNT, alpha-toxin signaling is associated with release of inflammatory chemokines and reactive oxygen species, which is responsible for the severity of gas gangrene symptoms. This signaling is mediated through GM1a, as treatment of cells with neuramidase to cleave the terminal sialic acid or an inhibitor of ganglioside synthesis, PPMP, dose-dependently reduces cytokine release upon exposure to alpha toxin¹⁴¹. Mutation of Trp84-Tyr85 attenuates GM1a binding, activation of TrkA signaling, and release of IL-8¹⁴¹. Once alpha-toxin binds the membrane, the phospholipase activity forms DAG which leads to alterations in the membrane that may induce vasculature permeability leading to edema¹³⁸.

AB5 Toxins and Ganglioside Cross-linking

Crosslinking gangliosides can induce endocytosis through receptor activation and can enrich lipid rafts for TrkA¹⁶⁵. Ctx clusters ganglioside GM1a by binding five molecules concurrently, leading to more efficient receptor-mediated internalization, and in neurons, the activation of TrkA^{43,166}. Stx binds up to 15 molecules of Gb3 at the plasma membrane, which activates the RTK Syk, which phosphorylates clathrin heavy chain^{150,167}. This leads to recruitment of adaptor protein 2 and clathrin-coated vesicles to the membrane, and may be responsible for the clathrin-dependent entry of Stx in some cell lines¹⁶⁸. In some cases, fatty acyl chain saturation and chain length are as important as the carbohydrate moiety for toxin binding, entry, and trafficking into productive pathways. One study utilized lipid probes of varying chemical species and acyl lengths to demonstrate that the ceramide portion of GM1a alters lipid trafficking within the cell⁴⁹; unsaturated ceramide chains retrograde trafficked from plasma membrane to the ER more efficiently than long, saturated ceramides, which were not localized with raft-associated proteins⁴⁹. In contrast, Ctx **B** pentamer did not alter lipid localization, which may explain how toxins exploit lipids trafficking to different compartments.

AB5 Toxins and Lipid trafficking

Lipids properties directly affect raft composition, which affect ganglioside dynamics by retaining certain lipids and GPI-anchored protein species¹⁶⁹. Ceramide composition and fatty acid acyl length also affects partitioning properties, cargo sorting, and further increase heterogeneity of the plasma membrane⁴⁹. For Stx1 and Stx2, in vitro binding affinity of Gb3 ganglioside was increased for shorter acyl chains compared to longer acyl chains^{170,171}. Gangliosides with shorter acyl chains partition differently within a cell membrane, leading to alterations in ligand accessibility¹⁷². Chain length may play a physiological role in the association of Stx with lipid rafts in cells, which are also required for clathrin-independent Stx uptake^{167,168}. The disruption of Gb3 localization to lipid rafts protects cells against Stx intoxication, but does not affect binding of the toxin¹⁷³. LT-IIb binds non-raft associated GD1a in cultured T84 human colon carcinoma cells; LT-IIb binds, but does not enter a productive retrograde pathway unlike Ctx which binds GM1a¹⁷⁴. These studies may resolve how host glycolipids as ubiquitous as gangliosides serve to bind and target protein toxins to certain tissues, causing the hallmarks of disease.

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AB toxin organization

AB₅ toxin organization

Figure 1. AB toxin schematic

AB protein toxins contain two functional properties: a catalytically active **A** domain and a receptor binding **B** domain. **AB** protein toxins include alpha-toxin (Atx), ricin toxin (Rtx), and clostridial tetanus and botulinum neurotoxins (TeNT/BoNT). Alpha-toxin does not contain an interchain disulfide, but contains a ganglioside binding loop between the **A** and **B** domains. TeNT/BoNT contain a **B** domain, which includes a receptor binding domain (HCR) and a translocation domain (HCT). HCT facilitates delivery of **A** domain, also known as the light chain (LC). **AB₅** protein toxins include four families: cholera toxin (Ctx), Shiga toxin (Stx), and subtilase cytotoxin (SubAB) contain an **A** domain that can be divided into **A1**- and **A2**- subdomains, and five identical **B** binding domains. Pertussis toxin (Ptx) contains non-identical **B** binding components consisting of four homologous subunits S2:S3:S4:S5 in an 1:1:2:1 molecular organization.

