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Holger Barth *Editor*

Uptake and Trafficking of Protein Toxins

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Uptake and Trafficking of Protein Toxins

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Preface

Bacterial protein toxins of the AB-type are the most toxic substances known today. They cause a variety of severe diseases in humans and animals either after uptake of the isolated toxins into the body, e.g. in the context of food-borne diseases such as botulism, or after uptake of toxin-producing bacteria or their spores into the body in the context of infectious diseases. Here, the released toxins represent the virulence factors which cause the clinical symptoms. Clinically relevant examples for the latter are diphtheria, anthrax, *Clostridium* (C.) *difficile* associated diseases (CDIs) and further severe enteric diseases caused by clostridia that produce binary toxins. Moreover, some of these toxins (e.g. *C. botulinum* neurotoxins) or toxin-producing bacteria (e.g. *Bacillus anthracis*) are considered as biological warfare and play emerging roles in the context of bioterrorism.

The remarkable toxicity of AB toxins is due to their unique structure and mode of action: A specific binding/transport (B) subunit of the toxin mediates the transport of an enzymatically active (A) subunit into the cytosol of mammalian target cells. There, the A subunit modifies its specific cellular substrate molecule, which changes the morphology of cells or interferes with cell signaling. In any case, the substrate-modification leads to the clinical symptoms which are characteristic for each toxin-induced disease. This volume reviews the current knowledge on the cell surface receptors as well as the molecular mechanisms underlying cellular uptake and intracellular transport of *C. botulinum* neurotoxins, *C. difficile* toxins A (TcdA) and B (TcdB), the Rho-modulating *C. botulinum* C3 toxin, the binary clostridial ADP-ribosylating enterotoxins including *C. botulinum* C2 toxin, *C. perfringens* iota toxin and *C. difficile* CDT, the binary anthrax toxins and diphtheria toxin. After receptor-binding and internalization into cells by receptor-mediated endocytosis, the before mentioned toxins deliver their A subunits from acidified endosomal vesicles into the host cell cytosol. This transport across endosomal membranes is pH-driven and requires specific translocation subunits of the toxins that insert as pores into the endosomal membrane and facilitate the translocation of the respective A subunits into the cytosol. Moreover, for some toxins a crucial role of specific host cell factors during this membrane transport step was described in past years. ADP-ribosylating toxins such as diphtheria toxin and the binary

clostridial actin ADP-ribosylating toxins exploit the components of the cellular Hsp90 chaperone machinery including Hsp90, Hsp70, cyclophilins and FK506 binding proteins for the translocation of their A subunits across endosomal membranes.

Some chapters of this volume point out that a detailed understanding of the molecular mechanisms underlying this extremely efficient and highly sophisticated transport of bacterial protein toxins into the cytosol of mammalian cells is not scientifically interesting but also clinically relevant. The novel knowledge from this basic research can be directly transferred to develop and optimize novel compounds for the targeted pharmacological inhibition of the uptake of clinically important toxins into cells. By preventing the uptake of the A subunits into the host cell cytosol, the cells are protected from the cytotoxic effects caused by the toxins and therefore, the toxin-induced clinical symptoms should be prevented or at least decreased in humans and animals. Thus, novel specific anti-toxins as described in this issue might result in novel therapeutic strategies to prevent and/or cure some toxin-associated diseases including food-borne intoxications such as botulism as well as severe infectious diseases including diphtheria, anthrax and enteric diseases caused by clostridial toxins. Anti-toxins including pharmacological inhibitors of relevant host cell chaperones or multivalent and heterocyclic molecules which specifically bind into the translocation channels of the toxins and inhibit translocation of the A subunits directly inhibit the mode of action of some toxins, even after their internalization into cells. Therefore, such compounds could be combined with antibiotics to target the toxins in addition to the toxin-producing bacteria. This strategy might be of particular interest if the toxin-associated disease is caused by bacteria that are (multi-)resistant towards antibiotics.

This volume includes eight chapters from experts in this field. I thank all contributors and I am confident that scientists from the fields of Toxinology, Toxicology, Pharmacology, Microbiology, Biochemistry and Cell Biology will enjoy this up-to-date resource.

Ulm, Germany Holger Barth

Contents

Two Feet on the Membrane: Uptake of Clostridial Neurotoxins

Andreas Rummel

Abstract The extraordinary potency of botulinum neurotoxins (BoNT) and tetanus neurotoxin (TeNT) is mediated by their high neurospecificity, targeting peripheral cholinergic motoneurons leading to flaccid and spastic paralysis, respectively, and successive respiratory failure. Complex polysialo gangliosides accumulate BoNT and TeNT on the plasma membrane. The ganglioside binding in BoNT/A, B, E, F, G, and TeNT occurs via a conserved ganglioside-binding pocket within the most carboxyl-terminal 25 kDa domain H_{CC}, whereas BoNT/C, DC, and D display here two different ganglioside binding sites. This enrichment step facilitates subsequent binding of BoNT/A, B, DC, D, E, F, and G to the intraluminal domains of the synaptic vesicle glycoprotein 2 (SV2) isoforms A-C and synaptotagmin-I/-II, respectively. Whereas an induced α-helical 20-mer Syt peptide binds via side chain interactions to the tip of the H_{CC}-domain of BoNT/B, DC and G, the preexisting, quadrilateral β-sheet helix of SV2C-LD4 binds the clinically most relevant serotype BoNT/A mainly through backbone–backbone interactions at the interface of H_{CC} and H_{CN}. In addition, the conserved, complex N559-glycan branch of SV2C establishes extensive interactions with BoNT/A resulting in delayed dissociation providing BoNT/A more time for endocytosis into synaptic vesicles. An analogous interaction occurs between SV2A/B and BoNT/E. Altogether, the nature of BoNT-SV2 recognition clearly differs from BoNT-Syt. Subsequently, the synaptic vesicle is recycled and the bound neurotoxin is endocytosed. Acidification of the vesicle lumen triggers membrane insertion of the translocation domain, pore formation, and finally translocation of the enzymatically active light chain into the neuronal cytosol to halt release of neurotransmitters.

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1 Introduction

Clostridial neurotoxins (CNT) exert the highest toxicity (parenteral LD_{50} ~1 ng/kg body weight) (Gill 1982) of all natural compounds due to their extraordinary target cell specificity. They bind specifically to nonmyelinated areas of cholinergic motor nerve terminals (Dolly et al. 1984). Here, gangliosides, complex poly sialic acid containing glycolipids, adhere the CNTs to the cell surface. A protease sensitive interaction of CNT with neuronal membranes provoked the dual receptor hypothesis postulating an interaction with gangliosides and a proteinaceous receptor (Montecucco 1986). In the last decade, enormous progress was seen on the identification of protein receptors and characterisation of the mode of receptor interaction which will be discussed here in detail.

2 How to Categorise CNT Variants: Serotypes and Subtypes

The family of CNT comprises tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNT). Whereas only a single isoform of TeNT was isolated and sequenced so far (Kitasato 1889; Eisel et al. 1986), a growing number of BoNT variants have been identified. Historically, they are grouped by the absence of cross-neutralisation in an animal bioassay by type-specific monovalent botulinum antitoxin (Leuchs 1910, 1919) into the seven serotypes BoNT/A-G. In 1895, Emile van Ermengem was the first to isolate a *Clostridium botulinum* strain (van Ermengem 1897) which later was ascribed as producing serotype BoNT/B (Leuchs 1910). In the following 75 years six further serotypes were discovered: BoNT/A in (1904), BoNT/C in 1922 (Bengtson 1922; Bengtson 1923), BoNT/D in 1928 (Meyer and Gunnison 1929), BoNT/E in 1937 (Hazel 1937), BoNT/F in 1960 (Møller and Scheibel 1960),

and BoNT/G in 1970 (Gimenez and Ciccarelli 1970). Of them, BoNT/A, B, E, and F are predominantly causing foodborne (intoxication), infant (colonisation of colon) and wound (infection) botulism in humans. BoNT/C and BoNT/D mainly evoke botulism in birds and cattle, respectively, while BoNT/G is rarely causing botulism. It took almost a century to decipher their encoding DNA sequences (CDS) (Binz et al. 1990; Whelan et al. 1992a, b; Thompson et al. 1990; Hauser et al. 1990; Poulet et al. 1992; East et al. 1992; Campbell et al. 1993) which revealed 37–69 % between-serotype difference (Niemann et al. 1994). Recombination events in the BoNT gene (Smith et al. 2015) led to the occurrence of interserotype mosaic BoNT like BoNT/CD and BoNT/DC (Moriishi et al. 1996a, b) which questions the universal validity of the historic serotype definition (Moriishi et al. 1989). Very recently, a novel mosaic BoNT was isolated (Barash and Arnon 2014) which can only be neutralised by anti-BoNT/A antibodies (Maslanka et al. 2016), although its CDS revealed high homology to BoNT/A only for the C-terminal third, the cell-binding domain H_C (Dover et al. 2014; Gonzalez-Escalona 2014). The remaining part of the mosaic BoNT is considered as a novel toxinotype designated H, but the next closest relative BoNT/F caused its temporary naming as BoNT/FA, although anti-BoNT/F antiserum does not neutralise it (Maslanka et al. 2016; Pellett 2016).

In course of BoNT gene sequencing studies also genetic variants of BoNT/A, B, E, and F were identified (Poulet et al. 1992; Thompson et al. 1993; Willems et al. 1993; Hutson et al. 1994, 1996; East et al. 1998; Santos-Buelga et al. 1998). Enormous progress in sequencing technology allowed publication of the first *C. botulinum* genome (Sebaihia et al. 2007). Subsequent worldwide systematic efforts in genome sequencing of *C. botulinum* strain collections and screening for novel clinical and environmental isolates boosted the number of genetic BoNT variants to >40 (Hill and Smith 2013; Peck and Smith 2016). They can differ up to 36 % in amino acid (AA) sequence as in case of BoNT/F variants. In contrast, apart from the two BoNT/CD and DC mosaics, no other variants of the serotypes BoNT/C, D, G as well as TeNT are known. Functional differences such as in antigenicity led to the introduction of the term subtype: a genetic BoNT variant with minimum 2.6 % difference in AA sequence (Smith et al. 2005). An alternative phylogenetic approach defines subtypes as corresponding to clades formed by the clustering of bont sequences (Hill et al. 2007; Raphael et al. 2010; Chen et al. 2007). Applying either of these definitions, the currently identified genetic variants have been ascribed to the subtypes BoNT/A1-A8 with 2.9–15.6 % between-subtype differences, BoNT/B1-B9 (1.6–7.3 %), BoNT/E1-E12 (0.9–10.9 %), and BoNT/F1-F9 (3.0–36.2 %) (Hill and Smith 2013; Peck and Smith 2016; Kull et al. 2015; Wangroongsarb et al. 2014; Kalb et al. 2012; Raphael et al. 2012; Weedmark et al. 2014; Giordani et al. 2015; Sikorra 2016; Smith et al. 2015; Mazuet et al. 2015). It is highly plausible that the between-subtype differences will also cause functional diversity, e.g., with respect to receptor recognition.

3 How Are CNT Molecules Structured?

Each CNT is initially synthesised as ~150 kDa single chain protein, which is subsequently cleaved by specific bacterial or host proteases. The resulting ~50 kDa light chain (LC) and ~100 kDa heavy chain (HC) remain attached via a single disulfide bond and non-covalent interactions mediated by a HC-derived peptide loop wrapping around the LC within the substrate cleft. The LC represents the active component which operates as zinc endoproteases with strict substrate specificities (Binz 2013). Their apo structures have been all determined (reviewed in (Brunger and Rummel 2009)). The structural differences among the LC are mostly limited to solvent-exposed loops and potential substrate interaction sites. Without linkage to their HC the LC are ordinary proteases, i.e., nontoxic molecules, but become highly poisonous agents upon linkage. The HC ensure that the catalytic LC come across their neuronal target cells and conquer the plasma membrane to reach the site of action, the cytosol. In order to fulfil these tasks, the HC comprise two functional subunits, a ~50 kDa largely α–helical domain at the N-terminus, called H_N, and at the C-terminus the ~50 kDa H_C-fragment, in which the two ~25 kDa domains H_{CN} and H_{CC} can be defined. The attachment of H_C to H_N is rigid in case of BoNT/A and B (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000), but flexible in BoNT/E (Kumaran et al. 2009). Nevertheless, pH-induced binding of the respective nontoxic non-hemagglutinin (NTNHA) to either BoNT/A or BoNT/E causes a 140° rearrangement of H_C via the H_N-H_C linker (Eswaramoorthy et al. 2015; Gu et al. 2012). Structural comparison among the H_C-fragments of BoNT/A [PDB code: 2VUA (Stenmark et al. 2008) 4RJA (Benoit et al. 2014)], B [2NM1 (Jin et al. 2006)], C [3R4S (Strotmeier et al. 2011)], DC [4ISR (Berntsson et al. 2013)], D [3OBT (Strotmeier et al. 2010)], E [3FFZ (Kumaran et al. 2009)], F [3FUQ (Fu et al. 2009)], G [2VXR (Stenmark et al. 2010)], and TeNT [3HMY (Chen et al. 2009)] showed that there is a varying twist between H_{CN} and H_{CC} culminating in H_{CC} by about 17.2°. Nevertheless, separate pairwise structure comparisons of all eight H_{CN}- and H_{CC}-domains demonstrated that the structures are conserved within each domain. H_{CN} folds as lectin-like jelly role, whereas H_{CC} builds up a β-trefoil domain which is assembled by 60 conserved residues in 12 conserved structural motifs which comprise six β-strands forming a barrel and six β-strands forming hairpins that close the bottom of the barrel (Ginalski et al. 2000). In spite of the conserved core structure, large structural differences are found in many surface-exposed loops. Five of such areas reside in H_{CN} and nine loops in the H_{CC}-domain (Fig. 1) providing sufficient degree of freedom to accommodate each H_C to specific receptor structures.

The function of the H_{CN} domain connecting H_N and H_{CC} is still not fully resolved. A low affinity binding of BoNT/A H_{CN} to phosphatidylinositol monophosphate incorporated in sphingomyelin enriched microdomains of the immortalised motor neuron cell line NSC-34 was reported (Muraro et al. 2009). Very recently, some contribution to the protein receptor binding of BoNT/A and E was exhibited (Yao et al. 2016; Mahrhold et al. 2013). Nevertheless, a direct

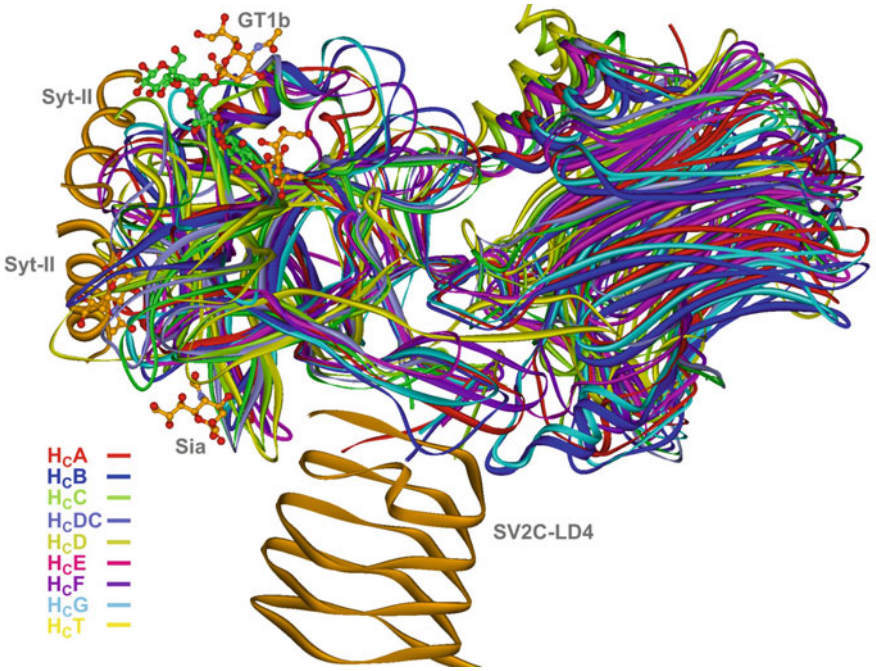


Fig. 1 Superimposition of the H_C-fragment crystal structures of TeNT, seven BoNT serotypes and BoNT/DC. Rat Syt-II (*orange ribbon*) bound to H_CB (*dark blue ribbon*, PDB code 2NM1) was superimposed with H_CA (*red ribbon*) in complex with GT1b (ball & stick, 2UV9) and human SV2C (*orange ribbon*, 4RJA), H_CC (*dark green*) in complex with sialic acid (*ball & stick*, 3R4S), H_CDC (*grey blue*) in complex with mouse Syt-II (*orange ribbon*, 4ISR), H_CD (*light green*) in complex with sialic acid (ball & stick, 3OBT), H_CE (pink, 3FFZ), H_CF (*orange*, 3FQU), H_CG (*light blue*, 2VXR) and H_CT (*yellow ribbon*) in complex with disialyllactose (*ball & stick*, 1YYN)

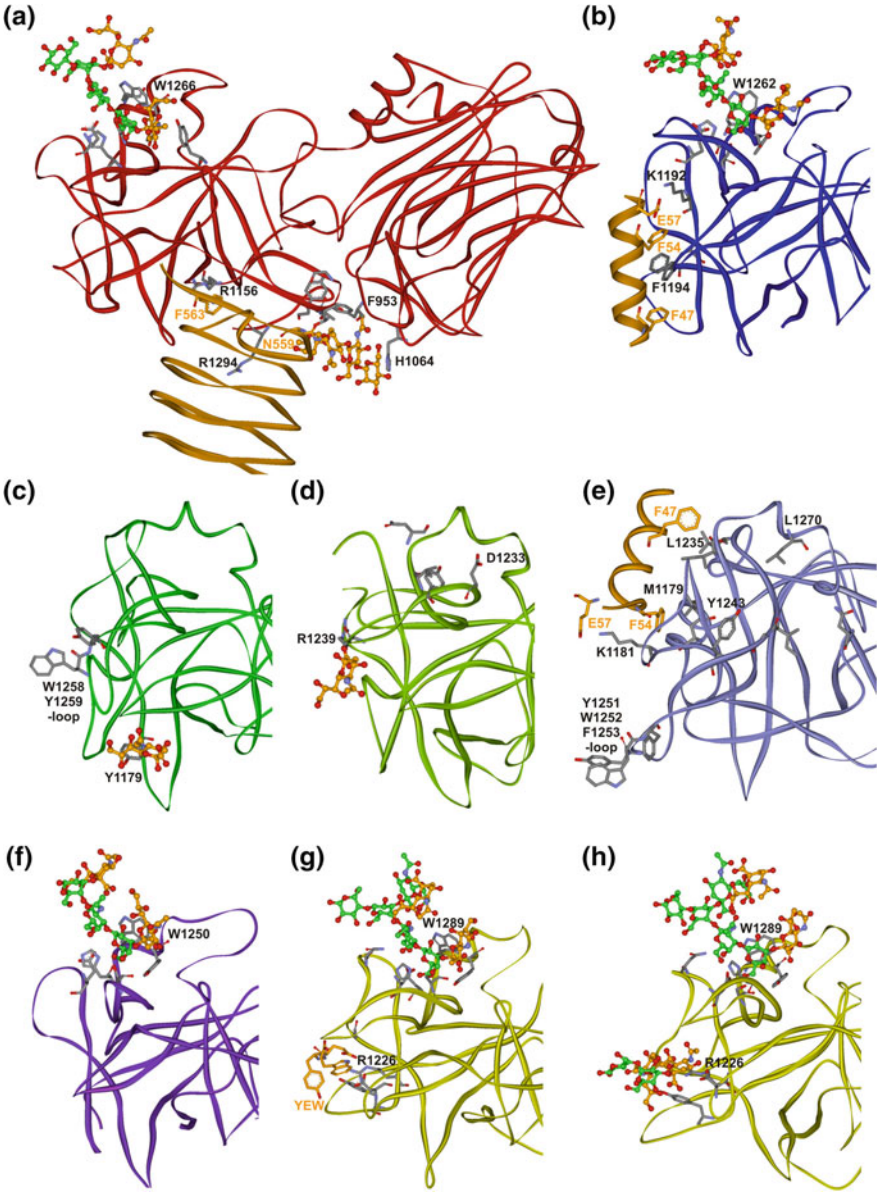
involvement of H_{CN} of BoNT/A in the translocation step could be ruled out lately (Fischer et al. 2008). On the other hand, the H_{CC}-domain harbours the main features required for target cell recognition and internalisation (see below).

