JB Minireview—Membrane Traffic in Physiology and Pathology

J. Biochem. **140**, 155–160 (2006)
doi:10.1093/jb/mvj161

Transport of Bacterial Toxins into Target Cells: Pathways Followed by Cholera Toxin and Botulinum Progenitor Toxin

Yukako Fujinaga*

International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Yamada-oka 3-1, Suita, Osaka 565-0871, and PRESTO, JST, 4-1-8 Honcho Kawaguchi, Saitama

Received March 16, 2006; accepted March 20, 2006

A number of bacterial toxins have sophisticated mechanisms for reaching their specific targets in mammalian cells, to exert their toxicity. This review focuses on the transport mechanisms of cholera toxin and botulinum neurotoxin complex. Cholera toxin is an ADP-ribosyltransferase toxin, and the covalent modification of heterotrimeric Gs protein in the cytosol leads to the activation of adenylyl cyclase and a sequence of events culminating in massive diarrheal disease. Here, we describe the structural features of this toxin and the transport pathway followed by this toxin from the plasma membrane to the cytosol of intestinal epithelial cells. Botulinum neurotoxin is a metalloprotease toxin that enters neurons, where it cleaves core proteins of the neuroexocytosis apparatus and elicits the inhibition of neurotransmitter release. The food-borne botulism is manifested when the neurotoxin is absorbed from the digestive tract, enters the blood stream, and reaches the cytosol of the peripheral nerves. We overview the structural organization and the long journey followed by this toxin.

Key words: botulinum toxin, cholera toxin, ganglioside, intestinal epithelial cell, raft, trafficking.

Many bacterial toxins are able to cause severe damage to the host, even at very low concentrations. Most bacterial toxins are enzymes, which act catalytically and with high specificity on the functional molecules of host cells, resulting in the marked modulation of the host homeostasis. Also, they have mechanisms by which they efficiently reach their target molecules in the host cells; in other words, they have ingenious transport systems. These toxin transport systems are often based upon the fundamental machinery of membrane trafficking and the functions of intracellular organelles (*1*, *2*).

A number of bacterial toxins have their target molecules in the cytosol of host cells. Most of these toxins are endocytosed before translocation to the cytosol. There are two major routes by which the toxins can enter the cytosol (Table 1). Several toxins, such as diphtheria toxin and botulinum neurotoxin translocate from endosomes into the cytosol in response to the acidification of the endosomes. Other toxins, such as cholera toxin and Shiga toxin retrogradely enter the ER *via* the golgi, and translocate from the ER.

This review is focused on the transport mechanisms of two bacterial toxins, cholera toxin and botulinum neurotoxin complex.

Cholera toxin (CT) is an enterotoxin released by *Vibrio cholerae*, and is responsible for the massive secretory diarrhea caused by infection with *V. cholerae* (*3*). The enzymatically active A subunit must enter the cytosol of the intestinal epithelial cells, to reach their target molecule, the α subunit of heterotrimeric Gs proteins (Gsα). The process by which the cholera toxin enters the cells and is translocated to the cytosol, where it exerts its toxicity remains obscure, although retrograde transport of the toxin to the ER has been implicated (*4*, *5*). Recently, the pathway followed by the cholera toxin from the plasma membrane to the ER lumen has been clarified (*6*). The first half of this review focuses on the molecular structure and trafficking of the cholera toxin into the cytosol *via* the golgi and the ER.

Botulinum neurotoxin (BoNT / NTX / 7S toxin; 150 kDa) produced by *Clostridium botulinum* is a metalloprotease toxin (*7*). BoNT binds highly specifically to peripheral nerve terminals, such as the motor neuron nerve endings, then enters the cytosol of the neuron. In the cytosol, the BoNT cleaves the SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor) proteins involved in the fusion process of synaptic vesicles with the presytaptic membrane, thereby blocking the release of acetylcholine, consequently causing a flaccid paralysis. Human and animal food-borne botulism are caused by the ingestion of food or feed contaminated with this toxin. After absorption from the small intestine, the toxin enters the lymphatic system, then the blood stream, and eventually reaches the peripheral nerves (*8*). In the second half of this review, we will provide an overview of the structural organization of the botulinum neurotoxin complex and the long journey followed by this toxin, starting from the lumen of digestive tract to the final destination, the cytosol of the neuron.