Figure 2. Endocytosis of AB protein toxins

AB5 protein toxins (Ctx, SubAB, Stx, and Ptx) enter by clathrin-mediated endocytosis (CME) and clathrin-independent lipid-raft (LR) pathways. Stx enters endosomal tubules (TB) and retrograde traffics with GPP130 through a bypass pathway towards the Golgi apparatus (GA). Other **AB5** protein toxins enter a retrograde trafficking pathway towards the endoplasmic reticulum (ER). The **A** domain of **AB5** protein toxins, excluding SubAB, retrograde translocate into the cytosol to modify a host substrate. SubAB modifies a resident ER protein. Atx associates with the plasma membrane (PM) phospholipids or lipid raft glycosphingolipids. Rtx and clostridial neurotoxins (CNT) enter early endosomes (EE) (in neurons); Rtx can retrograde traffic towards the ER or recycle back to the cell surface. CNTs escape acidifying endosomes (synaptic vesicles for BoNTs and endosomes in inhibitory neurons for TeNT) into the cytosol to cleave SNARE proteins. Other protein toxins (not shown) enter degradative pathways in late endosomes (LE).

Figure 3. Simple- and complex- gangliosides

Gangliosides are glycosphingolipids which contain a ceramide and oligosaccharides that are complexed with one or more sialic acid (ceramide not depicted). Simple gangliosides are depicted on the top row and more complex gangliosides are depicted on bottom row. Gangliosides are enriched in neurons.

Figure 4. AB5 toxin-aromatic sugar interactions

Crystal structures of the **B** pentamers of AB5 toxins LT-I, Ctx, SubAB, Ptx, and Stx1 are shown in blue on the left of each panel. Sugar ligands (carbohydrates, gangliosides, or globosides) are shown in yellow. Aromatic residues in the **B** domain are shown in green and numbered on the right. Polar groups, hydrophobic interactions, and inter-domain interactions are left out for clarity. Selected oxygen atoms are shown in red and nitrogen atoms shown in blue. **A)** LT-I **B** pentamer (PDB: LTT1) contains an oligosaccharide binding fold (OBF) shown in green and is bound to lactose. The oligosaccharide binding site is shown magnified on the right. **B)** Ctx **B** pentamer (PDB: 3CHB) shown in blue with one subunit bound to GM1a. A single subunit is shown on right with aromatic residues involved in binding. **C)** SubAB **B** pentamer (PDB: 3DWP) is bound to Neu5Gc, with magnification of this interaction shown on right. **D)** Ptx **B** pentamer (PDB: 1PTO) is shown with S3 bound to GM1a. S2 and S3 interactions with GM1a are similar; S3-GM1a is shown on the right. **E)**

Stx1 **B** pentamer (PDB: 1BOS) is shown bound to three Gb3 molecules. One **B** subunit interacts with three Gb3 molecules shown on the right.

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Table 1:**AB Protein Toxin Receptors, Action, and Disease**

Organism	Toxin	Receptor(s)	Substrate	Enzyme action	Disease
<i>C. tetani</i>	TeNT	GT1b	VAMP	Zn ²⁺ metalloprotease	spastic paralysis
<i>C. botulinum</i>	BoNT/A	GT1b, SV2C	SNAP-25	Zn ²⁺ Metalloprotease	flaccid paralysis
<i>C. perfringens</i>	Atx	GM1a	Phosphatidyl choline	Phospholipase C	gas gangrene
<i>V. cholerae</i>	Ctx	GM1a	Gα _s	ADP-ribosyl transferase	diarrheal disease
ETEC	LT-I LT-IIa LT-IIb LT-IIc	GM1a, LPS, A/B blood GD1b>GD1a>GM1a GD1a GM1a, long acyl	Gα _s	ADP-ribosyl transferase	diarrheal disease
<i>B. pertussis</i>	Ptx	glycolipids, glycoproteins; galactose, lactose, GD1a	Gα _i	ADP-ribosyl transferase	whooping cough
<i>R. communis</i>	Rtx	glycolipids, glycoproteins; galactose	A4256 28S rRNA	RNA N-glycosidase	pulmonary edema, seizures
<i>S. dysenteriae</i>	Stx	Gb3	A4256 28S rRNA	RNA N-glycosidase	hemorrhagic colitis, HUS
STEC	Stx1 Stx2	Gb3 Gb3, Gb4	A4256 28S rRNA	RNA N-glycosidase	hemorrhagic colitis, HUS
STEC	SubAB	glycolipids, glycoproteins; Neu5Gc	BiP	Subtilase-like serine protease	HUS