4 How Do Complex Polysialo Gangliosides Accumulate CNT on the Neuronal Membrane?

TeNT was first identified to bind polysialo gangliosides, glycosphingolipids that are found particularly in the outer leaflet of neuronal cell membranes (van Heyningen 1959; van Heyningen and Miller 1961). A decade later BoNT/A, B, D or E were de-toxified by preincubation with gangliosides, especially GT1b (Simpson and Rapport 1971a, b). Extensive overlay binding assays employing ganglioside mixtures separated by thin layer chromatography demonstrated binding of BoNT/A, B,

C, E, and F to GT1b, GD1b, and GD1a with varying affinities (Kozaki et al. 1987; Takamizawa et al. 1986; Tsukamoto et al. 2005; Kamata et al. 1986; Ochanda et al. 1986; Kitamura et al. 1980). BoNT/A, B, and E adhered to GT1b better than to GD1a and much less to GM1, and as the ionic strength increased, less binding was observed (Schengrund et al. 1991). Employing surface plasmon resonance (SPR), however, BoNT/A bound to isolated GT1b when the ionic strength was increased from 0.06 to 0.16 with a similar K_D ($\sim 10^{-7}$ m) for each ionic strength (Yowler and Schengrund 2004). Use of isolated, individual gangliosides coated on polystyrene microtiter plates complemented the understanding of ganglioside preference. TeNT prefers the b-series gangliosides GT1b, GD1b, and GQ1b (Chen et al. 2008; Angstrom et al. 1994; Rummel et al. 2003). Isolated GT1b also binds BoNT/A, B and with higher affinity BoNT/G (Rummel et al. 2004; Schmitt et al. 2010). In addition, BoNT/G interacts equally well with GD1a, 10-fold weaker with GD1b, 250-fold weaker with GM3 and hardly with GM1a (Willjes et al. 2013), somewhat similar to BoNT/F which predominantly binds GD1a and GT1b but hardly GD1b or GM1 (Fu et al. 2009). Comparing different serotypes, GD1a is bound best by BoNT/F, followed by BoNT/E and A (Benson et al. 2011) thereby supporting the GD3S-KO mice data (Rummel 2013). In contrast, BoNT/C is efficiently immobilised by GD1b and to a lesser extent by GT1b and GD1a while the closely related mosaic serotype BoNT/DC preferentially binds GM1 and much weaker GD1a, but hardly GT1b and GD1b (Karalewitz et al. 2010). BoNT/D, like TeNT, displays a ganglioside preference for GT1b, GD1b, and GD2 pinpointing the requirement of the disialyl moiety (Kroken et al. 2011). Furthermore, MALDI-TOF MS demonstrated binding of isolated GT1b to BoNT/A, B, and D (Strotmeier et al. 2010; Rummel et al. 2004). Co-crystallisation studies exhibited that BoNT/A binds GT1b-oligosaccharide (Stenmark et al. 2008), BoNT/B interacts with sialyllactose (Swaminathan and Eswaramoorthy 2000) and GD1a-oligosaccharide (Berntsson et al. 2013), BoNT/C complexes two, BoNT/D and DC one sialic acid molecule (Strotmeier et al. 2010, 2011; Karalewitz et al. 2012), BoNT/F interacts with GD1a-oligosaccharide (Benson et al. 2011) and TeNT binds lactose, GT1b-analogue, GT2-oligosaccharide and disialyllactose (Chen et al. 2009; Emsley et al. 2000; Fotinou et al. 2001; Jayaraman et al. 2005) (Fig. 2).

At the cellular level, removal of sialic acid residues by neuraminidase treatment of cultured cells isolated from spinal cord (Bigalke et al. 1986) and adrenergic chromaffin cells (Marxen et al. 1989) reduced BoNT/A potency as well as TeNT action (Critchley et al. 1986). Also binding of BoNT/C to neuroblastoma cell lines as well as rat brain synaptosomes was diminished upon neuraminidase treatment (Tsukamoto et al. 2005; Yokosawa et al. 1989) indicating interactions between sialic acid moieties and BoNT/A, C and TeNT. Conversely, bovine chromaffin cells lacking complex polysialo gangliosides were rendered sensitive to TeNT and BoNT/A by pretreatment with gangliosides (Marxen and Bigalke 1989; Marxen et al. 1991). In addition, a monoclonal antibody to GT1b antagonised the action of BoNT/A on rat superior cervical ganglions (Kozaki et al. 1998). The inhibition of ganglioside biosynthesis with fumonisin in primary spinal cord neurons or with D, L-threo-l-phenyl-2-hexadecanoylamino-3-morpholino-propanol in the mouse



neuroblastoma cell line Neuro-2a resulted in insensitivity toward TeNT and BoNT/A, respectively (Williamson et al. 1999; Yowler et al. 2002).

Employing a genetic approach, mice lacking the genes encoding NAcGal-transferase and/or GD3-synthetase were created. NAcGal-transferase deficient mice only expressing lactose ceramide (Lac-Cer), GM3 and GD3

◀ **Fig. 2** Receptor binding sites in the H_{CC}-domain of clostridial neurotoxins. Important neurotoxin and receptor residues in binding sites are displayed as stick presentations with *grey* and *orange* carbon atoms, respectively, whereas bound carbohydrates are displayed in *ball* and *stick* presentation. **a** Synthetic GT1b binds via its terminal NAcGalβ3-1Galβ moiety (Gal carbon scaffold in *green*, NAcNeu carbon scaffold in *orange*) to the conserved ganglioside binding site, (GBS) within the BoNT/A H_{CC}-domain (2VU9.pdb). Glycosylated hSV2C binds via β-strands and the N559-glycan to H_CA (5JLV.pdb). **b** Binding of GD1a in the conserved GBS within the H_{CC}-domain of BoNT/B (Gal carbon scaffold in *green*, NAcNeu carbon scaffold in *orange*) (4KBB.pdb). Mouse Syt-II 44-60 folds as α-helix (*orange ribbon*) and binds via F47, F54 and E57 to the H_{CC}-domain. **c** BoNT/C H_{CC} lacks the conserved ganglioside binding site, but instead displays the WY-loop (W1258, Y1259) and a unique sialic acid binding site. The coordination of sialic acid (carbon scaffold in orange) by Y1179 is of hydrophobic nature and an arginine like in BoNT/D and TeNT is missing (3R4S.pdb). **d** BoNT/D H_{CC} possesses a GBS at the homologous position, but with different amino acid configuration (D1233.Y1235…V1251.N1253; 3OBT.pdb). In addition, H_{CC}D displays a sialic acid binding site like in TeNT where R1239 coordinates the carboxyl group of the sialic acid. **e** BoNT/DC H_{CC} possesses a GBS at the homologous position, but with different amino acid configuration (Y1243, L1270; 4ISR.pdb). Mouse Syt-II 44-60 folds as α-helix (*orange ribbon*) and binds via F47, F54 and E57 to a unique site in the H_{CC}-domain. **f** Synthetic GD1a binds via its terminal NAcGalβ3-1Galβ moiety (Gal carbon scaffold in *green*, NAcNeu carbon scaffold in *orange*) to the conserved GBS within the BoNT/F H_{CC}-domain (3RSJ.pdb). **g** Synthetic GT1b binds via its terminal NAcGalβ3-1Galβ moiety (Gal carbon scaffold in *green*, NAcNeu carbon scaffold in *orange*) to W1289 in the conserved GBS within the TeNT H_{CC}-domain (1FV2.pdb). The tripeptide YEW interacts via the ε-carboxyl group of glutamate with R1226 in the sialic acid binding site in TeNT H_{CC}-domain (1YXW.pdb) mimicking nidogen G2-domain binding. **h** Disialyllactose interacts via its NAcNeuα8-2NAcNeuα element (sialic acid carbon scaffold in orange) to the sialic acid binding site in TeNT H_{CC}-domain (1YYN.pdb). R1226 mediates the main salt bridge to the carboxyl group of the terminal NAcNeu

resisted treatment with TeNT as well as BoNT/A, B, D, and G in time-to-death experiments (Peng et al. 2011; Kitamura et al. 1999; Dong et al. 2007). Furthermore, nerve stimulation-evoked endplate potentials at isolated neuromuscular junctions derived from NAcGal-transferase deficient mice remained unaltered upon incubation with BoNT/A (Bullens et al. 2002). In addition, binding and entry of the seven BoNT serotypes was reduced in cultured hippocampal neurons of NAcGal-transferase deficient mice, but could be rescued by adding exogenous bovine brain ganglioside mix (Peng et al. 2011; Dong et al. 2007, 2008). In addition, in the murine P19 embryonal carcinoma cell line exhibiting high sensitivity to BoNT/C the NAcGal-transferase was knocked out employing the CRISPR/Cas9 system. The sensitivity of the NAcGal-transferase deficient P19 neurons to BoNT/C was decreased considerably, but was restored upon exogenous addition of either GD1a, GD1b or GT1b (Tsukamoto et al. 2015). On the other hand, GD3-synthase knockout mice expressing only Lac-Cer, GM3, GM2, GM1 and GD1a were resistant to TeNT, but kept their sensitivity towards BoNT/A, B and E (Kitamura et al. 2005) which indicates that the disialyl moiety of GT1b plays a minor role in binding of BoNT/A, B and E. A conclusive combination of both gene knock-outs resulted in GM3-only mice which displayed high resistance toward all seven BoNT serotypes at motor nerve terminals using an ex vivo phrenic nerve hemidiaphragm preparation (Strotmeier et al. 2010; Rummel 2013; Rummel

et al. 2007, 2009; Strotmeier et al. 2014). Hence, the complex polysialo gangliosides GD1a, GD1b and GT1b are essential to specifically accumulate all BoNT serotypes on the surface of neuronal cells as the first step of intoxication.

5 How Do CNT Bind to Complex Gangliosides?

The specific binding of CNT to peripheral nerve endings at the neuromuscular junction only involves the H_C-fragment (Simpson 1984a, b, 1985; Evinger and Erichsen 1986; Fishman and Carrigan 1987; Lalli et al. 1999) and complex polysialo gangliosides. First for TeNT, cross-linking experiments and first mutagenesis studies allocated one ganglioside binding site (GBS) close to H1293 and Y1290 in a large cavity within the TeNT H_{CC}–domain (Shapiro et al. 1997; Sinha et al. 2000; Sutton et al. 2001). Independently, in silico docking of doxorubicin, an anthracycline antibiotic, to TeNT H_C confirmed this pocket (Lightstone et al. 2000). First structural insights were revealed by co-crystallisation of the TeNT H_C-fragment with four carbohydrate subunits of GT1b which exhibited four distinct binding sites, including the one in the proximity of H1293, W1289 and Y1290 binding lactose. A separate site comprising R1226 as the key residue coordinated either a molecule of NAcGal or sialic acid (N-acetylneuraminic acid, NAcNeu) (Emsley et al. 2000). The other Gal and the NAcGal complexing sites were not confirmed by a subsequent co-crystallisation approach using a synthetic GT1b-β analogue lacking the ceramide portion. Here, the terminal disaccharide NAcGalβ3-1Galβ bound to the lactose binding site next to W1289, while the disialic acid branch of another GT1b-β molecule interacted with the sialic acid binding site comprising R1226 (Fotinou et al. 2001). Independent co-crystallisation of TeNT H_C-fragment with disialyllactose as well as the carbohydrate portion of GT2 confirmed the binding of the disialic acid branch to the sialic acid binding site (Chen et al. 2009; Jayaraman et al. 2005). Mutation of residues D1222, H1271 and W1289 to alanine in the lactose binding site reduced binding of TeNT H_C-fragment to GT1b in SPR experiments and NGF-differentiated PC12 cells (Louch et al. 2002). The importance of the lactose binding site for binding and entry of TeNT was conclusively demonstrated for corresponding, recombinant full-length TeNT mutants using the mice phrenic nerve hemidiaphragm (MPN) assay leading to a 350-fold reduction in neurotoxicity in the case of the single amino acid mutation W1289L (Rummel et al. 2003). Furthermore, these experiments also demonstrated that the sialic acid binding site is essential for TeNT action, since e.g. the TeNT mutant R1226L possesses a 70-fold reduced activity in the MPN assay. Mass spectroscopy experiments indicated simultaneous binding of two molecules GT1b to the TeNT H_C-fragment (Rummel et al. 2003). Employing the above mentioned W1289A and R1226L mutants the individual ganglioside specificity of the lactose and sialic acid binding sites were biochemically defined. The lactose site binds preferentially GM1a and GD1a over GT1b and GD1b via the terminal disaccharide NAcGalβ3-1Galβ while the sialic acid site prefers the disialyl group of GT1b and

GD1b but hardly accepts GD1a and GM1a displaying only a monosialyl branch (Chen et al. 2008). Although binding of a ganglioside to the sialic acid binding pocket was conclusively shown, the binding of the tripeptide YEW to this site leaves the option of a subsequent substitution by or a direct interaction with a protein receptor (see below) (Jayaraman et al. 2005; Cosman et al. 2002).

The lactose binding site in TeNT is built by the peptide motif D…H…SXWY…G (Fig. 2g, h; Table 1) which is conserved among BoNT/A, B, E, F and G: E(Q)…H(K/G)…SXWY…G. This cavity displays the typical features necessary for carbohydrate interaction found also in other protein toxins such as ricin and cholera toxin. An aromatic residue, preferable tryptophan or tyrosine, stack parallel to the hydrophobic face of the sugar ring. Polar residues like aspartate/glutamate/glutamine, histidine/lysine and serine locate opposite to interact with the sugar hydroxyl groups. Co-crystallisation studies with BoNT/B and sialyllactose or doxorubicin suggested that the cavity homologous to the TeNT lactose binding site is the GBS in BoNT (Swaminathan and Eswaramoorthy 2000; Eswaramoorthy et al. 2001). Detailed mutational analyses defined the contribution of various residues within the homologous lactose binding pocket of BoNT/A and B (Fig. 2a and b). Like for TeNT, the mutations of the aromatic key residues, W1266 and W1262 in BoNT/A and B, respectively, to leucine lead to dramatic reductions of neurotoxicity using the MPN assay (Rummel et al. 2004). In contrast to TeNT, mass spectroscopy data revealed the binding of only a single GT1b molecule to the H_C-fragment of BoNT/A and B (Rummel et al. 2004). Thereafter, these biochemical and physiological data were confirmed by a co-crystal structure of a synthetic GT1b-analogue bound to the H_C-fragment of BoNT/A (Stenmark et al. 2008) (Fig. 2a) and a GD1a-oligosaccharide bound to BoNT/B H_C in a ternary complex with Syt-II (Berntsson et al. 2013) (Fig. 2b). Interestingly, whereas the mutational data of the lactose site suggests a shared ganglioside binding mode of BoNT/A and B differing to that of TeNT (Rummel et al. 2004), the crystallographic results indicate that BoNT/A and B like TeNT predominantly interact with the NAcGalβ3-1Galβ moiety and only BoNT/B displays additional contacts with the terminal α 2,3-linked NAcNeu (denoted NAcNeu-5) attached to Gal4 (Stenmark et al. 2008; Berntsson et al. 2013; Fotinou et al. 2001). The previously reported coordination of the terminal NAcNeu of sialyllactose by W1262 in BoNT/B (Swaminathan and Eswaramoorthy 2000) might be due to different crystallisation conditions and the use of sialyllactose instead of the entire oligosaccharide part of GD1a. Structural data of BoNT/E, BoNT/F H_C and BoNT/G H_C displayed the presence of a conserved GBS like in BoNT/A and B (Kumaran et al. 2009; Fu et al. 2009; Stenmark et al. 2010), but only the mutation of W1268 in BoNT/G, W1224 in BoNT/E and W1250 in BoNT/F, all part of the conserved motif E(D/Q)…H(K/G)…SXWY…G, to leucine demonstrated their key role in ganglioside interaction and biological activity (Mahrhold et al. 2013; Rummel et al. 2007, 2009) (Table 1). Again, the crystal structure of a GD1a-oligosaccharide—BoNT/F H_C complex confirmed the previous biochemical identification of the conserved GBS in BoNT/F (Fig. 2f). Here, like in BoNT/B, W1250 mainly coordinates the Gal4

Table 1 Identified neurotoxin receptors and corresponding binding sites

	Ganglioside binding site (GBS)		Protein receptor[#]			Protein receptor binding site (PBS)		
	Conserved GBS AA motif preferred ganglioside	Sialic acid site Key AA pref. ganglioside		Segment	N-glycan	Location of PBS	Key AA of PBS	
BoNT/A	E…H…SXWY…G GT1b > GD1a = GD1b > GM1	n.a.	SV2C* SV2A/SV2B	473–567 487–581^{+}/ 430–524^{+}	559 573/516	H_{CN}-H_{CC}	T1145, T1146, Y1149, R1156, R1294 %	F953, H1064, G1292[β]
BoNT/B	E…H…SXWY…G GT1b > GD1a > GD1b	n.a.	Syt-II[$] Syt-I	44–60 36–52		H_{CC}	K1192, F1194, F1204	
BoNT/C		WY-loop[$]/Y1179 GD1b > GT1b > GD1a > GM1a	?	?		?	?	
BoNT/DC	Y1243, L1270	YWF-loop[$] GM1a > GD1a > GD1b = GT1b>	Syt-II[$]/Syt-I	44–60/ 36–52		H_{CC}	K1181, M1179, V1191, L1235, I1264	
BoNT/D	DXY…VXN	R1239 GD2 > GT1b = GD1b	SV2B/C/A	?		?	?	
BoNT/E	E…K…SXWY…G GD1a/GQ1b/GT1b ≫ GM1	n.a.	SV2A/B	506–583/ 449–526^{+}	573/516	H_{CN}-H_{CC}	F1160, R1183, Y879, Y1041, E1246, K1084, K1126	
BoNT/F	E…H…SXWY…G GT1b = GD1a ≫ GM3 ≫ GD1b/GM1	n.a.	SV2A/C/B	?		?	?	
BoNT/G	Q…G…SXWY…G GT1b = GD1a > GD1b > GM3 > GM1	n.a.	Syt-I Syt-II[$]	36–52 44–60		H_{CC}	Q1200, F1202, F1212	
TeNT	D…H…SXWY…G GM1a > GD1a > GT1b = GD1b	R1226 GT1b > GD1b > GD1a > GM1a	Nidogen-1/2 SV2[&]	G2 domain?		$H_{CC?}$	R1226?	

[$]no conserved ganglioside binding site present; [#]order expresses decreasing neurotoxin affinity; *N–glycosylation of LD4 is not obligatory; $^{+}$extrapolated from main isoform; [%]SV2C protein—BoNT/A1 protein interactions; [β]SV2A-C N-glycan—BoNT/A1 protein interactions; [$]human and chimpanzee Syt-II are low affinity receptors; [&]only accounts for central neurons; n.a., not applicable

moiety and R1111 and R1256 create additional contacts with NAcNeu-5 (Benson et al. 2011).

In contrast, the conserved GBS is not present in the BoNT/C and D H_C structures (Strotmeier et al. 2010, 2011; Karalewitz et al. 2010) (Fig. 2c and d). Surprisingly, mutation of W1258 in BoNT/C, which aligns in the similar motif GXWY, clearly reduced binding of H_CC to isolated gangliosides and to synaptosomal membranes as well as biological activity (Rummel et al. 2009; Tsukamoto et al. 2008). However, the BoNT/C H_C structure taught one that W1258 is part of a long loop extending out of the core structure of $H_{CC}C$ and therefore termed ganglioside binding loop (GBL). Molecular dynamics simulations indicate that this WY-loop is not a crystallographic artefact (Strotmeier et al. 2011). However, the mode of ganglioside coordination at this peptide remote from the $H_{CC}C$ core structure remains unclear. Also the closely related H_C-fragment of BoNT/DC, displaying 75 % amino acid sequence identity to H_CC, lacks the ganglioside binding motif but presents an analogous YWF-loop (Karalewitz et al. 2010; Nuemket et al. 2011) (Fig. 2e). Accordingly, H_CDC mutant W1252A completely lost binding to its preferred ganglioside GM1 although the WF-loop backbone of the mutant superimposes well with the loop in the H_CDC wild-type structure (Karalewitz et al. 2010). However, partial electron density of sialyllactose was detected at a position homologous to the conserved GBS, but completely missing the corresponding motif. Subsequent mutational analysis identified residues within this cavity (N1114A, I1240A, G1241A, Y1243A, and L1270A; Fig. 2e) as well as in the YWF-loop important for binding of BoNT/DC H_C to liposomes endowed with GM1 and P19 embryonal carcinoma cells (Nuemket et al. 2011). Furthermore, a second ganglioside binding site within $H_{CC}C$ was exhibited by co-crystallisation of sialic acid with H_CC (Fig. 2c). This pocket called Sia-1 site locates at the tip of the H_{CC}-domain in the neighbourhood of the WY-loop, but constitutes an autonomous ganglioside binding pocket and displays a mode of sialic acid binding different to the one observed in the sialic acid binding site of TeNT (Strotmeier et al. 2011). Thereafter, a molecule of sialic acid was identified at the place homologous to the conserved GBS in a BoNT/C H_C crystal structure, but coordination only occurred via H-bonds. Biochemical analysis revealed that this site called GBP2 binds the NAcNeu-5 moiety of GD1a/GT1b and the Sia-1 site binds the NAcNeu-7 of GD1b/GT1b (Karalewitz et al. 2012). It remains open if BoNT/C even employs up to three molecules of ganglioside to specifically enter neurons and can afford to lack high-affinity binding to a protein receptor.