Cholera toxin

A. structure and function. Cholera toxin consists of one A (active) subunit that has an enzymatic activity and five B (binding) subunits that have a role in binding the

*For correspondence. Tel: +81-6-6879-4250, Fax: +81-6-6879-4252, E-mail: yukafuji@biken.osaka-u.ac.jp

Table 1. **Intracellular transport pathways of bacterial toxins after endocytosis.**

Transport pathway	Toxin	Activity	Target
Endosome	Diphtheria toxin	ADP-ribosyl transferase	Elongation factor-2
⇓ Acidification Cytosol	Anthrax toxin	Edema factor: Adenylate cyclase	Proteins of which function are changed by the intracellular cAMP
		Lethal factor: metalloprotease	MAPKK1, 2
	Clostridium neurotoxins (botulinum and tetanus toxins)	metalloprotease	SNARE (VAMP/synaptobrevin, SNAP-25, syntaxin 1)
	Botulinum C2	ADP-ribosyl transferase	G-actin
	Clostridium difficile toxin B	Glucosyl transferase	Rho
	Escherichia coli cytotoxic necrotizing factor (CNF)	Deamidase	Rho
ER	Cholera toxin	ADP-ribosyl transferase	Heterotrimeric G protein (Gs)
⇓ ERAD system	Shiga toxin	RNA *N*-glycosidase	28S rRNA
Cytosol	*Pseudomonas* exotoxin A (PE)	ADP-ribosyl transferase	Elongation factor-2

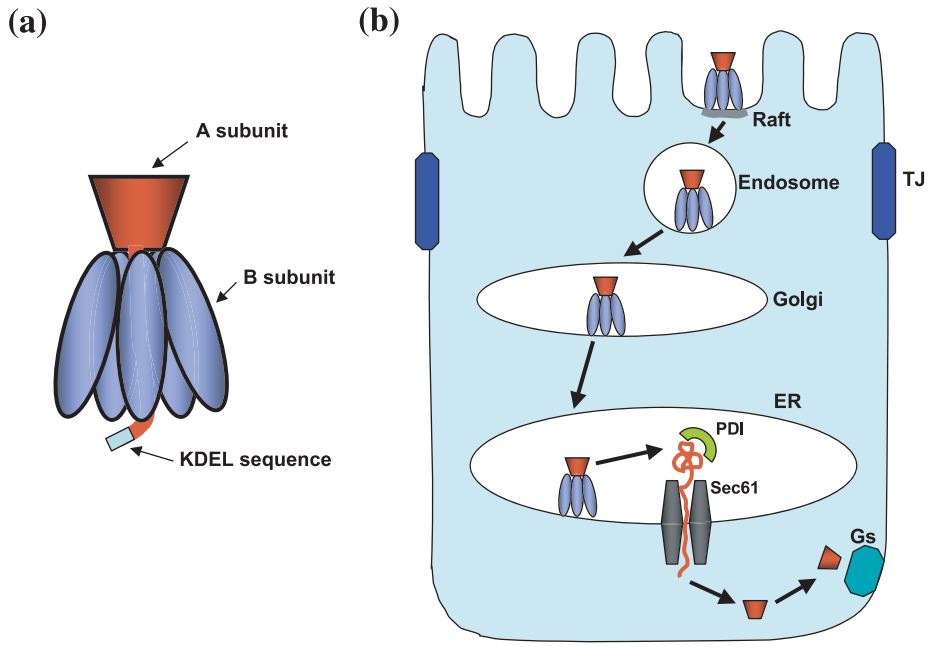

Fig. 1. **(a) Schematic structure of cholera toxin. (b) The pathway followed by the cholera toxin from the apical cell surface through the Golgi and the ER to the cytosol.** Cholera toxin bound to the cell surface GM1 in the lipid raft is endocytosed, and transported retrogradely to the Golgi and the ER as a holotoxin. At the ER, PDI (protein disulfide isomerase) unfolds and dissociates the A1 fragment from the B subunits. Then, at least the A1 subunit is translocated into the cytosol, probably through the Sec61 channel.

host cells (Fig. 1). The B moiety forms a highly stable ring-like assembly with five identical B subunits, and one B subunit-ring is able to bind up to five molecules of monosialoganglioside GM1 (*9*). The A subunit is cleaved by a bacterial endoprotease to form two fragments, A1 and A2, linked by a disulfide bond. This cut occurs after secretion from the microbe and is required for toxicity (*10*). The A1 fragment is ADP-ribosyltransferase, which ADP-ribosylates Gsα, thereby constitutively activating adenylyl cyclase and increasing the level of cAMP (*3*). The A2 fragment bores through the center of the B subunit ring, and its C-terminal portion protrudes (*11*). In the extreme C-terminus of the A2 fragment, there exists a KDEL (Lys-Asp-Glu-Leu) sequence, which is known to be an ER retrieval motif.

B. Transport from plasma membrane to ER. It was suggested that the binding of cholera toxin to a lipid raft via GM1 is important for the internalization and activation of the toxin (*12–14*). Recently, Lencer's group has elucidated the pathway followed by the cholera toxin from the plasma membrane to the ER lumen (*6*, *10*) (Fig. 1). We found that the GM1-cholera toxin complex in the lipid raft brings the holotoxin (A/B_5 subunits toxin) into the ER via the Golgi, whereas non-raft ganglioside does not carry the toxin to the Golgi or ER. The A2 fragment of the cholera toxin A subunit has an ER retrieval KDEL sequence. An amino acid substitution that inactivates this motif reduces the activity of the toxin (*5*). This sorting motif is not involved in the toxin transport from the Golgi to ER, but is involved in stemming the outflow of the toxin that has reached in the ER (*6*). These studies suggest that transport of the cholera toxin from the plasma membrane to the Golgi and ER is dictated by gangliosides existing in the lipid raft. The synthesis and forward transport of the ganglioside has long been established: the ceramide moiety is synthesized in the ER and

the carbohydrate chain in the Golgi, and the mature molecules then travel on to the plasma membrane (*15*). Our data demonstrated that the GM1 also exists in the ER, even when the cells have not been exposed to the toxins (*6*). These data suggest that some GM1 on the plasma membrane is able to go back to the ER in its physiological condition, and the cholera toxin may utilize this physiological pathway. There are a number of pathogens (viruses and toxins) that need to be transported to the ER to exert their pathogenicity, and many pathogens in this class were recently found to utilize raft gangliosides as carriers (*2*, *16*). Therefore, this retrograde transport pathway of raft ganglioside seems to be one of the general pathways utilized by the pathogens. However, a detailed mechanism that brings the toxin-raft GM1 complex to the Golgi and ER are still unknown. Further studies are required to clarify this process.

C. Translocation from the ER to the cytosol. Our data demonstrate that cholera toxin goes into the ER as a holotoxin (*6*). In the ER, ER chaperone protein disulfide isomerase (PDI) binds to the A1 fragment, unfolds it and releases it from the rest of the toxin (*4*, *17*). Then, the unfolded A1 fragment is transferred across the ER membrane (retro-translocation). However, the details of this retro-translocation process are not entirely clarified. There is evidence that this translocation occurs through the Sec61 channel (*18*). This pathway of the A1 fragment is assumed to utilize ERAD (ER-associated degradation), which is one of the physiological cell functions for the exclusion of misfolded proteins (*19–21*). The ERAD system is a complex process involving the recognition of misfolded proteins, ER retention, retro-translocation of proteins into the cytosol, followed by ubiquitination and subsequent proteolysis by the proteasome. The A1 fragment is not degraded by the proteasome (*22*). The mechanisms by which cholera toxin avoids proteasomal degradation may be based on the paucity of lysine residues, which are necessary for ubiquitination (*23*), and the rapid refolding of the toxin in the cytosol (*22*). These studies suggest that the A1fragment exploits a part of the ERAD system to enter the cytosol from the ER, but avoids the proteosomal degradation to reach the target molecule, Gs.

Botulinum toxin

Structure and function. BoNT is classified into seven serotypes, A through G, on the bases of their immunological properties. These toxins are synthesized as single-polypeptide chains of ~150 kDa, but must be posttranslationally modified by a bacterial or tissue protease for activation (*7*). The active form of the toxin consists of a light chain (L, 50 kDa) and a heavy chain (H, 100 kDa). The chains remain covalently and reversibly linked by a disulphide bond until exposed reducing conditions, such as the nerve cytosol. The L chain is a zinc endopeptidase which cleaves the SNARE proteins VAMP (vesicle-associated membrane protein/synaptobrevin; cleaved by BoNT type B, D, F and G), located in SVs (synaptic vesicles), and SNAP-25 (25 kDa synaptosome associated proteins; cleaved by BoNT type A, C and E) and syntaxin (cleaved by BoNT type C), expressed mainly in the plasma membrane (*7*). The H chain is composed of two domains and serves as the vehicle that delivers the L chain into the cytosol of neuronal cells. The carboxy-terminal part of the H chain (H_C) is mainly responsible for the neurospecific binding, and the amino-terminal part of the H chain (H_N) is thought to mediate translocation of the L chain from the lumen of an acidic intracellular compartment into the cytosol.