Co-crystallisation studies of BoNT/D H_C with sialic acid revealed a sialic acid binding site around R1239 in a position homologous to the sialic acid binding site in TeNT (Strotmeier et al. 2010) (Fig. 2d; Table 1). Structural data of two H_CD mutants exhibited that mutation W1238A disorders the backbone thereby also influencing binding of NAcNeu-5 to R1239, whereas mutation F1240A seems to reduce ganglioside binding by direct interaction (Kroken et al. 2011). Mutagenesis studies demonstrated for BoNT/D a binding mode similar to TeNT and identified a second carbohydrate binding site at the location of the conserved GBS, but with completely different configuration of amino acids, which might explain the low

affinity of BoNT/D to gangliosides (Strotmeier et al. 2010). Structural analysis of the closely related BoNT/CD H_C exhibited a sialic acid binding site consisting of W1242, R1243 and F1244 homologous to the one of BoNT/D (Zhang et al. 2011).

In conclusion, BoNT/A, B, E, F, G and TeNT harbour one ganglioside binding site made up of the conserved amino acid motif E(D/Q)…H(K/G)…SXWY…G at a homologous location within the H_{CC}-domain accommodating the terminal NAcGalβ3-1Galβ moiety (Fig. 2; Table 1). In addition, TeNT comprises a second site complexing groups like NAcNeu-2,8-α-NAcNeu. BoNT/D contains a closely related sialic acid site whereas its other carbohydrate binding site locates similarly to the conserved GBS, but is different with respect to its AA configuration. Although BoNT/C also displays minimum two ganglioside binding sites like TeNT and BoNT/D plus an exposed loop rich in aromatic AA, neither their AA configuration nor their positions are related to any of the known carbohydrate binding pockets of CNT.

6 Which Synaptic Vesicle Proteins Are Receptors of CNT?

As mentioned above, many evidences like a much higher affinity of BoNT in vivo compared to in vitro binary interaction studies or the marked reduction of TeNT binding to rat brain membranes upon protease pretreatment argued against gangliosides as the sole receptors of CNTs at nerve terminals (Lazarovici and Yavin 1986; Pierce et al. 1986). To account for these findings, a dual receptor model involving two sequential binding steps was proposed (Montecucco 1986) which suggests an initial low affinity accumulation of CNTs by the abundant polysialo gangliosides and a subsequent binding to the sparsely distributed protein receptor(s). Simultaneous interaction with ganglioside and protein receptor results in a high-affinity binding and is requisite for the subsequent specific endocytosis step of the neurotoxin.

Nevertheless, the nature of protein receptors for BoNT remained obscure for a long time although several studies demonstrated an accelerated uptake of TeNT (Simpson 1985; Schmitt et al. 1981), BoNT/A (Black and Dolly 1986; Hughes and Whaler 1962; Simpson 1980), BoNT/C (Rummel et al. 2009; Simpson 1982), BoNT/D (Rummel et al. 2009), BoNT/E (Rummel et al. 2009; Lawrence et al. 2007) and BoNT/F (Rummel et al. 2009) into the phrenic nerve prepared together with the adjacent diaphragm upon electrical stimulation which resulted in an earlier onset of neurotransmitter blockade. Others reported that high K^+ concentration stimulating neurons leads to an accelerated uptake of BoNT/A, B, D, E, F and G into spinal cord and hippocampal preparations (Fu et al. 2009; Peng et al. 2011; Dong et al. 2007, 2008; Keller et al. 2004). As neuronal stimulation causes increased rates of exo- and endocytosis of synaptic vesicles, the intravesicular domains of synaptic vesicle membrane proteins are more frequently extracellularly exposed and thus easier accessible for the membrane associated BoNT.

However, unbiased biochemical separation techniques of total rat brain protein identified the synaptic vesicle protein synaptotagmin I (Syt-I) as the protein receptor for BoNT/B (Nishiki et al. 1994). The two homologous synaptotagmin isoforms I and II of this type I transmembrane protein (Perin et al. 1990; Geppert et al. 1991) trigger synaptic vesicle fusion upon Ca^{2+} influx (Südhof 2002; Chapman 2002; Südhof 2013). Subsequent binding studies using recombinantly expressed Syt–I and Syt-II isoforms showed that BoNT/B exhibits a ten times higher affinity to Syt-II (Nishiki et al. 1996). Stable transfection of CHO cells with Syt-II identified the amino-terminal, intraluminal domain of Syt-II as the binding region for BoNT/B (Nishiki et al. 1996) (Table 1). Deletion mutants of Syt-II lacking the two cytosolic Ca^{2+} binding C2 domains could still act as protein receptor of BoNT/B (Kozaki et al. 1998). A decade later the physiological role of this neurotoxin-Syt interaction was reinforced by loss- and gain-of-function approaches in PC12 cells as well as BoNT neutralisation assays in mice employing a Syt-II fragment containing the luminal and transmembrane domain and a ganglioside mix (Dong et al. 2003). Isothermal titration calorimetry revealed that binding of BoNT/B to Syt-II luminal domain is tight at neutral pH (K_D < 34 nM) and acidic pH (K_D = 32 nM), stoichiometric (~1:1), endothermic (ΔH < 31 kJ/mol) and entropy driven (ΔS < 37.6 J/mol K) while the dissociation constant between H_CB and the luminal domain of Syt-I is at least two orders of magnitude larger (Jin et al. 2006). The binding kinetics H_CB-Syt-II 1-61 are characterised by very fast association and medium fast dissociation yielding a K_D of 75 nM as revealed by SPR assays (Weisemann et al. 2016). The dissociation constants of H_CB to the minimal peptide Syt-II 40-60 of 0.14 ± 0.05 μM and 0.18 ± 0.06 μM in the absence and presence of GD1a, respectively, are virtually identical and indicate that despite the topographical neighbourhood of both binding sites ganglioside and Syt binding occurs as independent, autonomous events (Berntsson et al. 2013).

Also BoNT/G, whose cell binding domain H_C shares 42 % AA sequence identity with that of BoNT/B, interacts with Syt-II and additionally with Syt-I in vitro (Table 1). Inhibition of BoNT/G toxicity at MPN hemidiaphragm preparations upon preincubation with the luminal domains of either Syt-I or Syt-II substantiated the finding that Syt-I and -II act as protein receptor for BoNT/G (Rummel et al. 2004). In contrast to BoNT/B, BoNT/G exhibits lower but similar affinities to both Syt-II and Syt-I in vitro (Rummel et al. 2007) which is caused by a different binding mode of the Syt peptides in the H_{CC}-domain of BoNT/G vs. B (Willjes et al. 2013). Final confirmation for activity-dependent uptake and Syt-mediated neuronal cell entry of BoNT/B and G was provided by mouse hippocampal Syt-I knockout neurons and restoration of toxin sensitivity by Syt-I/Syt-II expression in those neurons (Dong et al. 2007).

Surprisingly, the mosaic BoNT/DC, a hybrid comprising LC and H_N of BoNT/D and a cell binding domain H_C closely related to BoNT/C, also employs Syt–II and to a lesser extent Syt–I as protein receptor (Peng et al. 2012) (Table 1). While its H_{CN}-domain is 92 % identical to that of BoNT/C, its H_{CC}-domain shares only 61 % identity with that of $H_{CC}C$ which does not interact with any Syt isoform. On the other hand, the 28 % sequence identity to the Syt binders $H_{CC}B$ and $H_{CC}G$ is even lower.

That might be the reason for the unique Syt binding site (see below) as well as the higher apparent dissociation constants of BoNT/DC for Syt-II 1-87 compared to BoNT/B for Syt-II 1-267 each comprising the Syt-II transmembrane domain (TMD) in the presence of gangliosides (172 ± 14 nM versus 7.0 ± 0.6 nM) (Peng et al. 2012; Chai et al. 2006). However, in the absence of gangliosides the K_D for H_CDC is doubled (330 ± 56 nM) like for BoNT/B and G binding to membrane incorporated Syt-I and Syt-II in the absence of gangliosides (Rummel et al. 2007).

To identify protein receptors of the remaining BoNT serotypes synaptic vesicle proteins were preselected on the basis that their intravesicular segments exceeded 20 amino acids in length and comprised both intravesicular segments of the tetraspanin proteins synaptophysin, synaptoporin, synaptogyrin-I and -III as well as the large intravesicular domain of the synaptic vesicle glycoprotein 2 (SV2) isoforms A, B, and C. GST pull-down experiments employing their luminal domains discovered the interaction between the large luminal domain 4 (LD4) of SV2 and BoNT/A (Dong et al. 2006; Mahrhold et al. 2006) (Table 1). The function of this integral membrane glycoprotein with twelve putative transmembrane domains (Bajjalieh et al. 1992, 1993; Janz and Sudhof 1999) is likely linked to synaptic vesicles priming or rendering primed vesicles fully Ca^{2+} responsive (Chang and Südhof 2009; Custer et al. 2006; Xu and Bajjalieh 2001). Recent studies showed that absence of SV2 results in elevated Ca^{2+} levels in the presynaptic terminals and also reduces the rate of compensatory membrane retrieval after synaptic vesicle release (Wan et al. 2010). Interestingly, it has been demonstrated that SV2 associates with Syt and may regulate the endocytosis of Syt (Yao et al. 2010; Nowack et al. 2010). The unglycosylated, isolated LD4 of SV2C exhibits the highest affinity to BoNT/A, inhibits binding and entry of BoNT/A into hippocampal neurons and motor nerve terminals and efficiently decreases BoNT/A neurotoxicity (Weisemann et al. 2016; Dong et al. 2006; Mahrhold et al. 2006). Loss-of-function and gain-of-function studies in hippocampal neurons (Dong et al. 2006), RNAi experiments using wild-type PC12 and Neuro-2a cells as well as transiently SV2 expressing PC12 and Neuro-2a knockdown cell lines verified that all three SV2 isoforms can act as physiological receptors for BoNT/A (Dong et al. 2006). Since all three SV2 isoforms are expressed in varying levels in α-motoneurons (Dong et al. 2006), it is still not clear which SV2 isoform is most relevant for the physiological uptake of BoNT/A. Dissociation constants for the isolated human (h) SV2C-LD4 to BoNT/A H_C at neutral pH were determined by fluorescence anisotropy experiments (260 nM) (Benoit et al. 2014) and SPR (400 nM) (Weisemann et al. 2016). In contrast to the tight BoNT/B-Syt-II interaction at pH 5.0 prevailing in SV lumen, the K_D of hSV2C-LD4 to BoNT/A H_C increases more than 20-fold. Rat (r) SV2C-LD4 differing in 11 AA to hSV2C-LD4 displays slightly reduced affinity to H_CA while it binds H_CA approximately 5- and 7-fold stronger than rat SV2A and SV2B, respectively (Weisemann et al. 2016).

Sequence comparison of the LD4 s of all three SV2A-C isoforms of mouse, rat and human origin revealed the presence of three conserved putative N–glycosylation (PNG) sites. In hSV2C, these sites are N484, N534 and N559. SV2C displays two additional unique PNG sites at N480 and N565, suggesting that SV2C contains

two additional N-glycans which explains why recombinant N-glycosylated (g) SV2CLD + TMD migrates at higher molecular weight in SDS-PAGE than gSV2ALD + TMD (Mahrhold et al. 2013). Indeed, N–glycosylation of N534, N559 and N565, the latter two residing at the BoNT/A interface (see below), in LD4 of a hSV2C-Fc fusion protein secreted by HEK293 cells was confirmed by LC-MS analysis (Mahrhold et al. 2016). Detailed mutational analysis exhibited that only the N559-glycan, but not the neighbouring the N565-glycan increases affinity of BoNT/A to gSV2C–LD4. The N559-glycan was characterised as complex core-fucosylated type with a heterogeneity ranging up to tetra-antennary structure with bisecting N–acetylglucosamine (NAcGlc) which can establish extensive interactions with BoNT/A (see below). The mutant gSV2CLD-Fc N559A displayed a 50-fold increased dissociation rate k_d resulting in an overall 12-fold decreased binding affinity in SPR experiments (Mahrhold et al. 2016). The delayed dissociation of BoNT/A provides extended time for its endocytosis into synaptic vesicles. Hippocampal/cortical neurons cultured from SV2A(–/–) SV2B(–/–) mice and infected with lentiviruses that express either the SV2A N573A, SV2B N516A or SV2C N559A deglycosylation mutants showed decreased sensitivity towards BoNT/A. These data demonstrate that N-glycosylation of the third conserved PNG site in all SV2 isoforms increases efficiency of BoNT/A binding and uptake and compensates the low affinity protein–protein interaction of BoNT/A with SV2A and SV2B-LD4 (Yao et al. 2016).

Different to BoNT/A, BoNT/D and BoNT/E exclusively co-immuno-precipitated SV2B and SV2A, respectively, from rat brain Triton X-100 extracts (Peng et al. 2011) (Table 1). In contrast, Barbieri et al. showed limited binding of H_CD to SV2A, SV2B, and Syt-II from CHAPS-extracted SVs, but no detectable binding to SV2 or Syt from rat brain Triton X-100 extracts (Kroken et al. 2011). In turn, Mahrhold demonstrates precipitation of SV2B and minor fractions of Syt-I and Syt-II by H_CD from rat brain SV Triton X-100 extracts (Fig. 3) thereby confirming the SV2B IP data of Dong et al., but also raising the question as to whether Syt was co-precipitated due to the Syt-SV2 interaction or an yet unknown H_CD–Syt interaction. However, BoNT treatment of hippocampal neurons derived of SV2A/B knockout mice individually expressing one of the three SV2 isoforms revealed that BoNT/D requires the presence of SV2B and to a lesser extent of SV2A and SV2C to exert its biological activity (Peng et al. 2011). In contrast, BoNT/E sensitivity is rescued only by expression of SV2A or SV2B, but not by SV2C (Dong et al. 2008). Exclusively at central neurons also TeNT is supposed to employ SV2A and SV2B (Yeh et al. 2010) (Table 1). However, TeNT, BoNT/A, D and E utilise seemingly diverse SV2-binding mechanisms. Whereas BoNT/A already binds the unglycosylated LD4 of SV2C with sufficient affinity and N-glycosylation of N559 in SV2C additionally enhances its uptake rate, BoNT/E does not bind to any bacterial/non-glycosylated LD4-SV2 peptide, but relies for productive uptake on glycosylation of N573 in SV2A and N516 in SV2B, the carboxyl-terminal of three conserved PNG sites (Dong et al. 2008). BoNT/D and TeNT appear to have a SV2-recognition strategy distinct from BoNT/A and BoNT/E. First, mutation of any of the three PNG sites in SV2A has no significant effect on the entry of BoNT/D and

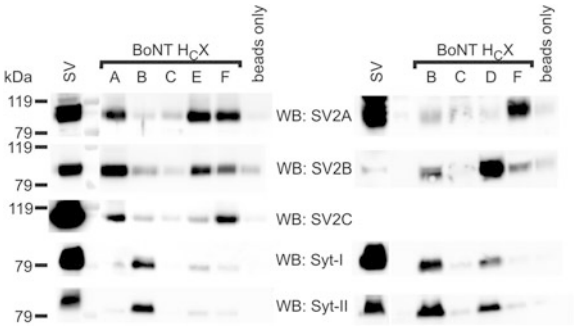


Fig. 3 Co-purification of proteins from detergent solubilised synaptic vesicles with One-strep-tagged H_C-fragments derived from BoNT/A, B, C, D, E and F. BoNT H_C-fragments (15 µg each) were incubated with highly purified synaptic vesicles solubilised with Triton X-100 (25 µg total protein) in a total volume of 200 µl and subsequently purified using Streptactin Superflow beads. Co-purified proteins were visualised by Western blotting using specific antibodies against the three isoforms of the synaptic vesicle glycoprotein 2 (SV2A, B and C) and synaptotagmin-I and -II (Syt-I and -II). The lane labelled "beads only" displays background binding of synaptic vesicle proteins to Streptactin Superflow beads

TeNT (Peng et al. 2011; Yeh et al. 2010). Second, the SV2-LD4 domain expressed in low density lipoprotein receptor-based or synaptogyrin-based chimeric proteins can function as the receptor for BoNT/A and E, but failed to mediate the entry of BoNT/D. Furthermore, BoNT/D does not compete for uptake in hippocampal neurons with BoNT/A or E (Peng et al. 2011). On the other hand, TeNT H_C inhibits the action of BoNT/E and BoNT/D at MPN hemidiaphragm preparations (Rummel et al. 2009) as well as SNAP-25 cleavage by BoNT/E in hippocampal neurons (Yeh et al. 2010). A direct protein–protein interaction between SV2 and BoNT/D or TeNT remains to be shown to draw a final conclusion.

In addition, there is dispute about the involvement of SV2 as protein receptor for BoNT/F whose cell binding domain H_C displays 55 and 43 % AA sequence identity with BoNT/E and A, respectively, which allows the assumption that also BoNT/F harnesses SV2 for cell entry. Although BoNT/F uptake at cultured hippocampal neurons was reported to occur independent of synaptic activity (Verderio et al. 1999), increased stimulation of motoneurons accelerates uptake of BoNT/F (Rummel et al. 2009) indicating the involvement of synaptic vesicular structures. Furthermore, BoNT/A H_C inhibits neurotoxicity of BoNT/F and BoNT/F H_C inhibits neurotoxicity of BoNT/E at MPN hemidiaphragm preparations (Rummel et al. 2009) due to cross-competition for SV2. Moreover, two independent studies co-purified all three SV2 isoforms by BoNT/F H_C from Triton X-100 solubilised synaptic vesicle lysates (Fu et al. 2009; Rummel et al. 2009) (Table 1). On the other hand, cleavage of synaptobrevin 2 by various concentrations of BoNT/F is not impaired in SV2B and SV2B/SV2A knockout hippocampal neurons (Peng et al. 2011; Yeh et al. 2010). Residual SV2C present in subpopulations of hippocampal neurons and a SV2 expression pattern in motoneurons different to hippocampal

neurons might explain these discrepancies (Peng et al. 2011; Dong et al. 2006). An ultimate experiment employing pan-SV2 knockout motoneurons has not been conducted yet. In conclusion, these data demonstrate that the CNT-SV2-binding mode is still not fully understood.

For BoNT/C, Kozaki and colleagues concluded that proteins are not a receptor component, as protease treatment or boiling of solubilised rat brain synaptosomes had no effect on binding (Tsukamoto et al. 2005). The binding of BoNT/C H_C to phosphoinositide-containing liposomes has been described, but is unlikely with respect to specificity and affinity to replace protein receptor interaction (Zhang and Varnum 2012). Along this line are observations that BoNT/C H_C failed to precipitate the synaptic vesicle proteins Syt-I, Syt-II, SV2A/B/C from Triton X-100 solubilised synaptic vesicle lysates (Fig. 3) (Rummel et al. 2009; Baldwin and Barbieri 2007). In addition, cleavage of SNAP-25 and syntaxin 1A by BoNT/C is not impaired in SV2B and SV2B/SV2A knockout hippocampal neurons (Peng et al. 2011). However, as mentioned above the stimulation dependent uptake of BoNT/C at MPN hemidiaphragm preparations points towards the involvement of synaptic vesicular structures (Rummel et al. 2009; Simpson 1982). Clearly, thorough studies are required to clarify this issue. If entry of BoNT/C turns out to depend upon proteinaceous receptors, it will be interesting to find out whether proteins different than the synaptic vesicle proteins Syt or SV2 act as receptors.