When produced by the bacterium, the neurotoxin is found as complex forms (progenitor toxins) associated with nontoxic components (non–toxic neurotoxin–associated proteins, NAPs), which are coded by genes adjacent to the BoNT gene (*8*, *24*, *25*) (Fig. 2). Three different forms of progenitor toxins have been identified; 12S toxin (M toxin/M-TC), 16S toxin (L toxin/L-TC), and 19S toxin (LL toxin/LL-TC) (*8*). 12S toxin is composed of a BoNT and a non-toxic non-HA (NTNH, also known as NTNHA; 130 kDa). 16S toxin is composed of a BoNT, an NTNH and two hemagglutinin (HA) components. 19S toxin has the same components as 16S toxin, and is presumed to be a dimer of two 16S toxins linked by one of the HA subunits, HA1 (*26*). The *C. botulinum* type A strain produces 12S, 16S and 19S toxins. Type B, C and D strains produce 16S and 12S toxins. Type E and F strains produce 12S toxin. Type G strain produces 16S toxin. The HA component has four different subunits; HA1 (also referred to as HA-33 in type C and D, HA-34 in type B and HA-35 in type A, based on their molecular weight), HA2 (also referred to as HA-15 in type A, HA-17 in type C and D and HA-18 in type B), HA3a (also referred to as HA-19-20 in type A, HA-19-23 in type B and HA-22-23 in type C and D), and HA3b (also referred to as HA-51 in type B, HA-52 in type A and HA-53 in type C and D). HA3a and 3b are generated by cleavage of their precursor polypeptide, HA3 (also referred to as HA-70) (*27*). All these components of the neurotoxin complex are associated with each other with non-covalent binding. The BoNT dissociates from the nontoxic components in slightly alkaline conditions (higher than pH 7.2) (*8*).

It is very important to elucidate how the subunits interact with each other to form the progenitor toxin complexes, to understand the role of each subunit in botulinum intoxication. Ohyama's group has recently elucidated the precise stoichiometry of each subunit in the type D 16S toxin (*28*, *29*), and proposed an assembly model for the arrangement of each subunit in the 16S toxin through the susceptibility test to trypsin proteolysis (*30*) (Fig. 2).

Penetration of intestinal epithelial barrier. The BoNT must pass down the digestive tract, and cross the epithelial barrier lining the intestine to cause food-borne botulism. It was long known that bare BoNT prepared from the progenitor toxins is only weakly toxic to mice when administrated orally. The oral toxicity increases with the incremental association of the BoNT with the nontoxic components (*8*). It has been proposed that their greater efficacy is due to the protective effect toward BoNT by the nontoxic proteins from the low pH and proteases in the digestive tract (*8*). HA1 has also reported to carry out the role of enhancing the endopeptidase activity of BoNT, by a different mechanism from its protective effect against the external environment (*31*).

There is a fundamental question still to be resolved; how does this large protein toxin breach the intestinal epithelial barrier? To answer this question, we have studied the interaction of progenitor toxins with the intestinal epithelium, and found that the HA component of type C