While BoNT exert their effects at the neuromuscular junction (NMJ) causing flaccid paralysis TeNT leads to spastic paralysis despite entering the α-motoneuron, too. The reason for the opposite symptoms is caused by different sorting of TeNT in the synapse and its subsequent retrograde transport upon clathrin-mediated endocytosis in axonal signalling endosomes with neutral lumen towards the spinal cord and transcytoses into inhibitory neurons (Bergey et al. 1987; Osborne and Bradford 1973; Bohnert and Schiavo 2005; Deinhardt et al. 2006). Shortly after internalisation of TeNT into Rab5-containing endosomes they undergo slow short-range movements. Rab5 is then exchanged with Rab7, a LE/lysosome marker, in a process that is required for fast axonal retrograde transport of these organelles to the soma (Deinhardt et al. 2006). Here, TeNT remains in nonacidic compartments and is transcytosed to adjacent interneurons while the co-transported activated neurotrophin receptors are mainly sent to degradation (Bercsenyi et al. 2013; Debaisieux et al. 2016).

The molecular basis of the differential sorting of TeNT and BoNT in α-motoneurons is most likely associated with their specific protein receptors at the motoneuron. Recently, a novel concept with the direct involvement of the extracellular matrix proteins nidogen-1 and -2 (also known as entactins) as primary receptors for TeNT at the NMJ was demonstrated (Bercsenyi et al. 2014) (Table 1). Based on the structure of the tripeptide YEW bound to the sialic acid binding site in TeNT H_C (Fig. 2h) (Jayaraman et al. 2005) 9-mer peptides of nidogen-1 and -2 comprising the motifs YQW and WSY, respectively, were identified as TeNT H_C binders as well as inhibitors of TeNT interaction with full-length nidogens in vitro. The N1 peptide also blocks tetanic paralysis by inhibiting TeNT binding at the NMJ. Furthermore, neurons as well as mice deficient in nidogen-1 and -2 display

reduced TeNT binding and increased resistance to TeNT, respectively, while BoNT/A H_C remained unaffected (Bercsenyi et al. 2014). Following this pre-membrane step, a nidogen-TeNT complex might bind to gangliosides via the lactose site and to an undefined glycosylphosphatidylinositol membrane anchored protein (Herreros et al. 2001; Schiavo et al. 1991; Munro et al. 2001) or to surface receptors known to bind nidogens, such as the protein phosphatase LAR (Stryker and Johnson 2007; O'Grady et al. 1998) to become endocytosed. In primary motoneurons a fraction of TeNT H_CT co-localises with SV2A, but hardly with SV2C. Addition of exogenous nidogen-1 increased uptake of H_CT but decreased the proportion of H_CT co-localised with SV2A (Bercsenyi et al. 2014). Hence, it seems that the crosstalk between the two entry routes in motoneurons deciding about intracellular transport of TeNT might be mediated by the presence of the extracellular matrix proteins nidogen-1 and -2. Such a sorting seems to be absent in case of central neurons where TeNT employs SV2A and B for productive uptake (Yeh et al. 2010).

7 How Do CNT Bind to Synaptotagmin and SV2?

Since BoNT/A, B, E, F and G do not possess a second carbohydrate binding site, it became apparent to check whether protein receptors such as Syt-II for BoNT/B bind in a pocket that is homologous to the sialic acid binding site within the H_{CC}-domain of TeNT or BoNT/D. The different affinities of H_C-fragment hybrids comprising H_{CN}- and H_{CC}-domains of BoNT/B strains Okra and 111, respectively, to GT1b/Syt-II endowed liposomes point in that direction (Ihara et al. 2003).

The binding mode of BoNT/B to its protein receptor Syt-II was resolved by two parallel co-crystallisation approaches. In one study full-length BoNT/B was co-crystallised with a 20-mer peptide corresponding to the interacting segment of Syt-II 40-60 (Chai et al. 2006). In the second study, a recombinant fusion protein consisting of the H_C-fragment of BoNT/B linked via a strep affinity tag to the complete luminal domain of Syt-II was crystallised (Jin et al. 2006). Analysis of both crystals revealed that Syt-II involving AA 44-60 bound to a saddle like crevice at the distal tip of the H_{CC}-domain in the direct neighbourhood of the GBS (Fig. 2b; Table 1). Interestingly, the in solution unstructured luminal domain of Syt-II formed an α-helix upon binding to BoNT/B. Hydrophobic binding of F47 and F54 into two adjacent pockets and a salt bridge between E57 and K1192 of BoNT/B constitute the main interactions. Also a computer-assisted binding pocket prediction followed by mutational analyses identified the Syt-I and Syt-II binding site in the BoNT/B and G H_{CC}-domain (Rummel et al. 2007). The two orders of magnitude lower affinity of BoNT/B to Syt–I versus Syt–II are caused only by the two conservative replacements M47 and L50 in Syt–I instead of F55 and I58 in Syt–II. Their individual conversion increases the binding affinity of Syt-I to BoNT/B, and the corresponding double mutation converted Syt-I to a Syt-II-like high-affinity receptor which argues for a conserved binding mode for both Syt isoforms in

BoNT/B (Jin et al. 2006). Following the experimental approach of Jin et al. the structure of a ternary complex consisting of GD1a-oligosaccharide and mouse luminal domain of Syt-II fused to H_CB was solved and displayed a virtually identical positioning of the Syt-II helix in the presence of GD1a. Hence, simultaneous ganglioside binding does not cause any significant structural changes in BoNT/B H_C and both receptors interact independently of each other with BoNT/B on the neuronal cell surface despite of their close (15 Å) neighbourhood (Berntsson et al. 2013).

Structural analysis of Syt-II derived from different species revealed that F54, key hydrophobic residue in mouse and rat Syt-II mediating high-affinity binding to BoNT/B, DC and G corresponds to L51 in human and chimpanzee Syt-II. Introducing the corresponding mutation F54L into Syt-II of mouse or rat origin resulted in complete loss of binding of BoNT/B, DC and G (Peng et al. 2012; Strotmeier et al. 2012) and could not be rescued in the presence of gangliosides. These data explains the observed disparity of BoNT/B potency in human and mice at the molecular level. Syt-II is present in every endplate in diaphragm muscle whereas a subpopulation ($\sim$40 %) of NMJ additionally expresses Syt-I (Pang et al. 2006). Interaction with human Syt-I whose corresponding residue F46 is strictly conserved in all sequenced species only partially compensates this single mutation. As consequence rimabotulinumtoxinB has to be administered in 40-fold higher protein dosages than, e.g., onabotulinumtoxinA. In contrast, the observed autonomic effects of BoNT/B (Kranz et al. 2011) might be explained by the predominant presence of Syt-I in autonomic and sensory neurons as shown for the rat peripheral nervous system (Li et al. 1994).

The binding of Syt-I and Syt-II to BoNT/G occurs at the homologous position as in BoNT/B (Rummel et al. 2007). However, only the general shape of the pocket and a few amino acids forming the surface of that binding area are conserved (Stenmark et al. 2010). Multiple mutations in Syt-II showed comparable effects on binding to BoNT/G except for mutant Syt-II E57 K which did not bind to BoNT/B, but exhibited wild-type like binding affinity in the case of BoNT/G (Willjes et al. 2013). Replacement of Q1200 in BoNT/G, the residue homologous to K1192 in BoNT/B, to lysine did not enhance binding to Syt-II, but clearly diminished binding and neurotoxicity (Rummel et al. 2007) (Table 1). The detailed mutational data along with molecular modelling experiments revealed atomistic models of BoNT/G − Syt recognition. Here, the Syt-I helix is shortened at the C–terminus whereas the Syt-II helix displays a bend after F55 compared to the Syt-II-BoNT/B complex (Willjes et al. 2013).

The surface area in H_CDC greatly differs to the homologous Syt-II site in H_CB. Hence it was not too surprising that of the three Syt-II mutants F47A, F54A and F55A ceasing BoNT/B binding only Syt-II F54A lacked interaction with BoNT/DC. Similarly, the Syt-I triple mutant F46A/M47A/E49 K halted synaptobrevin cleavage in neurons by BoNT/DC (Peng et al. 2012). Crystal structures of BoNT/DC H_C in complex with Syt-I and Syt-II 20-mer peptides revealed a novel binding site at the tip of the H_{CC}-domain which is rotated clockwise by 90° compared to the cleft in BoNT/B and G (Berntsson et al. 2013) (Fig. 2e). It shows a

conserved binding mode for both Syt isoforms which is in agreement with the above mutational data. Mutation of BoNT/DC M1179, V1191, and I1264 to serine at the hydrophobic core of the interface abolished binding of both Syt-I and Syt-II (Berntsson et al. 2013). Like K1192 in BoNT/B, residue K1181 of BoNT/DC forms a salt bridge with E49 and E57 of Syt-I and Syt-II, respectively (Table 1). Comparison with the most related BoNT/C reveals that it cannot bind to Syt due to a lack of the important hydrophobic patch found in BoNT/DC as well as steric clashes elsewhere (Berntsson et al. 2013).

In conclusion, Syt-I and -II always adopt an α-helical structure of 14–17 residues upon binding at homologous sites albeit in different modes in the H_{CC}-domains of BoNT/B and G and at a closely related site with a common recognition motif in the H_{CC}-domain of BoNT/DC.

The SV2 binding site was first identified in BoNT/E (Mahrhold et al. 2013) which requires N-glycosylated LD4 of SV2A or SV2B for neuronal uptake. Establishment of a stable SV2ALD expressing HEK293 cell based system allowed mapping of the SV2-binding interface of BoNT/E at an expanded surface area comprising H_{CN} and H_{CC} located on the back of the conserved GBS and extending up to the tip of the H_{CC}-domain corresponding to the Syt-binding site of BoNT/B. Mutations impairing the affinity also reduced the neurotoxicity of full-length BoNT/E at MPN hemidiaphragm preparations and are part of an epitope of a monoclonal antibody neutralising BoNT/E activity (Mahrhold et al. 2013) (Table 1).

Shortly thereafter, two independent studies identified the SV2 binding site in BoNT/A (Benoit et al. 2014; Strotmeier et al. 2014). Strotmeier et al. employed neutralising monoclonal antibodies with defined epitopes used as therapeutic antitoxins (Mullaney et al. 2001; Garcia-Rodriguez et al. 2007) and subsequent site-directed mutagenesis to localise the SV2-binding site at a position homologous to the SV2A binding site in BoNT/E at the interface of H_{CN} and H_{CC}. The key mutation G1292R, e.g., decreased binding to rat SV2C-LD4, ablated the co-immunoprecipitation of SV2A and SV2B from Triton X-100 solubilised rat brain synaptosomal membranes und diminished the biological activity of BoNT/A at the MPN hemidiaphragm ∼300-fold (Strotmeier et al. 2014). Benoit et al. co-crystallised the hSV2C-LD4 (AA 456–574) expressed in *E. coli* in complex with the H_C-fragment of BoNT/A. Here, hSV2C-LD4 folded into a right-handed, quadrilateral β-helix bound mainly through backbone-to-backbone interactions at open β-strand edges at the interface of H_{CN} and H_{CC} with a distance of approximately 40 Å opposite to the GBS (Fig. 1). Only the few side chains of T1145, T1146, Y1149, R1294 and R1156 of BoNT/A play a critical role in this interaction (Table 1), the latter mediating an important cation–π-stacking interaction with F563 of hSV2C which, however, is unique to human, chimpanzee and chicken SV2C (Benoit et al. 2014). Indeed, a virtually identical co-crystal of rSV2C-LD4 P455–Y577 with H_CA lacked this cation–π-stacking interaction due to L563 (Yao et al. 2016). SPR experiments showed that mutant hSV2C-LD4 F563L mimicking the residue in mouse, rat and bovine SV2C displayed a 1.6-fold higher binding constant for H_CA than hSV2C-LD4 wild-type. In contrast to BoNT/B, DC and G inducing

α-helix formation of the luminal portion of Syt upon binding, circular dichroism spectroscopy revealed that the quadrilateral helix of hSV2C-LD4 already exists in solution prior to BoNT/A binding. Nevertheless, the association rates of rSyt-II to H_CB and hSV2C to H_CA are virtually identical (3–5 × 10^5 $M^{-1}s^{-1}$) (Weisemann et al. 2016).

The above SV2 studies did not exhibit the interaction of the SV2C N559-glycan contributing to BoNT/A binding (Yao et al. 2016; Mahrhold 2016). To elucidate the N559-glycan binding the core of the hSV2C-LD4 (AA V473–T567) was secreted as glycosylated protein mixture (gSV2C-LD) with a molecular weight ranging from 25 to 37 kDa indicative of heterogeneous glycoforms (Yao et al. 2016). Its co-crystallisation with H_CA yielded a complex whose structure was determined at 2.0 Å resolution and is overall similar to that of H_CA in complex with rat and human SV2C of bacterial origin, respectively. The N559-glycan is clearly visible as quadruple-saccharide core made up of two NAcGlc, a mannose (Man), and a fucose (Fuc) (Fig. 2a) (Yao et al. 2016). The N–glycans of the two other conserved PNG sites (N484 and N534) hardly visible in the co-crystal structure are located far away from the BoNT/A-binding interface and thus are unlikely to directly participate in toxin binding. The N559-glycan directly interacts through a network of hydrogen bonds and van der Waals contacts with H_CA, notably stacking interactions between residues F953 and H1064 of H_CA and the hydrophobic faces of the two NAcGlc (Table 1). In addition, ten well-defined water molecules act as molecular 'glue' in the glycan–H_CA interface. These interactions almost double the contact area between H_CA and gSV2C from 557 to 925 $Å^2$. Accordingly, mutants BoNT/A F953G and F953 displayed 50-fold reduced and no detectable toxicity at MPN hemidiaphragm preparations, respectively (Yao et al. 2016). The extensive SV2 binding area varies substantially among the eight different BoNT/A subtypes. E.g., residue R1156 is unique to BoNT/A1, R1294 is found only in BoNT/A1 and A4 and H1064 is present only in BoNT/A1, A4, A5 and A7. Their counterparts in other BoNT/A subtypes (R/Q1064, E/M1156 and K/S1294) might disrupt their interactions with SV2C. Interestingly, the three single mutants BoNT/A1 H_C H1064R, R1156E and R1294S still bound substantially to neurons despite showing clearly decreased binding to gSV2C-LD in vitro and in case of R1156E and R1294S also to bacterial SV2C-LD4 (Yao et al. 2016). These data suggest that loss of individual side chain–mediated interactions of BoNT/A is tolerated on neuronal surfaces because of the tripartite binding to the peptide moiety and the N-glycan of SV2 and to complex polysialo gangliosides. However, systematic analysis of BoNT/A subtypes binding to its well-defined receptor structures is mandatory to understand the consequences of sequence variations in the receptor binding sites.

Knowing the key residues of the receptor binding sites in BoNT/A provide powerful tools to prove the dual receptor hypothesis for BoNT/A analogously to BoNT/B and G (Rummel et al. 2007) the single mutant BoNT/A G1292R with eliminated SV2 protein receptor recognition (Strotmeier et al. 2014), the single mutant BoNT/A W1266L lacking ganglioside binding (Rummel et al. 2004), a combination thereof as well as ganglioside deficient mice (Kawai et al. 2001). The sensitivity of hemidiaphragm tissue expressing GM3 only to BoNT/A wild-type is

similar to wild-type mice hemidiaphragm tissue towards BoNT/A W1266L bearing a deactivated GBS whereas the neurotoxicity of BoNT/A W1266L at GM3-only tissue remained unaltered. BoNT/A G1292R with inactivated SV2 binding site (= 300-fold reduced neurotoxicity at wild-type tissue) showed further reduced neurotoxicity at GM3-only tissue, similar to the double mutant BoNT/A W1266L/G1292R having both receptor sites inactive which displayed a 250,000-fold reduced biological activity in the MPN assay using wild-type tissue. This synergistic reduction clearly proves the double receptor model also for BoNT/A and explains its extraordinary neuronal tropism (Strotmeier et al. 2014).

In conclusion, SV2 is bound as preformed quadrilateral β-helix at a novel site in the H_C-fragments of BoNT/A and E mainly via peptide backbone–backbone interactions and the core of a conserved N-glycan branch. Whether BoNT/F closely related to BoNT/E interacts with SV2 in a similar manner has to be determined in the future.

As outlined above, the sialic acid binding site of TeNT located in the area that corresponds to the Syt binding site of BoNT/B and G comprises the central R1226 by which it accommodates the tripeptide YEW and most likely also nidogen-1 and -2 (Jayaraman et al. 2005; Bercsenyi et al. 2014). Since a similar pocket was described for BoNT/D (R1239) (Strotmeier et al. 2010) and both, BoNT/D and TeNT, were reported to employ SV2 as protein receptor (Peng et al. 2011; Yeh et al. 2010), it is imaginable that the sialic acid binding site interacts with terminal sialic acids of the N-glycan branches as well as with residues of the SV2 LD4 which would mediate the neuronal specificity. Also a binding of SV2 to the surface area homologous to the extended SV2 site in BoNT/A and E cannot be ruled. As long as a direct binding of the three SV2 isoforms to BoNT/D and TeNT and the definition of their interacting segments and residues is pending neither hypothesis can be proven.

8 How Does BoNT Approach the Membrane?

Although ganglioside and protein receptor binding sites share the same sub-domain (Fig. 4) they function independently and do not require preformation of a ganglioside/protein receptor complex (Berntsson et al. 2013; Rummel et al. 2007; Strotmeier et al. 2014). Moreover, mutants of BoNT/A, B and G with both the ganglioside and protein binding sites (individually or in combination) deactivated, do not exhibit appreciable toxicity excluding any significant contributions of other cell surface molecules to binding and entry of BoNT/A, B and G (Rummel et al. 2007; Strotmeier et al. 2014). A recent study investigated the vesicle membrane approach by making it to occur at the surface of neurons due to blockade of the vesicular H^+-ATPase by bafilomycin A1. Also here, the anchorage via two receptors is a strict prerequisite for a productive low pH-induced conformational change followed by membrane translocation, and TeNT, BoNT/B, C and D change structure and interact with the membrane in the same range of pH values as of the

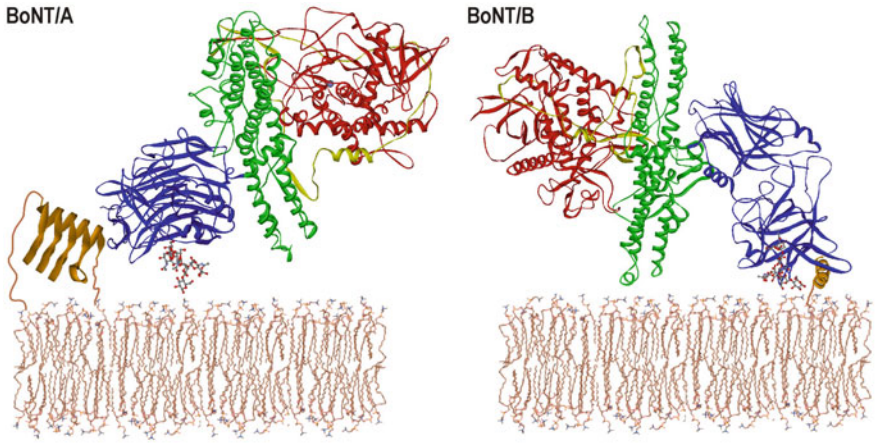


Fig. 4 Membrane approach of receptor bound BoNT (taken from (Rummel 2015)). BoNT/A (*left*) bound via its C-terminal H_C-fragment (*blue ribbons*) to GD1a (*ball* and *stick*) and luminal domain 4 of human glycoprotein 2C (hSV2C; *gold ribbons*) incorporated into the membrane. A tip of the translocation domain H_N (*green ribbons*) approaches the membrane whereas the catalytic light chain (red ribbons) is fixed by the belt (*yellow ribbons*) covering the active site coordinating Zn^{2+} (*grey sphere*). BoNT/B (*right*) bound to GD1a and half of the luminal domain of mouse synaptotagmin-II (mSyt-II; *gold ribbons*). In these models H_{CN} of BoNT/A might interact with membrane phospholipids whereas H_{CN} of BoNT/B is too far away. Models were built based upon superimposition of pdb coordinates of 3BTA, 2VU9 and 4JRA for BoNT/A as well as 1F31 and 4KBB for BoNT/B, respectively

SV lumen pH (Pirazzini et al. 2011). However, analogous data were not obtained for BoNT/A and E probably because of limited SV2 expression, low pH-sensitive SV2-BoNT interaction and low affinity ganglioside binding in case of BoNT/A. It has to be mentioned that binding only to GT1b enables BoNT/B to sense low pH, undergo a significant change in secondary structure, and transform into a hydrophobic oligomeric membrane protein. Imaging of the toxin on lipid bilayers using atomic force microscopy revealed donut-shaped channel-like structures that resemble other protein translocation assemblies (Sun et al. 2011).

The position of the toxin on the membrane might be important for the subsequent endocytosis and translocation steps. Geometric restrictions are imposed by the simultaneous adherence to the ganglioside and the protein receptor. Different binding modes are conceivable (Stenmark et al. 2008; Benoit et al. 2014; Jin et al. 2006; Berntsson et al. 2013; Rummel 2015). It was suggested that the predominant negatively charged molecular surfaces of BoNT/B H_N favour a perpendicular orientation of the translocation domain and thus bend Syt into a membrane tangential direction. In this scenario, four solvent-exposed lysine residues conserved among Syt-I and Syt-II might interact with the phospholipid head groups of the membrane (Jin et al. 2006). This view has recently been supported by modelling GT1b into the Syt-II/BoNT/B complex based on its binding to the conserved GBS of BoNT/A

(Stenmark et al. 2008) and was experimentally supported further by the ternary Syt–II-H_CB-GD1a complex (Berntsson et al. 2013).

Further evidence for this model was derived by employing the monoclonal antibody 4E17 which shows efficient neutralisation of BoNT/A, B and E in vivo indicating involvement in an important, universal step in the mechanism of action. It recognises a conserved epitope in the H_N translocation domain of BoNT/A (Y750NQYTEEEK758), BoNT/B (Y737NIYSEKEK745) and BoNT/E (Y723NSYTLEEK731) (Garcia-Rodriguez et al. 2011) residing in the tip of the helix bundle, which according to the model described above, is proximal to the membrane. Montal et al. demonstrated that preincubation of BoNT/E with 4E17.1 destroyed channel activity in an electrophysiological single molecule assay. Thus, these mAb precludes insertion of H_N into the membrane and selectively disrupts the BoNT translocation activity (Fischer et al. 2008).

Following the perpendicular orientation of the BoNT/B translocation domain, the face exposing those negatively charged carboxylates might become protonated in acidic pH environment and can now approach the negatively charged vesicular membrane surface as a preliminary step towards insertion of the H_N-domain. Indeed, protonation of conserved surface carboxylates (E48, E653 and D877) in BoNT/B were shown to increase neurotoxicity due to membrane translocation at less acidic pH and hence faster cytosolic delivery of the enzymatic domain. Thus, neutralisation of specific negative surface charges facilitates membrane contact permitting a faster initiation of the toxin membrane insertion (Pirazzini et al. 2013). Knowledge of the SV2 orientation on the BoNT/A surface also allows some conclusion with respect to geometric restrictions (Benoit et al. 2014; Yao et al. 2016). However, it has to be noted that linkers of 22 and 24 residues connect the SV2C quadrilateral β-helix to TMD 7 and 8, respectively, and provide it with sufficient flexibility on the surface. Thus, also BoNT/A can accommodate an orientation with the translocation domain perpendicular to the negatively charged membrane surface (Rummel 2015). Hereby also H_{CN} of BoNT/A might interact with membrane phospholipids which fits to previous results of Montecucco et al. (Muraro et al. 2009). Moreover, analogous to BoNT/B, conserved carboxylates which become neutralised upon protonation inside acidifying synaptic vesicles are also present in BoNT/A: E47, E666 and D890. Corresponding experimental data to prove this model are outstanding.

This model also complies with the role predicted for an extended loop of BoNT/B, that contains a very hydrophobic tip (G1246 - F1249) and extends out between the ganglioside and Syt binding pockets, to be additionally involved in the interaction with the membrane. Similar loops are found in the Syt-binders BoNT/G and DC. The H_CD crystal structure revealed a corresponding loop whose hydrophobic segment F1242 - Y1246 is likely to be involved in membrane association because its mutation leads to a clear loss in toxicity of BoNT/D (Strotmeier et al. 2010; Kroken et al. 2011). A similar observation was made for the WY-loop in BoNT/C and WF-loop in BoNT/DC (Strotmeier et al. 2011; Kroken et al. 2011). Hence several low affinity interactions are postulated beside the proven double

receptor interaction to support BoNT binding to the neuronal surface and trigger its uptake and translocation.

The fact that the BoNT/A and E H_C-fragments reorientate 140° in the pH-resistant M-PTC and mutation of the H_N-H_{CN}-linker helix clearly decreased the biological activity of BoNT/A indicate that such rearrangement could also occur in the absence of NTNHA within the acidic environment of the vesicle lumen (Eswaramoorthy et al. 2015; Gu et al. 2012). The results of future experiments have to link the geometric restrictions of BoNT membrane anchorage with the mode of membrane contacts and insertion of the translocation domain H_N to form LC translocating channels.

9 Outlook

The double receptor interaction of BoNT/A and B, the two most relevant serotypes, has been deciphered in great detail with respect to ganglioside binding, protein receptor identification and molecular description of receptor binding sites. The recent finding that simultaneous recognition of an SV2 protein segment and its neighbouring N–glycan as a composite binding site constitutes a novel host recognition strategy which uses a conserved post-translational modification as an evolutionarily static recognition site, in addition to protein–protein interactions that encode the location and specificity information.

Now, this comprehensive knowledge enables the targeted search for and generation of potent inhibitors of BoNT to prevent botulism as well as the development of receptor-based capturing tools to detect BoNT in vitro. For pharmaceutical purposes, modification of the BoNT binding sites allows the optimisation of BoNT uptake to decrease dosages and/or extend treatment intervals. Furthermore, retargeting of modified BoNTs to different neuron types or non-neuronal cells to treat novel medicinal indications becomes more feasible. Currently, eight BoNT/A and nine BoNT/B subtypes are known (Peck and Smith 2016) with substantial variations in their receptor binding sites. Their systematic characterisation is still outstanding and will contribute to an improved exploitation of BoNT as active pharmaceutical ingredient.

The remaining serotypes are far less characterised. Although the physiological ganglioside dependence of TeNT and BoNT as well as their ganglioside preference and mode of binding has been exhibited thoroughly for all serotypes in the last two decades, still the protein receptor of BoNT/C is unknown. Moreover, a direct interaction between SV2 and BoNT/D and F, respectively, remains to be demonstrated, not to mention their respective binding sites in BoNT/D and F. Structural information is missing for BoNT/E-SV2 and TeNT-nidogen complexes. In case of TeNT, the candidate attaching the TeNT-nidogen complex on the neuronal surface awaits its identification. Also numerous subtypes of BoNT/E and BoNT/F, the latter with up to 36 % variation in AA sequence, exist and probably display differences in receptor recognition. It is imaginable that analogous to BoNT/DC, whose H_C-fragment differs

by 25 % from BoNT/C and employs Syt–I and Syt–II as protein receptors, also e.g. BoNT/F7 whose H_C-fragment is 22–29 % dissimilar from the other seven BoNT/F subtypes employs a protein receptor other than SV2 or binds SV2 in a different way. Along that line, the identification of novel BoNT serotypes either from *Clostridium* species, but also homologues from other species like *Weissella oryzae* (Zornetta 2016) will occur in the future and awaits characterisation of their receptor binding.

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Uptake of Clostridial Neurotoxins into Cells and Dissemination

Chloé Connan and Michel R. Popoff

Abstract Clostridial neurotoxins, botulinum neurotoxins (BoNT) and tetanus neurotoxin (TeNT), are potent toxins, which are responsible for severe neurological diseases in man and animals. BoNTs induce a flaccid paralysis (botulism) by inhibiting acetylcholine release at the neuromuscular junctions, whereas TeNT causes a spastic paralysis (tetanus) by blocking the neurotransmitter release (glycine, GABA) in inhibitory interneurons within the central nervous system. Clostridial neurotoxins recognize specific receptor(s) on the target neuronal cells and enter via a receptor-mediated endocytosis. They transit through an acidic compartment which allows the translocation of the catalytic chain into the cytosol, a prerequisite step for the intracellular activity of the neurotoxins. TeNT migrates to the central nervous system by using a motor neuron as transport cell. TeNT enters a neutral pH compartment and undergoes a retrograde axonal transport to the spinal cord or brain, where the whole undissociated toxin is delivered and interacts with target neurons. Botulism most often results from ingestion of food contaminated with BoNT. Thus, BoNT passes through the intestinal epithelial barrier mainly via a transcytotic mechanism and then diffuses or is transported to the neuromuscular junctions by the lymph or blood circulation. Indeed, clostridial neurotoxins are specific neurotoxins which transit through a transport cell to gain access to the target neuron, and use distinct trafficking pathways in both cell types.

Contents

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1 Introduction

Clostridial neurotoxins, including botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT), are the most potent toxins known among all the bacterial toxins and toxins from other kingdoms. BoNTs and TeNT are responsible for severe neurological diseases which are characterized by paralysis. While BoNTs induce a flaccid paralysis, TeNT causes a spastic paralysis. In the final intoxication step, both types of toxins induce respiratory failure and death. Despite these two opposite clinical symptoms, BoNTs and TeNT retain a similar molecular mode of action. BoNTs and TeNT are zinc-dependent proteases, which block the evoked exocytosis of neurotransmitter in target neurons. The main difference between BoNT and TeNT activity is that BoNTs essentially act at the peripheral nervous system, notably at the neuromuscular junctions, whereas TeNT enters the central nervous system (CNS) and targets specific inhibitory interneurons. The high specific neurotoxic activity of BoNTs and TeNT accounts for the extreme potency of these toxins. Specific clostridial neurotoxin trafficking in the organism and entry into specific cells are the initial and prerequisite steps of their neurotoxicity.

In contrast to TeNT, of which, up to now, only a unique isoform is known, BoNTs show a high level of diversity. Thereby, BoNTs are divided into 7 serotypes (A to G) based on their antigenicity properties in neutralization assay (Popoff 1995; Peck et al. 2011; Hill et al. 2015; Smith et al. 2015; Williamson et al. 2016). A new BoNT type called H has been reported but was characterized as a hybrid between BoNT/A heavy (H) chain and a light (L) chain related to that of BoNT/F5. Antiserum against BoNT/A neutralizes the novel BoNT type but not the antiserum against BoNT/F (Barash and Arnon 2014; Maslanka et al. 2016). Based on the unique characteristics of BoNT/F5 L chain, the novel BoNT variant could be considered as BoNT/HA and BoNT/F5 as BoNT/HF (the reviewer could be cited as personal communication). All BoNT types induce similar pharmacological effects, which are characterized by flaccid paralysis, but with some differences between toxinotypes like duration and intensity of symptoms. For example, BoNT/A induces the longest and most severe forms of botulism, compared to BoNT/E which

leads to short duration symptoms and BoNT/B which is most often responsible for mild botulism illness (Eleopra et al. 1998; Keller et al. 1999; O'Sullivan et al. 1999; Foran et al. 2003; Meunier et al. 2003; Keller 2006). BoNT/B causes predominantly dysautonomic signs, whereas BoNT/A has a pronounced paralytic effect on the respiratory muscles and most often leads to acute respiratory distress (Jenzer et al. 1975; Hughes et al. 1981; Merz et al. 2003; Sobel 2005; Potulska-Chromik et al. 2013). Albeit all BoNT types share a similar mechanism of entry into cells and intracellular activity, each toxinotype retains molecular specificity regarding recognition of cell surface receptor, intracellular substrate, and cleavage site. In addition, each toxinotype is subdivided into several subtypes according to amino acid sequence variations (Hill and Smith 2013; Hill et al. 2015; Smith et al. 2015; Williamson et al. 2016). Variations in BoNT amino acid sequences might affect some aspects of their activity such as binding to target cells, efficiency of entry into cells, potency of enzymatic activity, duration of effects, or neutralization efficiency by type- or subtype-specific antibodies.

2 Clostridial Neurotoxins

2.1 Botulinum Neurotoxins and Tetanus Neurotoxin Share a Common Structure

BoNTs and TeNT share a common structure (Lacy and Stevens 1999; Rossetto et al. 2013). They are synthesized as a precursor protein (about 150 kDa), which is inactive or weakly active. The precursor which does not contain a signal peptide is released from the bacteria possibly by a yet misunderstood cell wall exfoliation mechanism (Call et al. 1995). The precursor is proteolytically activated in the extra-bacterial medium either by *Clostridium* proteases or by exogenous proteases such as digestive proteases in the intestinal content. The active neurotoxin consists of a light chain (L, about 50 kDa) and a heavy chain (H, about 100 kDa), which remain linked by a disulfide bridge.

The structure of BoNTs shows three main distinct domains: L chain containing α-helices and *β*-strands and including the catalytic zinc binding motif; the N-terminal part of the H chain forming two unusually long and twisted α-helices; and the C-terminal part of the H chain (H_C) consisting of two distinct subdomains (H_{CN} and H_{CC}) involved in the recognition of the receptor. While the three domains are arranged in a linear manner in BoNT/A and BoNT/B, both the catalytic domain and the binding domain are on the same side of the translocation domain in BoNT/E in the crystal structure. However, BoNT/E shows a more flexible conformation in solution. This domain organization in BoNT/E might facilitate a rapid translocation process (Umland et al. 1997; Lacy et al. 1998; Lacy and Stevens 1999; Emsley et al. 2000; Swaminathan and Eswaramoorthy 2000; Fotinou et al. 2001; Breidenbach and Brunger 2005; Fu et al. 2006; Stenmark et al. 2008; Kumaran et al. 2009; Swaminathan 2011).

The overall sequence identity at the amino acid level between BoNTs and TeNT ranges from 34 to 97%. Several domains are highly conserved which account for the common mode of action of these toxins. Thereby, the central domains of L chains are closely related in all the clostridial neurotoxins and contain the consensus sequence (His-Glu-X-X-His…Glu) characteristic of zinc-metalloprotease active site. The N-terminal half of the H chains (H_N) is also highly conserved, and it is involved in the translocation of the L chain into the cytosol. Thus, a similar mechanism of internalization of the intracellular active domain into target cells is shared by all the clostridial neurotoxins. In contrast, the C-terminal half of H chain, mainly the H_{CC} subdomain, is the most divergent (Popoff and Marvaud 1999; Poulain et al. 2008). This accounts for the different receptors recognized by the clostridial neurotoxins (see below).

2.2 *Botulinum Neurotoxin Diversity*

BoNT genes have been sequenced from a large number of strains and sequence comparison has permitted to identify sequence variations in each toxinotype. Thereby, botulinum toxinotypes are divided into subtypes, which are defined as toxin sequences differing by at least 2.6% identity at the amino acid level [Smith, 2005 #1754; Peck et al. J. Bacteriol. submitted]. BoNT genes from type A strains show 92–95% nucleotide identities corresponding to 84–90% amino acid identities and are currently divided into eight subtypes termed A1 to A8 (Table 1). The locus of the toxin gene cluster also differs. Type B genes differ from 2 to 4% at the nucleotide level and 3–6% at the amino acid level. They are classified into at least eight subtypes, B1 to B8. In addition, some strains (bivalent strains) harbor two toxin gene clusters, notably a *bont/B* and another *bont* cluster such as (A(B), Ab, Ba, Bf). BoNT genes from non proteolytic type B strains (group II) form only one subtype (B4), whereas those from proteolytic strains (group I) show a greater variation leading to a seven subtype division. Sequences of BoNT/B genes show less variation than those of BoNT/A, but a higher within-subtype sequence variation is observed for *bont/*B subtype compared to *bont/*A. BoNT/E sequences from *C. botulinum* type E (group II) fit into multiple subtypes (E1 to E12) sharing 99% nucleotide identity and 97–99% amino acid identity, and are more distantly related to BoNT/E sequences from *C. butyricum* strains which are distributed into two subtypes (E4, E5) with 97–98% nucleotide and 95–96% amino acid identities between sequences from both *Clostridium* species. Gene diversity has also been evidenced in the other parts of the genome as tested by MLST (multilocus sequence typing), AFLP (amplified fragment length polymorphism) analysis, PFGE (pulse-field gel electrophoresis) and whole genome phylogenetic analysis, but most of *C. botulinum* E strains are conserved in a same clade. Subtype variation in *C. botulinum* E strains seems to result from recombination events rather than random mutations. High differences (up to 25%) have been found in nucleotide sequences of BoNT/F mainly in the region coding the light chain, and at least seven subtypes have been identified

Table 1 Receptors of clostridial neurotoxins on neuronal cell surface

Clostridial neurotoxin	Receptor ganglioside moiety	Receptor protein moiety	References
BoNT/A	GT1b, GD1a	SV2A/B/C	Dong et al. (2006)
		SV2C	Mahrhold et al. (2006), Yao et al. (2014)
BoNT/B	GT1b, GD1a	SynaptotagminI/II	Nishiki et al. (1996), Dong et al. (2003)
BoNT/E	GT1b, GD1a	Glycosylated SV2A/B	Dong et al. (2008)
BoNT/C	GD1b, GT1b, GD1a	nd	Tsukamoto et al. (2005), Karalewitz et al. (2012)
BoNT/D	Phosphatidylethanolamine	nd	Tsukamoto et al. (2005)
		SV2A/B/C	Peng et al. (2011)
BoNT/DC	Gangliosides	SynaptotagminI/II	Peng et al. (2012), Berntsson et al. (2013b) Nuemket et al. (2011)
BoNT/F	GT1b, GD1a	Glycosylated SV2A, SV2B, SV2C	Fu et al. (2009), Rummel et al. (2009)
BoNT/G	GT1b, GD1a, GD1b	Synaptotagmin II	Rummel et al. (2004a), Willjes et al. (2013)
TeNT	GT1b, GD1b, GQ1b, GM1a, GD3	GPI-anchored protein(s) SV2A/B? nidogen	Schiavo et al. (1991), Herreros et al. (2001), Munro et al. (2001) Chen et al. (2009), Yeh et al. (2011) Bercsenyi et al. (2014)

nd not determined

in proteolytic *C. botulinum* F (F1 to F6, F8). BoNT from *C. baratii* is assigned to subtype F7. In group III, mosaic genes between BoNT genes types C and D can be distinguished from classical types C and D strains. BoNT/F sequences from *C. botulinum* type F form a different cluster of those from *C. baratii* (Chen et al. 2007; Hill et al. 2007; Carter et al. 2009; Raphael et al. 2010; Macdonald et al. 2011; Carter et al. 2013; Hill and Smith 2013; Dover et al. 2014; Wangroongsarb et al. 2014; Giordani et al. 2015; Hill et al. 2015; Mazuet et al. 2015; Smith et al. 2015; Williamson et al. 2016; Peck et al. 2017; Zhang et al. 2017).

2.3 *Botulinum Neurotoxin Complexes*

BoNTs are produced by neurotoxigenic strains of *Clostridium* together with several associated non-toxic proteins (ANTPs, also called neurotoxin associated proteins, NAPs). BoNTs and ANTPs associate to form large complexes, also known as

progenitor toxins. ANTPs encompass a non-toxic non-hemagglutinin component (NTNHA), and several hemagglutinin components (HAs including HA33, HA17, and HA70) or OrfX proteins (OrfX1, OrfX2, OrfX3, P47) (Oguma et al. 1999; Popoff and Marvaud 1999; Sharma et al. 2003b; Gu 2013; Singh et al. 2014). Botulinum complexes, in which proteins are not covalently linked, are formed in cultures and naturally contaminated food, they are stable at acidic pH, and dissociate at alkaline pH ($\geq$ pH 7.5) (Eisele et al. 2011). It is noteworthy that the stoichiometry can vary according to the strain, culture conditions (culture media, temperature, period of culture, etc), and the method of complex preparation (Singh et al. 2014).

C. botulinum A produces three types of botulinum complexes (also called progenitor complexes or progenitor toxin complexes) designated M (12S, 300 kDa), L (16S, 500 kDa), and LL (19S, $\sim$760 kDa) (Benefield et al. 2013; Lee et al. 2013), whereas the other *C. botulinum* types yield only M and L complexes. The subunit structure of botulinum type D complex was extensively investigated and various stoichiometries have been proposed. Botulinum L complex (650 kDa) from strain 4947, which has the particularity to produce the botulinum complex proteins in the native forms without proteolytic processing, was initially determined as one BoNT/D, two HA70, four HA33, and four HA17 molecules. Intermediate complexes (540 and 610 kDa) constituted of various HA subunits, including an intermediate M complex (410 kDa) containing BoNT/D, NTNH, and two molecules of HA70 have also been determined (Kouguchi et al. 2002; Mutoh et al. 2003). The current model of botulinum type D complex assembly comprises the association of a single BoNT/D molecule with a single NTNH molecule resulting in the M complex. Three HA70 molecules bind to M complex via NTNH to form an intermediate L complex. Then, three HA33/HA17 trimers constituted of one HA17 molecule bound to two HA33 units, associate with the intermediate L complex through HA17/HA70 interactions. The resulting L complex is a 14-mer structure (Fig. 1) (Hasegawa et al. 2007; Mutoh et al. 2005; Suzuki et al. 2005, 2014). Mature L complex results from proteolytic fragmentation of some components, BoNT in L and H chains, NTNH nicking, and HA70 in HA53 and HA22.