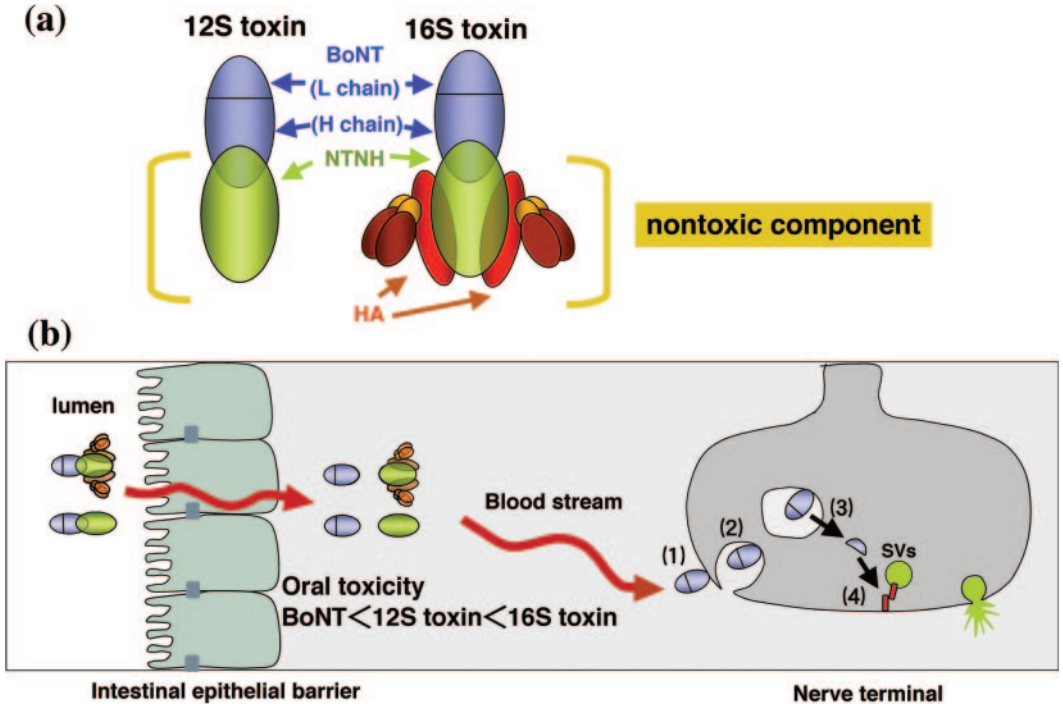

Fig. 2. **(a) Schematic model for the arrangement of individual subcomponents in botulinum 16S toxin (adopted from Suzuki *et al.* 2005, with minor alteration) and 12S toxin.** 16S toxin produced by *C. botulinum* type D strain 4947 consists of one molecule of neurotoxin (blue), one molecule of NTNH (green), two molecules of HA3 (red), four molecules of HA1 (brown), and four molecules of HA2 (orange) (*30*). 12S toxin is a heterodimer of BoNT and NTNH (nicked form) (*25*). **(b) The pathway followed by botulinum neurotoxin complexes from the lumen of the intestinal tract to the cytosol of the peripheral nerve terminal.** Orally ingested botulinum neurotoxin complexes (12S and 16S) must cross the intestinal epithelial barrier to cause the food borne botulism. After absorption from the small intestine, the botulinum neurotoxin complexes enter the lymphatic system, then the blood stream (*8*). In the lymphatic circulation and blood, BoNTs exist as a free form dissociated from the complex (*8*), and binds specifically to neurons (*7*). Inhibition of neurotransmitter release occurs *via* a four step mechanism: 1, binding; 2, internalization; 3, translocation; 4, cleavage of the SNARE proteins (*7*).

16S toxin has a selective binding activity to the microvilli of the upper small intestine *via* sialic acid residues in the cell surface glycoconjugates (*32*). Moreover, in an intestinal epithelial cell line (HT-29), the cell surface sialic acid containing *O*-linked glycoproteins recognized by the type C 16S toxin was shown to induce the internalization of the toxin into the cells (*33*, *34*). On the other hand, it was reported that bare BoNT is able to pass through the intestinal epithelial monolayer by transcytosis *in vitro* (*35*). *In vivo*, a substantial amount of ingested progenitor toxins seem to exist as un-dissociated forms, at least until when the progenitor toxins reach the luminal side of the intestine. This is because the BoNT does not dissociate from the nontoxic components in the intestinal juice (*8*). Therefore, it is intriguing to speculate that the HA associated with the BoNT may have the role of increasing the efficiency of the transcytosis of BoNT.

Studies using recombinant HA subcomponents showed that two distinct subcomponents, HA1 and HA3b, have binding activities with different specificities (*36*, *37*). For instance, the recombinant type C HA1 recognizes NeuAcα2-3 Galβ1- and Galβ1-4 GlcNAcβ1-, whereas HA3b (and its precursor form, HA3) recognizes NeuAcα2-3 Galβ1- (*37*). These results reveal a complexity of the molecular mechanism by which the progenitor toxin recognizes the carbohydrate ligands. Although we do not know why the 16S toxin has two different carbohydrate-binding subcomponents with different ligand specificities, we speculate that the multi-step dependent activation of the 16S toxin binding *via* two different subcomponents is needed for passing through the barrier of intestinal epithelial cells.

Intracellular trafficking in neurons. After entering the systemic circulation, the BoNT exists as a free form dissociated from the complex (*8*), and binds specifically to neurons (*7*). A large number of studies have been focused on the identification of botulinum neurotoxin receptors, and a widely held view is that polysialogangliosides and certain proteins are involved in the specific binding of the toxin at the neuronal cell surface (*38*, *39*). It has been reported that synaptic vesicle proteins, synaptotagmin I and II interact with BoNT type A (*40*), B (*41*), E (*40*) and G (*42*). This evidence has been confirmed by studies showing that synaptotagmin I and II mediate the entry of the BoNT type B (but not type A or E) into PC12 cells (*43*). It is also demonstrated that the BoNT type B is co-internalized with the synaptotagmins when the cells were depolarized with high [K^+] to induce exocytosis of the synaptic vesicles (*43*). These results suggest that the synaptotagmins are functional receptors that mediate BoNT type B entry through the recycling of SVs.