Overall, the 12S or M complex results from the association of a BoNT molecule together with a NTNH at a 1:1 ratio and does not possess any hemagglutinating activity (Gu et al. 2012). But, the stoichiometry of the L complexes of *C. botulinum* A, B, and C, which exhibit a HA-BoNT gene locus (Fig. 1), is still controversial. The L complex consists of a BoNT, a NTNH, and several HAs (HA70, HA17, and HA33). Previous works based on gel electrophoresis and densitometry analysis have suggested several molar ratios such as 1:1:2:2:3 for BoNT/A L complex (Bryant et al. 2013). The HA33 are likely to be at the periphery of the complex. The LL complex produced only by serotype A is presumed to be a dimer of the 16S complex linked by an oligomeric HA33 consisting of four molecules and thus containing two molecules of BoNT/A (Inoue et al. 1996; Oguma et al. 1999; Sharma et al. 2003b). A refined stoichiometry of the LL (19S) complex indicated 1 BoNT/A, 1 NTNH/A, 5–6 HA17, 4–5 HA23, 3–4 HA48, and 8–9 HA34 (HA23 and HA48 resulting from HA70 nicking) with a molecular mass of 925 $\pm$ 45 kDa (Lietzow et al. 2008). More recently, an elegant negative stain electron microscopy

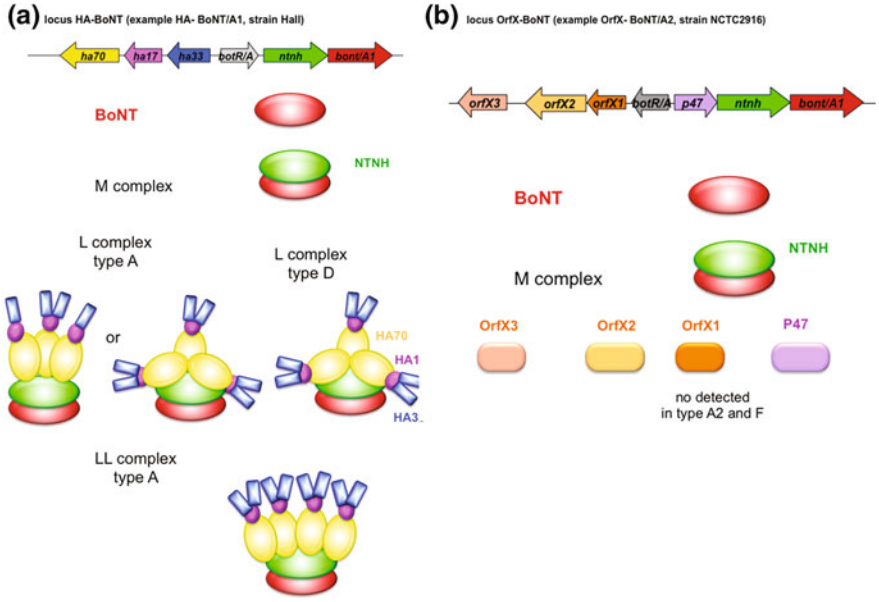


Fig. 1 Schematic representation of botulinum complexes (from Poulain et al. 2015). 1**a**, Schematic representation of *ha-bont* locus and the corresponding botulinum complexes containing BoNT and HA molecules. 1**b**, Schematic representation of *orfX-bont* locus and the corresponding botulinum complexes containing BoNT and OrfX molecules

and single particle averaging study showed that BoNT/A and BoNT/B complexes share a similar structure. BoNT/A and BoNT/B L complexes adopt an ovoid structure with three flexible appendages whereas the M BoNT/E complex is devoid of these arms. It is deduced that the L BoNT/A and BoNT/B complexes result of the assembly of BoNT/A or BoNT/B, NTNH, HA33, HA17, HA70 at a presumed 1:1:6:3:3 ratio (Benefield et al. 2013). Further crystal structure analysis corroborated this 14-subunit complex (∼760 kDa) of L BoNT/A (Lee et al. 2013).

The composition and organization of botulinum complex types A1, A2, and E, the proteins of which are encoded from a OrfX-BoNT locus (Fig. 1), remain undetermined (Dineen et al. 2003). *C. botulinum* A2, A3, A4, A6, A7, A8, E, and F only produce M complexes devoid of hemagglutinating activity, and *C. argentinense* produces only L complex (Oguma et al. 1999). M botulinum complex type A2 only contains BoNT/A2 and NTNH, although P47, Orfx2 and OrfX3 are produced in the culture supernatant but not OrfX1 or in very low amount (Lin et al. 2010). OrfX botulinum complexes are possibly unstable and can easily dissociate. OrfX1 has been detected in botulinum complex type E but not type F, whereas neither OrfX2 nor P47 has been evidenced in both toxinotypes (Li et al. 1998; Hines et al. 2005). The structural organization between BoNT/E and NTNH/E seems similar to that between BoNT/A and NTNH/A (Benefield et al. 2013; Eswaramoorthy et al. 2015).

NTNH/A has a cleavage site within the N-terminus and is separated into 13 and 106 kDa fragments as assessed by SDS-PAGE. NTNH/C and NTNH/D are cleaved at Lys127 by a trypsin-like protease with 7–13 amino acids removed from the N-terminus of the 115 kDa fragment that subsequently results in three proteins starting at Leu135, Val139, or Ser141. NTNH is only cleaved in the 12S (M) complexes from *C. botulinum* types A, C, D, and probably B and G but not in the L (16S) or LL (19S) complexes. The cleaved NTNH molecules constituted a nicked structure since the two fragments still remain together after NTNH purification (Sagane et al. 2000). In contrast, NTNH/E and NTNH/F show an identical deletion of 33 residues in the corresponding region of NTNH/A, NTNH/C, and NTNH/D encompassing the cutting site, and NTNH/G possesses a slightly different sequence in this region. It is presumed that the processing and additional sequence of NTNH in *C. botulinum* A, C, and D are responsible for forming 16S-, and 19S-sized complexes. The inability of *C. botulinum* A2–A8, E and F to form L complexes may result from the absence of HA or other related proteins that bind to NTNH, and from the absence of a putative binding site in NTNH/E and NTNH/F (Oguma et al. 1999; Popoff and Marvaud 1999).

NTNHs from different *C. botulinum* types possess a high identity level (76–83.5%), and are the most conserved proteins in various Botulinum complexes (Popoff and Marvaud 1999). Interestingly, albeit BoNT and NTNH from *C. botulinum* type A share a weak amino acid sequence identity ($\sim$20%), they retain a similar structure. Indeed, NTNH can be divided into three domains called nL, nHN, and nHC (n for NTNH) by analogy with BoNT structure (Gu et al. 2012). NTNH associates with BoNT by non-covalent bonds in a pH-dependent manner to form medium-size botulinum complex (12S), which is resistant to acidic pH and protease degradation (Gu et al. 2012). Indeed, NTNH and BoNT form an interlocked complex that protects BoNT from damaging. Thereby, NTNH is a non-toxic protein which acts as a chaperone protein to protect BoNT. NTNH does not contain the catalytic HExxH motif, but another putative zinc binding motif, KCLIK, at the same position. Inductively coupled plasma-mass spectrometry indicates one zinc atom per each molecule, but NTNH exhibits no proteolytic activity (Inui et al. 2012).

HA33–35 is the most abundant hemagglutinin component of the botulinum complex. Type A and B HA35 binds to oligosaccharides containing galactose-β1-4glucose-*N*-acetyl-D-neuraminic acid (Galβ1-4GlcNAc) (Inoue et al. 2001; Lee 2013 #2428, 2014 #2577]. Thereby, hemagglutination induced by 16S and 19S type A botulinum complex is mainly mediated through complexed HA35 binding to erythrocyte membrane glycolipids and glycoproteins containing Galβ1-4GlcNAc, such as paragloboside and glycophorin A (Inoue et al. 1999, 2001). Similarly, HA33 from types C and D botulinum complex binds to paragloboside on Galβ1-4GlcNAc, but also sialylglycolipids (GM3), as well as sialoglycoproteins (sialosylparagloboside) on the *N*-acetyl-D-neuraminic acid-α2-3-galactose-β1 motif (Fujinaga et al. 2004). The importance of HA33–35 in hemagglutination is also supported by monoclonal antibody studies. Type C-specific monoclonal antibodies against HA33 inhibit hemagglutination, contrary to those against HA50 and HA17

(Mahmut et al. 2002). However, type C HA70 and its derivative HA50 recognize sialosylparagloboside and GM3 at the *N*-acetyl-D-neuraminic acid-α2-3-galactose-*β*1motif in erythrocyte membranes, like the corresponding 16S botulinum complex. Thus, HA50 could also be involved in hemagglutination (Fujinaga et al. 2004). Also HA70-A binds monomeric sialic acids as well as terminal NeuNAc (Lee et al. 2013). HA35 purified from *C. botulinum* A is predominantly a dimeric, *β*-sheet protein in aqueous solutions. In *C. botulinum* A, five N-terminal amino acids are removed from HA35, but similar posttranslational modification has not been observed in HA33 from *C. botulinum C*. The significance of HA35 processing on its biological activity is not known (Sharma et al. 1999). It was first discovered that the 31 C-terminal amino acids, which contain a predicted carbohydrate recognition site, play an essential role in hemagglutination (Sagane et al. 2001). Structure of type C HA33 shows two *β*-trefoil domains consisting of a six stranded, antiparallel *β*-barrel capped on one side by three *β*-hairpins. Related *β*-trefoil structures bind to oligosaccharides and are found in other proteins, including various lectins like the ricin B-chain, cytokines, trypsin inhibitor, xylanase, as well as the C-terminal 200 amino acids of BoNTs. Type A HA35 retains a similar structure related to the carbohydrate binding site of ricin, a plant toxin. It is noteworthy that Asp263 and Asn285 of HA35/HA33 from type A, B and C, which are conserved in the lactose-binding site for ricin B-chain, are critical for carbohydrate binding (Inoue et al. 2003; Arndt et al. 2005; Amatsu et al. 2013; Lee 2013 #2428).

2.4 Tetanus Neurotoxin Forms No Complex

TeNT does not yield any complex, and related botulinum *antp* genes are not evident in the *C. tetani* genome (Brüggemann et al. 2003; Rossetto et al. 2013). Therefore, TeNT is inactive by oral route essentially because of absence of ANTPs (Singh et al. 1995).

3 Transport of Botulinum Neurotoxins Through the Intestinal Epithelial Barrier

3.1 BoNT Passage Through the Intestinal Epithelial Cell Barrier

There is evidence that BoNTs undergo a receptor-mediated transcytosis to cross the intestinal barrier. Indeed, BoNT/A transport through polarized intestinal cell monolayers is inhibited at 4 °C and occurs at 37 °C in a saturable manner over a 30–60 min period (Maksymowych and Simpson 1998, 2004; Ahsan et al. 2005; Couesnon et al. 2008), which is compatible with previously reported receptor-mediated transcytosis

through epithelial cells (Mostov et al. 2000; Ouzilou et al. 2002). This is further supported by the fact that BoNT/A is transported through epithelial cells within 2 h without either transelectrical epithelial resistance (TEER) alteration or any organization disturbance of tight and adherens junctions, thus excluding a paracellular passage of BoNT/A. In addition, the competition experiments showing that BoNT/A transport through intestinal cell monolayers was significantly prevented by an excess of recombinant BoNT/A Hc, but not by Hc from BoNT/B or tetanus neurotoxin (TeNT), strongly argue for a specific receptor-mediated transcytosis. This is in agreement with the fact that iodinated BoNT/A H chain as well as Hc domain are able to bind and cross epithelial cells (Maksymowych and Simpson 1998, 2004). Among the cell lines that have been tested, the mouse intestinal crypt cells m-IC_{cl2} showed the highest level of BoNT/A passage, whereas the colon carcinoma cells Caco-2 or T84, which retain a phenotype of enterocyte (Bens et al. 1996), showed a lower passage rate. Since many pathogens preferably use M cells to enter the intestinal barrier (Kerneis et al. 2000; Ouzilou et al. 2002), Caco-2 cells cocultured with murine lymphocytes differentiated in M cells have been tested (Kerneis et al. 1997). No significant difference in BoNT/A passage was observed through Caco-2 cell monolayers containing or not M-like cells (Couesnon et al. 2008). This strongly suggests that BoNT can pass more efficiently through certain cell types of the intestinal mucosa and that intestinal crypt cells may represent a preferential site of BoNT/A absorption. Indeed, using a mouse intestinal loop test, fluorescent BoNT/A Hc has been found to preferentially localize into neuroendocrine intestinal crypt cells, mainly serotonergic cells (Couesnon et al. 2012).

The rate of transcytosed BoNT/A through an intestinal cell monolayer is low, about 1% with m-IC_{cl2} and 0.1% with Caco-2 cell monolayers after 2 h incubation (Couesnon et al. 2008). These transport rates are in agreement with the translocation efficiency through epithelial cells of bacterial or viral enteric pathogens ranging from 0.1 to 10% of the inoculum within 2 h (Kerneis et al. 1997; Ouzilou et al. 2002), and also with that of BoNT/B from rat duodenum to the lymphatic circulation (0.01–0.1%) (Sugii et al. 1977). Therefore, the passage of BoNT trough an epithelial barrier is a weakly efficient process in the same range of that of enteric pathogens of much bigger size.

A transcytosis-mediated passage of BoNT/A has also been evidenced through human pulmonary adenocarcinoma (Calu-3) cell monolayer. BoNT/A transport was significantly higher through Calu-3 cell monolayers than through Madin Darby canine kidney (MDCK) cell monolayers suggesting a specific BoNT passage according to the epithelial barrier. A transcytosis mechanism instead of a paracellular BoNT passage across pulmonary cell monolayers was based on (1) no modification of the TEER of the cell monolayer, (2) no alteration of tight junctions as monitored by the absence of passage of small molecules, such as inulin, which are not endocytosed and which can only diffuse upon disruption of intercellular junctions, (3) prevention of BoNT passage at low temperature compared to 37 °C indicating that an active process rather than a passive diffusion is involved, and (4) inhibition of BoNT passage with specific antibodies. Transcytosed BoNT/A

molecules were found to be in their active undissociated form, L and H chains linked by a disulfide bridge, like the native toxin (Park and Simpson 2003).

The receptor-mediated transcytosis process raises the question whether BoNT receptors on intestinal cells are the same than those on neuronal cells? As for neuronal cells, the same toxin domain, H_C, is involved in the recognition of receptors on intestinal cell surface. Thereby, BoNT/A H_C is able to bind and cross epithelial cells (Maksymowych and Simpson 1998, 2004). Moreover, BoNT/A transport through intestinal cell monolayers is significantly prevented by an excess of recombinant BoNT/A H_C, but not by H_C from BoNT/B or TeNT (Couesnon et al. 2008). Gangliosides GD1b/GT1b are also part of BoNT receptor on intestinal cells. Indeed, addition of GD1b/GT1b impairs the binding of fluorescent BoNT/A Hc to neuronal cells as well as to intestinal cells as assayed by fluorescence-activated cytometry (FACS), and significantly decreases the transport of biologically active BoNT/A through intestinal cell monolayers. Furthermore, ganglioside depletion by DL-threo-1-Phenyl-2-palmitoyl-amino-3-morpholino-1-propanol (PPMP), an inhibitor of glucosyl ceramide synthase, strongly decreases BoNT/A transport through intestinal cells, and loading PPMP-treated cells with GD1b/GT1B reverses the effect (Couesnon et al. 2008). SV2C (synaptic vesicle protein 2), the protein part of BoNT/A receptor on neuronal cells (see below 6.1), is also expressed in intestinal cells such as CaCo-2 or m-IC_{cl2} cells. Recombinant intraluminal domain (L4) of SV2C was found to decrease BoNT/A transcytosis through CaCo-2 or m-IC_{cl2} cell monolayers suggesting that SV2C or a SV2C-related protein might be involved in the BoNT/A receptor on intestinal cells. Thus, BoNT receptors might be the same or consist of related molecules on neuronal and intestinal cells. However, BoNT/A H_C binding to intestinal cells is much lower than to neuronal cells as monitored by FACS, indicating that the affinity is weaker or the number of BoNT receptors is possibly smaller on intestinal cells when compared to neuronal cells (Couesnon et al. 2008). A different type of BoNT/A receptor on the colon carcinoma T84 cells has been suggested, based on the fact that a recombinant BoNT/A Hc mutated on the ganglioside binding site (W1266L, Y1267S) was impaired in binding to neuronal cells, but retained the ability to cross the intestinal cell monolayer (Rummel et al. 2004b; Elias et al. 2011). Based on the dual nature of BoNT receptor, a ganglioside associated to a membrane protein, mutation on the ganglioside binding site does not preclude that the receptor protein part or the global receptor organization is different on intestinal cells. A difference between BoNT receptors on neuronal and intestinal cells might be a distinct distribution in membrane subdomain or different exposition of the site(s) accessible to the toxin. However, the almost complete inhibition of BoNT/A H_C binding to cells depleted in gangliosides by PPMP treatment suggests that gangliosides are the main BoNT/A receptor on intestinal epithelial cells (Couesnon et al. 2008). Moreover, recent investigations with BoNT/B H_C also show that gangliosides (GD1a, GD1b, GT1b) are the main players in binding of this BoNT type to epithelial cells using competition between HcB and gangliosides in binding experiments to cells (Connan et al. 2017). Therefore, BoNT likely use only one type of receptors on intestinal epithelial cells consisting in gangliosides or related membrane glycoproteins.

3.2 BoNT Entry into Intestinal Epithelial Cells

Receptor-mediated transcytosis through epithelial barrier involves toxin uptake at the apical side and subsequent exocytosis of the whole active toxin at the basolateral side. Do BoNTs enter intestinal and neuronal cells via a same pathway? BoNT entry into target neuronal cells requires an endosome acidification which facilitates the translocation of L chain into the cytosol and its subsequent intracellular enzymatic activity. In contrast, transcytosis of active toxin molecules is mediated by a non-acidic intracellular pathway. Indeed, for example BoNT/A transport through intestinal cells is not prevented by Bafilomycin A1, an inhibitor of vesicular ATPase, or monensin, an alkalinising agent. Both drugs prevent acidification of endocytosis vesicles indicating that BoNT/A transits through a neutral pH compartment in intestinal cells (Maksymowych and Simpson 2004; Couesnon et al. 2008). In contrast, Bafilomycin A1 inhibits SNAP25 proteolytic cleavage in PC12 cells treated with BoNT/A by preventing L chain release into the cytosol (Simpson et al. 1994; Coffield et al. 1999; Keller et al. 2004). Moreover, BoNT/B Hc colocalizes with Lamp1, a marker of late endosome in cultured neuronal cells, whereas no significant association with late endosomes was observed in intestinal epithelial cells (Connan et al. 2017). This further supports a BoNT transport in acidic pathway in neuronal cells and in a non-acidic route in epithelial cells. In addition, Brefeldin A, which disrupts the Golgi apparatus, does not impair BoNT/A transcytosis, indicating a toxin trafficking via an intracellular pathway independent of this cellular compartment (Maksymowych and Simpson 2004; Couesnon et al. 2008).

A differential intracellular trafficking pathway of BoNT/A in intestinal cells versus neuronal cells has been identified based on distribution and morphology of endocytic vesicles carrying the toxin and on activity modulation of molecules involved in the main cell entry pathways. In neuronal cells, BoNT/A Hc enters endocytic vesicles, which progressively migrated to the perinuclear area, whereas endocytic vesicles containing BoNT/A Hc are more scattered in the cytoplasm of intestinal cells, supporting a different trafficking in both cell types. BoNT/A enters neuronal cells via a clathrin-dependent pathway, mostly mediated by the synaptic vesicle recycling but not exclusively (Keller et al. 2004; Verderio et al. 2007). BoNT/A Hc markedly colocalizes with transferrin, a marker of the clathrin-dependent endocytosis (Couesnon et al. 2009). In addition, potassium depletion, which prevents assembly of clathrin-coated vesicles, significantly inhibits BoNT/A Hc uptake into neuronal cells (Petro et al. 2006; Couesnon et al. 2009). In contrast, BoNT/A Hc does not significantly colocalize with transferrin in intestinal cells and the potassium depletion does not prevent its entry into these cells. The BoNT/A entry pathway into intestinal cells is also caveolin-independent, based on a non-significant colocalization between BoNT/A Hc and caveolin (Couesnon et al. 2009). The different entry route of BoNT/A into neuronal and intestinal cells is further supported by EM analysis. BoNT/A Hc is distributed in coated vesicles in neuronal cells, whereas it is shown in smooth vesicles in intestinal cell, which likely

correlate with caveolae endosomes (Couesnon et al. 2010). Therefore, clathrin-dependent pathway seems to be a preferential entry route of BoNT/A into neuronal cells and only an accessory entry pathway into intestinal cells.

A significant part of BoNT/A Hc was found in early endosomes, as evidenced by colocalization with (early endosome antigen) EEA1 in neuronal and m-IC_{cl2} cells (Couesnon et al. 2009). Early endosomes labeled with EEA1 are a common compartment reached by clathrin-dependent and several clathrin-independent routes (Lamaze and Johannes 2006a). This indicates that BoNT/A is transported in a common early compartment in neuronal and intestinal cells. Then, the toxin is sorted in distinct pathways according to the cell type: either BoNT/A passes through an acidic compartment which facilitates the translocation of the L chain into the cytosol of target neurons, or it is transcytosed in a neutral compartment in intestinal cells as TeNT in retrograde transport along motor neurons (Lalli et al. 2003).

Rho-GTPases, which are involved in the control of the actin cytoskeleton, differentially regulate several endocytic pathways. Thereby, Cdc42 controls the endocytosis of glycosyl-phosphatidyl-inositol anchored proteins (GPI-APs), fluid phase markers, and *Helicobacter pylori* VacA toxin into GPI-AP enriched early endosomal compartments (GEECs), whereas RhoA is involved in the internalization of interleukin-2 receptor (Mayor and Pagano 2007). Dominant-negative mutant of Cdc42 efficiently inhibited BoNT/A Hc entry into intestinal cells and to a lower extent into neuronal cells, whereas dominant-negative mutant of Rac1 or RhoA did not affect the entry pattern of BoNT/A Hc into both cell types (Couesnon et al. 2009). Thus, BoNT/A enters neuronal and intestinal cells by two distinct regulated endocytosis mechanisms. BoNT/A Hc probably follows a similar Cdc42-dependent endocytic route in intestinal cells than GPI-APs or VacA. However, the Cdc42-dependent pathway in intestinal cells delivers BoNT/A Hc in a different compartment than GEECs. Indeed, GEECs have been found to be devoid of transferrin and EEA1 markers (Sabharanjak et al. 2002; Gauthier et al. 2005), and BoNT/A Hc partially colocalizes with transferrin and EEA1 (Couesnon et al. 2009). In addition, cholesterol depletion with low concentration of MβCD (2 mM) which has been found to prevent GPI-AP uptake into GEECs (Chadda et al. 2007) did not impair BoNT/A Hc entry. Thereby, a distinct endocytic pathway than GEEC and controlled by Cdc42 seems to be involved in BoNT/A Hc entry into intestinal cells. Similarly BoNT/B Hc was also found to enter epithelial cells via a Cdc42-dependent pathway (Connan et al. 2017).

An important step in endocytosis is the fission process, which allows the individualization of endocytic vesicle from invaginated membrane containing a cargo and its subsequent migration. The GTPase dynamin is a key molecule implicated in the pinching mechanism of endocytic vesicle (Takei et al. 2005; Roux et al. 2006a). Dynamin was first described to be part of the clathrin-dependent pathway, but it was subsequently found to be involved in several clathrin-independent endocytosis pathways such as those mediated by caveolae or RhoA (Mayor and Pagano 2007). We observed that BoNT/A Hc endocytosis in both neuronal and intestinal cells was dynamin-dependent indicating a similar mechanism of endocytic vesicle formation in both cell types (Couesnon et al. 2009). Intersectin, a major binding partner of

dynamin, was first involved in clathrin-dependent endocytosis (Simpson et al. 1999), but it also regulates caveolae-mediated transcytosis in endothelial cells. Indeed, intersectin recruits dynamin through its SH3 domains to the caveolae neck (Predescu et al. 2003). Overexpression of the intersectin SH3A domain efficiently inhibits BoNT/A Hc entry into neuronal cells as well as in intestinal cells, indicating a same mechanism of entry in both cell types (Couesnon et al. 2009). However, several isoforms of intersectin have been described. Intersectin isoform 1 is neuron specific and is involved in synaptic vesicle formation, whereas isoform 2 is widely distributed in the different tissues (Evergren et al. 2007). In endothelial cells, intersectin 2 associates with dynamin and SNAP23 in the fission of caveolae and transcytosis mechanism (Predescu et al. 2003). Interestingly, the long isoform of intersectin 1 or 2 contains a DH (Dbl homology) domain which functions as a GEF (guanyl nucleotide exchange factor) for Cdc42 and a SH3 domain interacting with N-WASP (Evergren et al. 2007). This suggests a possible intersectin-mediated Cdc42 activation in the entry process of BoNT/A Hc into intestinal cells. The dynamin dependence in cell entry has also been evidenced for BoNT/B H_C. Its endocytosis is completely prevented by treatment with Dynasore, a dynamin inhibitor (Connan et al. 2017). A different regulation of entry mechanism might be involved in neuronal cells, in which BoNT/A Hc entry is dynamin- and intersectin-dependent, but not or only partially Cdc42-dependent. This does not rule out that several BoNT Hc entry pathways might coexist. It is noteworthy that Cdc42 is involved in the caveolae pathway and fluid phase endocytosis (Cheng et al. 2010), as well as in polarity and transcytosis in epithelial cells (Rojas et al. 2001). Cdc42 likely controls the BoNT transcytotic pathway in epithelial cells.

3.3 Role of ANTPs in BoNT Passage Through Intestinal Epithelial Barrier

HAs have been initially found to bind to carbohydrate structures thus mediating agglutination of red blood cells and then to facilitate the binding of botulinum complexes to intestinal mucus or directly to intestinal epithelial cells. In a guinea pig in vivo model, only L form of botulinum complex type C (16 s), which contains HAs, binds to epithelial cells of the upper small intestine prior to be absorbed from the intestine and then released into the serum, whereas botulinum complex type C lacking HAs (12 s) or BoNT/C alone are poorly absorbed into the serum (Fujinaga et al. 1997). Among HA components from type C, type A, or type B, HA33/35 and HA50 recognize distinct carbohydrate structures on erythrocytes and intestinal cells. HA33/35 binds to galactose moieties and HA50 to sialic acid moieties and is considered to be involved in botulinum complex binding and absorption through the intestinal mucosa (Fujinaga et al. 2000, 2004; Arimitsu et al. 2008). HA33 and HA50/70 also facilitate the binding and transport of botulinum complexes type C or

D through rat intestinal epithelial cell monolayers in a sialic acid-dependent manner. HA50/70 might bind to sialic acid on mucin and/or sialic acid-dependent receptor on cell surface, and it was speculated that HA33 might also interact with sialic acid on cell membranes (Inui et al. 2010; Niwa et al. 2010; Ito et al. 2011). Hence, sialic acid derived molecules seem to be important motifs for the binding of botulinum complexes on intestinal cells and/or mucin. Botulinum complex type A binds to Intestine-407 cells, which are derived from human intestinal epithelium, via *N*-acetyl-lactosamine (Kojima et al. 2005). In human colon carcinoma cells (HT-29), type C 16S botulinum complex binds to surface glycoproteins like mucin (Nishikawa et al. 2004). However, HA components are not absolutely required, since BoNT/C or D alone display binding and transport through intestinal cells, albeit to a lower extent (Inui et al. 2010; Niwa et al. 2010; Ito et al. 2011). Thus, HA50 and HA33 seem to facilitate the binding and transport of botulinum complexes through intestinal epithelial cell monolayers in a sialic acid-dependent manner. The binding of HAs from type A to carbohydrates has been confirmed by using highly purified recombinant proteins and a wide glycan array screening. Wild type HA complex (three HA70, three HA17, and six HA33 molecules) or the minimal HA complex (domain 3 of HA70, one molecule of HA17, and two HA33 molecules) specifically recognize glycans with a terminal galactose or sialic acid and mediate the binding to the luminal surface of mouse jejunum (Lee et al. 2013). In contrast, NTNH or M complex (NTNH/BoNT/A) shows no detectable binding to small intestine (Yao et al. 2014).

More recently, a novel function has been attributed to HA complexes consisting in the disruption of intercellular junctions between intestinal epithelial cells. Such evidence has been shown with HA from type B and type A that alters tight and adherens junctions of CaCo-2, T84 or MDCK cell monolayers, and enhances BoNT transport. HAs act specifically on the basolateral side of cultured intestinal cells and induce a disorganization of molecules of the tight junctions (occludin, ZO1) as well as those of adherens junctions (E-cadherin, catenin). HA-dependent alteration of the intercellular junctions result in a paracellular passage of BoNT, 12 s botulinum complex (lacking HAs), as well as other macromolecules without inducing cytotoxicity. In contrast, HAs from type C are ineffective in disrupting human intestinal cell barrier, although they are able to alter cell monolayer integrity from canine (MDCK cells) or rat origin via a cytotoxic mechanism (Matsumura et al. 2008; Fujinaga et al. 2009; Jin et al. 2009). The cytotoxic mechanism of type C HAs for the epithelial cells seems dependent of binding to the ganglioside GM3 (Sugawara et al. 2015). It is noteworthy that type C HA cytotoxicity and epithelial barrier disruption has only been observed in vitro with cell lines (Fujinaga et al. 2013).

Then, it was determined that HAs recognize E-cadherin, which plays a crucial role in basolateral junction. The interaction of HAs with E-cadherin is species and isoform specific. Thereby, HAs directly bind to the extracellular domain of (epithelial) E-cadherin, but not of (neural) N-cadherin, nor (vascular endothelial) VE-cadherin. Type B HAs specifically bind to human, bovine, and mouse E-cadherin but not to that of rat and chicken (Sugawara et al. 2010). This is

consistent with the fact that botulism type B is common in humans and is rarely observed in chickens. Type A BoNT complexes also recognize human E-cadherin whereas type C BoNT complexes do not (Sugawara et al. 2010).

The combination of HAs (HA33, HA17, and HA50/70) organized in complex is required for the optimum binding to E-cadherin, whereas the association of HA17 and HA50/70 is poorly effective and individual HAs do not interact with E-cadherin. Reconstitution of HA complexes with highly purified recombinant proteins gave additional detailed insights regarding the interaction with E-cadherin. HAs assemble in a threefold symmetric hetero-dodecameric structure and the whole HA complex exhibits the highest affinity to E-cadherin. The minimal HA complex interacting with E-cadherin consists of domain 3 of HA70 (Pro-378-Asn-626), one molecule of HA17, and two HA33 molecules (Lee et al. 2014). HAs bind to the distal extracellular domain (EC1) of E-cadherin near the cadherin trans-dimer interface (Sugawara et al. 2010). Thus, the HA binding sites to carbohydrates and E-cadherin are functionally and structurally distinct (Sugawara et al. 2014).

HAs bind to E-cadherin in a Ca^{++}-dependent manner without exerting a protease activity towards this substrate, but in changing its cellular localization. E-cadherin in HA treated cells is redistributed from the cell–cell contacts to clusters on cell surface and it is partially endocytosed (Sugawara et al. 2010). Structural analysis of HA-E-cadherin complex shows that HAs bind to extracellular domains 1 and 2 (EC1, EC2) of E-cadherin and stabilize E-cadherin in strained monomeric conformation (Lee et al. 2014). Thus, HA binding to E-cadherin prevents the interaction between E-cadherin molecules resulting in dissociation of the intercellular junctions.

Binding of HA complex to E-cadherin leads to intercellular junction alteration and thus facilitates the passage of BoNT or botulinum complexes by the paracellular way. Indeed, HA complexes increase the permeability of intestinal cell monolayers grown on filter as monitored by the measure of transepithelial electrical resistance and enhance the passage of BoNT by about 10 fold. In addition, MDCK cells expressing rat E-cadherin, which acts as dominant-negative since it does not interact with type B HA, maintain their intercellular junction integrity, although type B HA complex applied basolaterally binds to endogenous E-cadherin as in control cells (Sugawara et al. 2010). However, this effect is only efficiently observed after several hours, 10–48 h (Matsumura et al. 2008; Fujinaga et al. 2009). In mouse intestinal loop model, HA complexes also enhance the translocation of BoNT/A from the intestinal lumen to the intestinal mucosa (Lam et al. 2015b). In addition, the facilitating effect of HA complexes on BoNT transport through the intestinal barrier has also been evidenced in vivo. HAs mutated on the binding sites to E-cadherin decrease the oral toxicity of BoNT/A complexes in mouse (Lee et al. 2014). Therefore HAs might facilitate the transport of BoNT through an epithelial barrier, but they are not indispensable since BoNT/A alone can cross intestinal or pulmonary epithelial barrier in in vitro or in vivo models (Park and Simpson 2003; Maksymowych and Simpson 2004; Couesnon et al. 2008). A more recent study shows that BoNT/A and BoNT/E have an equivalent

absorption rate and toxin potency when administrated by inhalation route in mice as pure neurotoxin or complex form (Al-Saleem et al. 2012). In addition, disruption of the intestinal epithelial barrier usually results in digestive symptoms like, increased secretion, diarrhea, mucosal inflammation, which are not observed in botulism. This supposes that HAs might have a limited activity on the intestinal barrier, just enough to mediate a BoNT paracellular passage.

How HA can have access to E-cadherin from the intestinal lumen, since E-cadherin molecules are localized along the basolateral side of the epithelial cells? It is suggested that HAs bind to the apical surface and subsequently are endocytosed in early endosomes and then transcytosed through the basolateral side (Sugawara et al. 2010; Sugawara and Fujinaga 2011). This supposes that HA recognize specific receptor(s) on the apical side and it is not clear whether the carbohydrates recognized by HAs are involved. It has been hypothesized that HA mediates the transcytosis of whole botulinum complexes through intestinal epithelial cells, which are delivered on the basolateral side allowing the access of HA to E-cadherin (Fujinaga et al. 2013). E-cadherin is the major player of adherens junction but its role in controlling tight junctions is controversial (Gumbiner 1996; Chen and Gumbiner 2006; Capaldo et al. 2007; Hartsock and Nelson 2008; Popoff et al. 2009). It seems that botulinum complexes use a more specific entry pathway to cross the intestinal barrier. HAs have been found to bind to M cells and thus to facilitate the oral toxicity of BoNT/A complexes (Matsumura et al. 2015). This suggests that M cells mediate the transport of HA complexes which subsequently interact with the basolateral E-cadherin, and alter the intercellular junctions allowing the paracellular passage of the botulinum complexes. However, the most efficient absorption site was identified in the upper small intestine (Bonventre 1979), whereas M cells are mainly localized in the terminal ileum (Jepson and Clark 1998).

E-cadherin is not the unique target of botulinum HAs, this molecule is also the target of other bacterial toxins and virulence factors. For example, *Bacteroides fragilis* enterotoxin proteolytically cleaves the extracellular domain of E-cadherin, which subsequently induces a degradation of the intracellular domain probably by ATP-dependent cellular proteases, reorganization of actin filaments, and increase in cell barrier permeability (Sears 2001; Sears et al. 2006). Interestingly, the N-terminal part of HA70 shows sequence similarity with *Clostridium perfringens* enterotoxin, which binds to claudins, forms pores, and alters the intestinal barrier integrity (Fujinaga et al. 1994; McClane 2006; Robertson and McClane 2011; Yelland et al. 2014). But HA70 does not retain *C. perfringens* enterotoxin activity on intestinal cells. The cytotoxic mechanism of type C HAs for the epithelial cells seems dependent of binding to the ganglioside GM3 (Sugawara et al. 2015).

In contrast, OrfX proteins and OrfX complexes, the structure of which is still unknown, have not been reported to interact with E-cadherin or to alter the intestinal epithelial barrier. This raises the question whether OrfX complexes are involved in BoNT passage through epithelial barriers? In *C. botulinum* strains type

E, F, and some type A, BoNTs form complexes lacking HAs, and are responsible for food borne botulism, which is as severe as the classical type A and B botulism.

4 Dissemination of Botulinum Neurotoxins to the Central Nervous System

The basic explanation of BoNT dissemination in the organism includes that, once absorbed in the intestinal barrier, BoNTs are delivered in the extracellular compartment and partially reach the lymphatic and blood circulation which disseminate the toxin through the whole organism. BoNTs can persist for long periods in the general circulation (Cheng et al. 2009; Bagramyan et al. 2013), which is considered as a "holding compartment". Only a small proportion of toxin disseminated throughout the body enters the target neurons and are responsible for the neurological symptoms (Simpson 2013). However, it is still unclear whether BoNTs are transported through epithelial barrier in their complex or dissociate form. After oral administration, only 16S complex of BoNT/A and BoNT/B and 12S complex of BoNT/E are detected in the lymph and in case of naturally acquired botulism, only monomeric form of BoNT/A could be detected in blood (Kitamura et al. 1969; Sugii et al. 1977; Kobayashi et al. 2003). This suggests that either BoNTs are transported in their complex form or they are transported separately and re-associate into a complex once the intestinal barrier crossed. However, if bloodstream is considered as the main route for BoNT dissemination, BoNT should be distributed to the whole neuromuscular junctions of the organism leading to a generalized botulism which is not the case. Botulism symptoms are characterized by a descendent flaccid paralysis which starts by an attack of cranial nerves suggesting that BoNTs use another way of dissemination than the bloodstream or the cranial motor neurons are more susceptible to BoNTs.