Subsequent to endocytosis, the L chains of BoNTs must cross the vesicle membrane and enter the cytosol, where they exert their proteolytic activity. There is indirect but persuasive evidence that BoNTs need to be exposed to the

low pH of the endocytic vesicles before translocation of the toxins into the cytosol can occur; cells were protected against BoNTs intoxication by bafilomycinA1, an inhibitor of the proton-ATPase responsible for endosomal acidification (*44*). The low pH is assumed to trigger a conformational change of BoNT, converting the toxins from a water-soluble form to an integral membrane form with surface-exposed hydrophobic segments within the H_N domain, and their insertion into the lipid bilayer (*7*). After this membrane insertion, BoNTs form ion channels in lipid bilayers by the oligomerization of the H_N domain (*7*). These channels are believed to mediate the process of translocation of the L chain across the vesicle membrane into the nerve cytosol. Although the molecular details about the elusive translocation process that occurs at the cell membrane are still poorly understood, recent data provide evidence that translocation through reconstituted lipid bilayers occurs with a partially unfolded L chain conformation, whereupon the H-chain channel presumably functions as a pH-driven transmembrane chaperone (*45*).

It has long been known that nerve stimulation facilitates BoNT intoxication (*46*). After vigorous neurotransmitter release, the SVs undergo rapid recycling *via* endocytosis and refilling with neurotransmitter by proton-driven neurotransmitter transporters (*7*). The activity-dependence of botulinum intoxication, together with the evidence that synaptotagmins interact with specific types of BoNTs and that low pH induces membrane translocation of the BoNTs, converge on a hypothesis; the BoNT enters the SVs and translocates from the vesicles in response to the acidification of the SVs, by exploiting the recycling and refilling process of SVs. To prove this idea, further studies on the complete identification of the functional receptors for each type of BoNTs, as well as the characterization of the endocytic vesicles, are needed.

Perspectives

A number of protein toxins exploit the fundamental cellular machinery, such as membrane trafficking and functions of intracellular organelles, to reach their target molecule in the host cells. Therefore, studies on toxin trafficking, in some cases, provide us with valuable information on basic cellular function, in addition to important knowledge regarding the pathology and therapeutics of these toxins. To exploit such a wealth of information lying under the toxin studies, combined research based on the comprehension of toxin biochemistry, and appropriate views from the perspective of cell biology are indispensable. We believe that detailed studies on the trafficking of protein toxins, such as cholera toxin and botulinum toxin, will give us novel information on cellular processes in general.

REFERENCES

1. Schiavo, G. and van der Goot, F.G. (2001) The bacterial toxin toolkit. *Nat. Rev. Mol. Cell. Biol.* **2**, 530–537
2. Sandvig, K. and van Deurs, B. (2002) Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. *FEBS Lett.* **529**, 49–53
3. Spangler, B.D. (1992) Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev.* **56**, 622–647
4. Orlandi, P.A. (1997) Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line. *J. Biol. Chem.* **272**, 4591–4599
5. Lencer, W.I., Constable, C., Moe, S., Jobling, M.G., Webb, H.M., Ruston, S., Madara, J.L., Hirst, T.R., and Holmes, R.K. (1995) Targeting of cholera toxin and *Escherichia coli* heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J. Cell Biol.* **131**, 951–962
6. Fujinaga, Y., Wolf, A.A., Rodighiero, C., Wheeler, H., Tsai, B., Allen, L., Jobling, M.G., Rapoport, T., Holmes, R.K., and Lencer, W.I. (2003) Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to endoplasmic reticulm. *Mol. Biol. Cell* **14**, 4783–4793
7. Schiavo, G., Matteoli, M., and Montecucco, C. (2000) Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* **80**, 717–766
8. Sakaguchi, G., kosaki, S., and Ohishi, I. (1984) Structure and function of botulinum toxins. In *Bacterial Protein Toxins*, pp. 435–443, Academic Press, London
9. Merritt, E.A., Sarfaty, S., van den Akker, F., L'Hoir, C., Martial, J.A., and Hol, W.G. (1994) Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci.* **3**, 166–175
10. Lencer, W.I. and Saslowsky, D. (2005) Raft trafficking of AB5 subunit bacterial toxins. *Biochim. Biophys. Acta* **1746**, 314–321
11. Zhang, R.G., Scott, D.L., Westbrook, M.L., Nance, S., Spangler, B.D., Shipley, G.G., and Westbrook, E.M.he three-dimensional crystal structure of cholera toxin. *J. Mol. Biol.* **251**, 563–573
12. Orlandi, P.A. and Fishman (1995) T, P.H. (1998) Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* **141**, 905–915
13. Wolf, A.A., Jobling, M.G., Wimer-Mackin, S., Ferguson-Maltzman, M., Madara, J.L., Holmes, R.K., and Lencer, W.I. (1998) Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J. Cell Biol.* **141**, 917–927
14. Wolf, A.A., Fujinaga, Y., and Lencer, W.I. (2002) Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. *J. Biol. Chem.* **277**, 16249–16256
15. Kolter, T., Proia, R.L., and Sandhoff, K. (2002) Combinatorial ganglioside biosynthesis. *J. Biol. Chem.* **277**, 25859–25862
16. Tsai, B., Gilbert, J.M., Stehle, T., Lencer, W., Benjamin, T.L., and Rapoport, T.A. (2003) Gangliosides are receptors for murine polyoma virus and SV40. *EMBO J.* **22**, 4346–4355
17. Tsai, B., Rodighiero, C., Lencer, W.I., and Rapoport, T.A. (2001) Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell* **104**, 937–948
18. Schmitz, A., Herrgen, H., Winkeler, A., and Herzog, V. (2000) Cholera toxin is exported from microsomes by the Sec61p complex. *J. Cell Biol.* **148**, 1203–1212
19. Matlack, K.E., Mothes, W., and Rapoport, T.A. (1998) Protein translocation: tunnel vision. *Cell* **92**, 381–390
20. Tsai, B., Ye, Y., and Rapoport, T.A. (2002) Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat. Rev. Mol. Cell. Biol.* **3**, 246–255
21. Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell. Biol.* **4**, 181–191
22. Rodighiero, C., Tsai, B., Rapoport, T.A., and Lencer, W.I. (2002) Role of ubiquitination in retro-translocation of cholera toxin and escape of cytosolic degradation. *EMBO Rep.* **3**, 1222–1227
23. Hazes, B. and Read, R.J. (1997) Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry* **36**, 11051–11054

24. Minton, N.P. (1995) Molecular genetics of clostridial neurotoxins. *Curr. Top. Microbiol. Immunol.* **195**, 161–194
25. Oguma, K., Inoue, K., Fujinaga, Y., Yokota, K., Watanabe, T., Ohyama, T., Takeshi, K., and Inoue, K. (1999) Structure and function of *Clostridium botulinum* progenitor toxin. *J. Toxicol-Toxin Rev.* **18**, 17–34
26. Inoue, K., Fujinaga, Y., Watanabe, T., Ohyama, T., Takeshi, K., Moriishi, K., Nakajima, H., and Oguma, K. (1996) Molecular composition of *Clostridium botulinum* type A progenitor toxins. *Infect. Immun.* **64**, 1589–1594
27. Fujinaga, Y., Inoue, K., Shimazaki, S., Tomochika, K., Tsuzuki, K., Fujii, N., Watanabe, T., Ohyama, T., Takeshi, K., Inoue, K., and Oguma, K. (1994) Molecular construction of *Clostridium botulinum* type C progenitor toxin and its gene organization. *Biochem. Biophys. Res. Commun.* **205**, 1291–1298
28. Kouguchi, H., Watanabe, T., Sagane, Y., Sunagawa, H., and Ohyama, T. (2002) In vitro reconstitution of the *Clostridium botulinum* type D progenitor toxin. *J. Biol. Chem.* **277**, 2650–2656
29. Mutoh, S., Kouguchi, H., Sagane, Y., Suzuki, T., Hasegawa, K., Watanabe, T., and Ohyama, T. (2003) Complete subunit structure of the *Clostridium botulinum* type D toxin complex via intermediate assembly with nontoxic components. *Biochemistry* **42**, 10991–10997
30. Suzuki, T., Watanabe, T., Mutoh, S., Hasegawa, K., Kouguchi, H., Sagane, Y., Fujinaga, Y., Oguma, K., and Ohyama, T. (2005) Characterization of the interaction between subunits of the botulinum toxin complex produced by serotype D through tryptic susceptibility of the isolated components and complex forms. *Microbiology* **151**, 1475–1483
31. Sharma, S.K. and Singh, B.R. (2004) Enhancement of the endopeptidase activity of purified botulinum neurotoxins A and E by an isolated component of the native neurotoxin associated proteins. *Biochemistry* **43**, 4791–4798
32. Fujinaga, Y., Inoue, K., Watanabe, S., Yokota, K., Hirai, Y., Nagamachi, E., and Oguma, K. (1997) The haemagglutinin of *Clostridium botulinum* type C progenitor toxin plays an essential role in binding of toxin to the epithelial cells of guinea pig small intestine, leading to the efficient absorption of the toxin. *Microbiology* **143**, 3841–3847
33. Nishikawa, A., Uotsu, N., Arimitsu, H., Lee, J.C., Miura, Y., Fujinaga, Y., Nakada, H., Watanabe, T., Ohyama, T., Sakano, Y., and Oguma, K. (2004) The receptor and transporter for internalization of *Clostridium botulinum* type C progenitor toxin into HT-29 cells. *Biochem. Biophys. Res. Commun.* **319**, 327–333
34. Uotsu, N., Nishikawa, A., Watanabe, T., Ohyama, T., Tonozuka, T., Sakano, Y., and Oguma, K. (2006) Cell internalization and traffic pathway of *Clostridium botulinum* type C neurotoxin in HT-29 cells. *Biochim. Biophys. Acta* **1763**, 120–128
35. Maksymowych, A.B. and Simpson, L.L. (1998) Binding and transcytosis of botulinum neurotoxin by polarized human colon carcinoma cells. *J. Biol. Chem.* **273**, 21950–21957
36. Fujinaga, Y., Inoue, K., Nomura, T., Sasaki, J., Marvaud, J.C., Popoff, M.R., Kozaki, S., and Oguma, K. (2000) Identification and characterization of functional subunits of *Clostridium botulinum* type A progenitor toxin involved in binding to intestinal microvilli and erythrocytes. *FEBS Lett.* **467**, 179–183
37. Fujinaga, Y., Inoue, K., Watarai, S., Sakaguchi, Y., Arimitsu, H., Lee, J., Jin, Y., Matsumura, T., Kabumoto, Y., Watanabe, T., Ohyama, T., Nishikawa, A., and Oguma, K. (2004) Molecular characterization of binding subcomponents of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes. *Microbiology* **150**, 1529–1538
38. Montecucco, C., Rossetto, O., and Schiavo, G. (2004) Presynaptic receptor arrays for clostridial neurotoxins. *Trends Microbiol.* **12**, 442–446
39. Tsukamoto, K., Kohda, T., Mukamoto, M., Takeuchi, K., Ihara, H., Saito, M., and Kozaki, S. (2005) Binding of *Clostridium botulinum* type C and D neurotoxins to ganglioside and phospholipid. Novel insights into the receptor for clostridial neurotoxins. *J. Biol. Chem.* **280**, 35164–35171
40. Li, L. and Singh, B.R. (1998) Isolation of synaptotagmin as a receptor for types A and E botulinum neurotoxin and analysis of their comparative binding using a new microtiter plate assay. *J. Nat. Toxins* **7**, 215–226
41. Nishiki, T., Kamata, Y., Nemoto, Y., Omori, A., Ito, T., Takahashi, M., and Kozaki, S. (1994) Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes. *J. Biol. Chem.* **269**, 10498–10503
42. Rummel, A., Karnath, T., Henke, T., Bigalke, H., and Binz, T. (2004) Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. *J. Biol. Chem.* **279**, 30865–30870
43. Dong, M., Richards, D.A., Goodnough, M.C., Tepp, W.H., Johnson, E.A., and Chapman, E.R. (2003) Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J. Cell Biol.* **162**, 1293–1303
44. Simpson, L.L., Coffield, J.A., and Bakry, N. (1994) Inhibition of vacuolar adenosine triphosphatase antagonizes the effects of clostridial neurotoxins but not phospholipase A2 neurotoxins. *J. Pharmacol. Exp. Ther.* **269**, 256–262
45. Koriazova, L.K. and Montal, M. (2003) Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat. Struct. Biol.* **10**, 13–18
46. Hughes, R. and Whaler, B.C. (1962) Influence of nerve-ending activity and of drugs on the rate of paralysis of rat diaphragm preparations by *Cl. botulinum* type A toxin. *J. Physiol.* **160**, 221–233