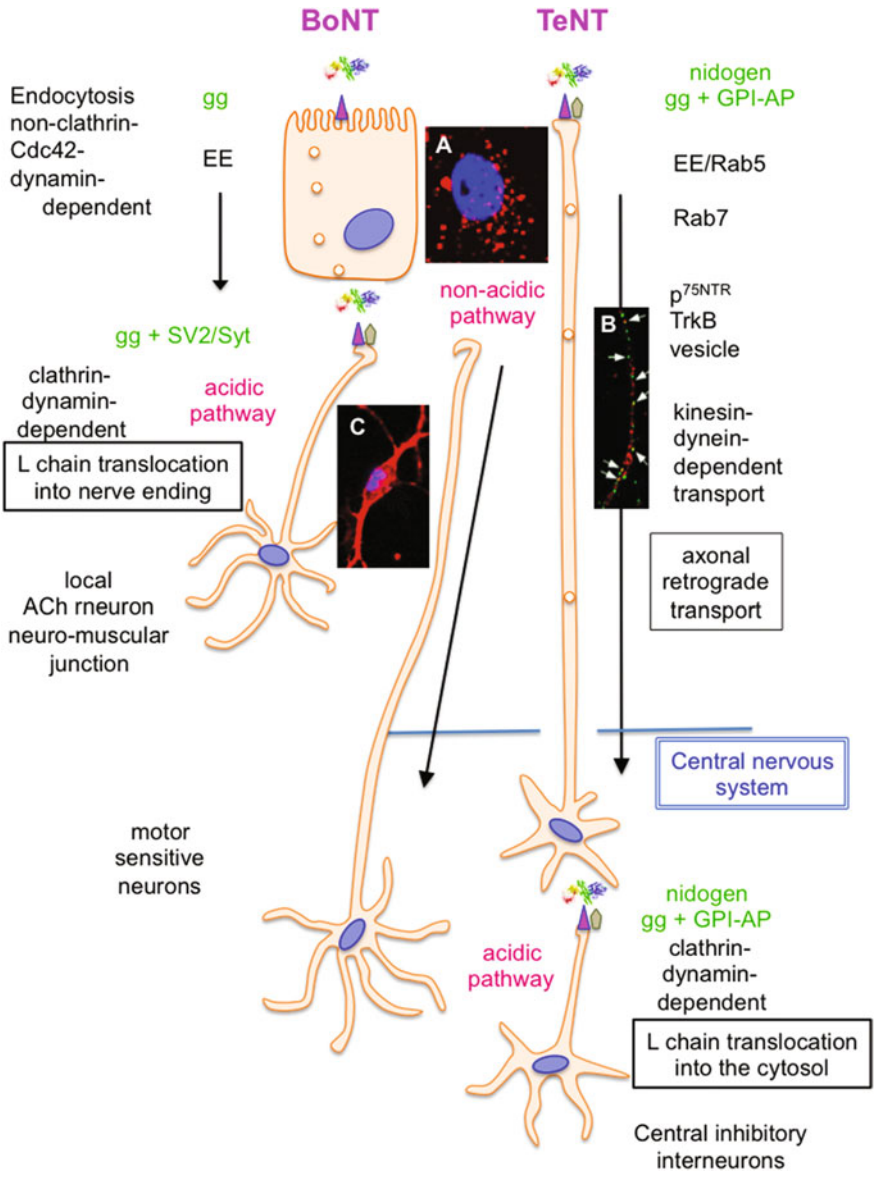
Since BoNTs share a closely similar structure/function with TeNT, do BoNT use certain neuronal cells to transcytose to target neuronal endings and to enter the central nervous system? It was first shown in 1963 by Tyler et al. that the effects of BoNTs are not restricted to the peripheral nervous system, but that they also have several effects on the central nervous system (Polley et al. 1965). Thus, another approach implies BoNT dissemination by axonal transport as it is the case for TeNT. More recently, Antonnuci et al. demonstrated that BoNT/A undergoes also a retrograde transport to the motor neurons and central nervous system and is then transcytosed to afferent neurons (Antonucci et al. 2008). Indeed, one day after injection of BoNT/A into the hippocampus, cleaved SNAP25 was detected in the ipsilateral hippocampus, and 3 days after injection in the contralateral hippocampus. Another experiment was performed in the superior colliculus also called optic tectum of the rat which receives projection from contralateral retina and from primary visual cortex ipsilateral to the injection. Three days after injection of BoNT/A, a small quantity of cleaved SNAP25 was detected in the retina which means that

BoNT/A underwent a retrograde transport from the optic tectum to retina. In addition, three days after BoNT/A injection in mouse pad, cleaved SNAP25 was detected in the facial nucleus ipsilateral to the injection site (Antonucci et al. 2008; Caleo et al. 2009; Caleo and Schiavo2009). Furthermore, injection of BoNT/A1 or BoNT/A2 in the gastrocnemius muscle of rat unilateral leg induced paralytic effects on both hind legs and cleaved SNAP25 was visualized in the bilateral dorsal and ventral horns of distal spinal regions that receive efferent projections from the injected muscles. Cleaved SNAP25 was more widely spread in the contralateral spinal cord subsequently to BoNT/A1 muscle injection than BoNT/A2 (Torii et al. 2011; Akaike et al. 2013; Koizumi et al. 2014). These experiments provided evidence that BoNT/A is transported from the peripheral to the central nervous system through motor neuron and/or sensory neurons. BoNT/A1 seems more potent than BoNT/A2 to undergo the retrograde neural pathway, whereas BoNT/A2 could preferentially use the blood circulation for its transport to the contralateral muscles when injected at high doses (Koizumi et al. 2014). Moreover, it has been shown that this transport is dependent of microtubules and is blocked after colchicine treatment (Restani et al. 2011, 2012b; Matak et al. 2012). Further experiments showed that BoNT/A, BoNT/E and TeNT are transported by the same non-acid axonal organelles and remain enzymatically functional in the soma of the motor neurons (Restani et al. 2012a). However, the quantities of BoNT used for these experiments are much higher than the usual therapeutic dosage.

Is BoNT entry through the digestive tract such as in food borne botulism, accompanied by toxin transport to the central nervous system? Investigations in mouse intestinal loop showed that BoNT/A and BoNT/B target different types of neurons in the intestinal mucosa, mainly including acetylcholinergic neurons but also, albeit to a lower extent, non-cholinergic neurons such as glutamatergic and serotonergic neurons (Couesnon et al. 2012; Connan et al. 2016, 2017). The effect of BoNT/A on non-cholinergic neurons is unknown, but they could be the key for the understanding of the retrograde transport of BoNTs to the spinal cord. BoNTs might use non-cholinergic neurons not only to contribute to the local paralytic effects, but also to disseminate to other target neurons locally or at distance from the intestine.

5 Transport of Tetanus Neurotoxin to the Central Nervous System

In contrast to BoNTs, which mainly target the motor neuron endings at the neuromuscular junctions, TeNT uses motor neurons through a transcytotic mechanism to target neurons in the central nervous system (Fig. 2). TeNT enters motor neurons via endocytic vesicles different than those in target central neurons. TeNT enters motor neuron as well as sensory neuron endings via clathrin-coated pits and is sorted into organelles successively controlled by Rab5 and then Rab7 GTPases, which move



retrogradely along the axon in a microtubule-dependent manner. Rab5 regulates the entry into early endosomes, and Rab7 is usually associated with the transport to lysozomes via acidic organelles. However, TeNT enters Rab5 early endosomes which mature slowly in a subpopulation of Rab7 endosomes. These are neutral since devoid of vacuolar (H^+)ATPase, and undergo a slow speed (0.2–1.2 μm/s) retrograde transport. In contrast, TeNT enters the small subpopulation of endosomes

◂**Fig. 2** Schematic comparison of the cell entry and dissemination pathways of botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT). BoNTs undergo transcytosis through intestinal epithelial cells via mainly recognition of gangliosides (GD1b/GD1a) as cell surface receptors and via a non-clathrin-, Cdc42-dependent and non-acidic pathway, whereas TeNT uses both gangliosides and protein receptor (nidogen1/2, GPI-anchored protein) to enter neuronal cells and for their axonal retrograde transport in non-acidified vesicles which is similar to that of neurotrophin until the central nervous system. BoNTs and TeNT recognize gangliosides and distinct protein receptors (nidogen/SV2/synaptotagmin) to enter target neuronal cells and neuromuscular junction via a clathrin-dependent pathway and subsequent delivery in acidified vesicles allowing the translocation of the L chain into the cytosol, where it exploits proteolytic activity towards SNARE proteins, and inhibits the synaptic vesicle fusion with presynaptic membrane and delivery of neurotransmitter. Passage of BoNT and botulinum complexes through the paracellular way is not represented. A. BoNT/B Hc-Cy3 transcytosis in mouse epithelial intestinal (m-IC_{CL2}) cells (unpublished). B. TeNT Hc-Alexa488 (*green*) retrograde transport in motor-neuron is associated with vesicles labeled with Rab7 (*red*) (modified from Deinhardt et al. 2006b). *Arrows* indicate colocalization of TeNT Hc and Rab7. C. BoNT/B Hc-Cy3 at the cell surface and in perinuclear endosomes of primary neuronal cells (unpublished). *Ach* acetylcholine; *EE* early endosome; *gg* gangliosides; *Syt* synaptotagmin; *SV2* synaptic vesicle protein 2; *GPI-AP* glycosyl-phosphatidyl-inositol anckored protein

which mature rapidly by acquiring Rab7, is driven into the degradation pathway (Bohnert and Schiavo 2005; Deinhardt et al. 2006b).

TeNT shares a retrograde transport mediated by neutral pH endosomes, with neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Thereby, the tubulo-vesicular organelles involved in TeNT retrograde transport are characterized by the presence of neurotrophin receptors such as $p75^{NTR}$ and TrkB (tyrosine receptor kinase B). BDNF which preferentially uses TrkB as receptor activates the efficiency and kinetics of TeNT internalization at neuromuscular junctions through receptor activation mechanism. TeNT H_C, BDNF, TrkB, and $p75^{NTR}$ have been found in the same endosomes (Deinhardt et al. 2006b). However, Roux et al. (2006b) did not show specific colocalization between TeNT H_C and TrkB. These contrasting observations indicate that TeNT transport is identical or similar to that of neurotrophin factors. TeNT axonal retrograde transport is mediated by dynein and kinesins, which are microtubule-dependent motor proteins (Lalli and Schiavo 2002; Lalli et al. 2003; Bohnert and Schiavo 2005; Bohnert et al. 2006; Deinhardt et al. 2006a; Roux et al. 2006b; Schmieg et al. 2015). TeNT H_C was assumed to drive the retrograde transport of the toxin, and can be used to transport heterologous protein in the same way (Li et al. 2001; Maskos et al. 2002). However, recent studies showed that the entire TeNT is required for efficient retrograde transport, indicating that regions outside the receptor-binding domain also contribute to the intracellular trafficking (Wang et al. 2012; Blum et al. 2014a). Transcytosis through motor neurons releases undissociated TeNT in the spinal cord or brain. When released in the extracellular space in the central nervous system, TeNT carries out a transsynaptic migration and reaches its final target neurons, which are preferentially inhibitory interneurons involved in the regulation of the motor neuron activity resulting in spastic paralysis. However, TeNT can inhibit the release of neurotransmitter in various neuronal cell

types (Popoff and Poulain 2010; Poulain et al. 2015). TeNT enters target inhibitory interneurons via vesicles that are acidified thus permitting the delivery of the L chain into the cytosol, where it inhibits the regulated release of glycine and GABA (Lalli et al. 2003; Bohnert and Schiavo 2005; Schmieg et al. 2015). Overall, the precise mechanism of translocation is not completely understood for BoNT and TeNT.

The dual mode of TeNT intracellular trafficking, retrograde transport in neutral pH endosomes in motor neurons and entry into acidified vesicles in target inhibitory interneurons, raises the question which are the receptors recognized on each type of cells to drive the two distinct intracellular pathways? The identity of the TeNT protein receptor(s) at the surface of the distinct neuronal cells remains to be defined (Bercsenyi et al. 2013).

6 Entry of Clostridial Neurotoxins into Target Neuronal Cells

BoNTs enter the organism by crossing mucosal barrier such as digestive tract mucosa following ingestion of preformed toxin in food or intestinal colonization by a BoNT producing *Clostridium,* lung mucosa following toxin inhalation, or skin through intentional medical injection or wound contamination (wound botulism). Then, BoNTs are transported to motor neuron endings, where they bind to high-affinity receptors. TeNT only uses the parenteral route subsequently to a rupture of tegument integrity and local proliferation of *C. tetani,* which releases the toxin in the surrounding tissues. TeNT also recognizes high-affinity receptors on neuronal cell endings, but in contrast to BoNTs, it enters a distinct trafficking pathway and is transported to the central nervous system (CNS) (Fig. 2).

6.1 Binding to Receptors

The first step of BoNTs or TeNT intoxication is the specific binding to target cells. BoNTs and TeNT bind specifically to non-myelinated areas of nerve terminals. The concept of dual receptor on neuronal cells for the clostridial neurotoxins was characterized at the molecular level in the recent years and involves gangliosides and various protein receptors depending on BoNT types and cell types.

BoNTs and TeNT bind to gangliosides preferentially from the b-series like GT1b and GD1b, as well as GD1a, but with varying affinity according to the neurotoxin type (Table 1) (reviewed in Rummel 2013; Lam et al. 2015a). Binding to ganglioside is essential to initiate the toxic process of clostridial neurotoxins. Indeed, binding to gangliosides (GD1b, GT1b, GD1a) is necessary and sufficient for TeNT entry into neurons (Chen et al. 2008, 2009). In addition, knockout mice

defective in b-series gangliosides or complex gangliosides and expressing only GM3, GM2, GM1 and GD1a were resistant to TeNT, but still sensitive to BoNT/A, B and E, and resistance to all BoNT types was achieved in mouse expressing only GM3 (Kitamura et al. 1999, 2005; Rummel et al. 2007, 2009; Rummel 2013). It is assumed that binding to gangliosides allow specific accumulation of the clostridial neurotoxins to target membranes.

The ganglioside binding sites are located on the H_{CC} domain of the H chain. Whereas TeNT, as well as BoNT/C and D contain two ganglioside binding sites, BoNT/A, B, E, F, and G exhibit only one. Indeed, using co-crystallization of toxin molecules with GT1b and mutation analysis, a ganglioside binding site comprising W1289 and Y1290 as key residues which form the WY loop and an additional one including R1226 were identified in TeNT. The WY loop motif (E(D)…H(K)…SXWY…G) is conserved in BoNT/A, B, E, F, and G and is the functional ganglioside binding site in these BoNT types. In contrast, the WY motif is not conserved in BoNT/C and D. In BoNT/C, a WY loop localized on W1252 and a second ganglioside binding site on the tip of the H_{CC} domain have been identified. The closely related BoNT/DC exhibits a WF loop analogous to the BoNT/C WY loop. BoNT/D shows two binding sites, one located on R1239 similarly to the corresponding TeNT site, and another one at the equivalent position of the WY loop in BoNT/A, B, E, F, G, and TeNT, but with a different conformation, which may explain the low affinity of BoNT/D to gangliosides (Karalewitz et al. 2012; Rummel 2013).

Distribution of BoNT receptor on cell membrane is not fully understood. Considering the lipid BoNT receptor moiety, BoNT receptor is presumed to be localized in distinct lipid microdomains, but the protein moiety (SV2 for BoNT/A, BoNT/E, and BoNT/F, synaptotagmin for BoNT/B and BoNT/G) (Nishiki et al. 1996; Dong et al. 2003, 2006, 2008; Rummel et al. 2004a, 2009; Mahrhold et al. 2006; Fu et al. 2009) supposes a distribution on synaptic vesicle membranes fused at active zones with the plasma membrane, but these proteins have a wider membrane repartition than vesicle membranes. Lipids are heterogeneously distributed in plasma cell membrane and the most well-known lipid organization is the lipid microdomains which are enriched in cholesterol and sphingolipids (Mayor and Rao 2004). Using the C-terminal domain of *Clostridium perfringens* Perfringolysin (PFO), an extensively characterized cholesterol-binding cytolysin (Ramachandran et al. 2002) fused to GFP (GFP-PFO), only a partial colocalization was observed with BoNT/A Hc on neuronal and intestinal cells. This correlates with the absence of colocalization between BoNT/A Hc and probe of glycosylphosphatidylinositol-anchored proteins (GPI-APs), which are also localized in lipid rafts, such as *Clostridium septicum* alpha toxin receptor or the decay-accelerating factor (DAF), as well as with the distribution of BoNT/A receptor (ganglioside + SV2) on soluble fractions of membrane treated with Triton X-100 rather than in detergent-resistant microdomains. In addition, depletion of plasma membrane cholesterol by a high concentration of methyl-β-cyclodextrin (MβCD;15 mM) only partially prevented BoNT/A Hc binding to neuronal and intestinal cells. But incubation of cells first with GFP-PFO for 5 min, prevented further binding of fluorescent BoHT/A Hc

(Couesnon et al. 2009). Thus, BoNT/A receptor seems to have a distinct membrane distribution, since it does not localize directly on cholesterol-enriched microdomains, but in a subset of microdomains from detergent soluble fractions closely connected to the cholesterol-enriched microdomains.

The nature and membrane localization of receptors are critical to drive a specific intracellular ligand trafficking (Lamaze and Johannes 2006b). Indeed, cholera toxin binds to ganglioside GM_1 localized on lipid rafts and is transported until the endoplasmic reticulum, whereas *E. coli* LTIIb (lethal toxin II), which is structurally and functionally related to cholera toxin, binds to GD_{1a}, a ganglioside not associated with lipid rafts, and it is not routed to the endoplasmic reticulum (Fujinaga et al. 2003). Lipid rafts seem not to be required for the BoNT/A entry into neuronal cells. Despite BoNT binding was greatly decreased to cells deficient in ganglioside synthesis (Kozaki et al. 1998; Yowler et al. 2002; Rummel et al. 2007; Strotmeier et al. 2014), disruption of lipid rafts with MβCD or filipin does not prevent, but on the contrary enhances BoNT/A activity on N2a cells (Petro et al. 2006). In addition, knockout mice lacking b-series gangliosides show a similar sensitivity to BoNT/A as wild type mice, indicating that b-series gangliosides may not be essential in the initial steps of BoNT/A intoxication (Kitamura et al. 2005). This suggests that BoNT receptors containing gangliosides GD_{1b}/ GT_{1b} are not restricted to lipid rafts but have a wider distribution, possibly on distinct microdomain structures within the cell membrane. Interestingly, such a heterogeneous localization on membrane is supported by the finding that the same glycosphingolipid is distributed on distinct and contiguous membrane microdomains according to its concentration, low or high (Sharma et al. 2003a). But the accurate localization of BoNT receptor on neuronal and intestinal cell membranes remains to be determined.

Whereas all BoNT types and TeNT bind to gangliosides, the interaction with the protein receptor moiety is BoNT or TeNT type-dependent and strengthens the specific recognition of the target neuronal cells (see below). Two classes of proteins mainly specific of neuronal cells, synaptotagmin and synaptic vesicle glycoprotein 2 (SV2), have been identified as BoNT receptors (Table 1). Synaptotagmin binding site has been localized at the tip of Hcc of BoNT/B, G, and DC in a pocket close to the ganglioside binding site. Interestingly, this pocket corresponds to the second carbohydrate binding site of TeNT (Rummel et al. 2007; Peng et al. 2012; Berntsson et al. 2013a, b; Rummel 2013; Willjes et al. 2013). BoNT/A, E, F, and possibly D, use SV2 isoforms as the receptor protein part. SV2 is a transmembrane glycoprotein with one large luminal domain (loop L4) which is the binding domain of BoNTs. Glycosylation of SV2 is essential for interaction with BoNTs. Thereby, BoNT/A binds to glycosylated SV2C L4 by recognition of both the protein part and an N-linked glycan, and BoNT/E interacts with glycosylated SV2A or SV2B (Dong et al. 2006, 2008; Mahrhold et al. 2006; Yao et al. 2016). SV2 binding site has been localized in a distinct area to that of synaptotagmin in BoNT/B. Indeed, the interface of H_{CN} and H_{CC} is crucial for the interaction of BoNT/A and BoNT/E with SV2 (Benoit et al. 2014; Strotmeier et al. 2014; Rummel 2015; Yao et al. 2016). In addition, the integrity of the H_{CN} and H_{CC} interface is required to induce efficient neutralizing antibodies against BoNT/A (Tavallaie et al. 2004). In contrast, TeNT

uses a different type of protein receptor. TeNT interacts with the extracellular matrix proteins nidogen-1 and -2 (Bercsenyi et al. 2014) which likely concentrate the toxin molecules for an efficient binding to cell surface receptor formed of gangliosides and a yet uncharacterized GPI-anchored protein (Schiavo et al. 1991; Herreros et al. 2001; Munro et al. 2001). SV2A and SV2B have been proposed to facilitate TeNT entry into neurons (Yeh et al. 2011), but these results have not been confirmed (Blum et al. 2012, 2014b).

6.2 Endocytosis into Neuronal Cells

Recognition of two distinct receptors results in high and specific binding to target membranes. By binding to gangliosides, which are abundant on neuronal cell membranes, BoNTs and TeNT are selectively concentrated on neuronal cell surface. Then, the interaction with the protein receptors, which are mainly localized in vesicle synaptic membrane, trap the clostridial neurotoxins into endocytic vesicles. SV2 and synaptotagmin are supposed to be transiently exposed to the external medium during neurotransmitter exocytosis (Lam et al. 2015a). However, synaptotagmin and SV proteins are localized not only in vesicle membranes and release sites, but also extend in plasma membrane beyond the release sites (Fernandez-Alfonso et al. 2006; Poulain et al. 2008). Therefore, membrane areas containing both gangliosides and synaptotagmin or SV2 proteins constitute high-affinity binding sites for clostridial neurotoxins and subsequent endocytosis.

Intravesicular domains of SV2 and synaptotagmin are exposed on the surface of neurons when the synaptic vesicles fuse with the plasma membrane, and thus facilitate the entry of clostridial neurotoxins into neurons. Indeed, it has been evidenced that BoNTs use the recycling pathway of synaptic vesicles as a Trojan horse for their entry into neurons. Thus, the enhancement of the synaptic activity induced for example by K^+ depolarization in the presence of Ca^{++} increases the uptake of BoNTs (Keller et al. 2004; Verderio et al. 2007; Poulain et al. 2008). However, BoNTs do not exclusively use the synaptic vesicle recycling and can also enter via the basal endocytosis entry, which is also mediated by toxin binding to ganglioside and SV2/synaptotagmin receptors. Thus, BoNTs are able to enter neurons under resting conditions, and the basal entry can represent more than half of the total toxin entry into stimulated motor neurons (Verderio et al. 2007; Meng et al. 2013). In addition, it has been shown that different BoNT types can enter neuronal cells already exposed to another BoNT type for a long period (48 h) thus impairing synaptic vesicle recycling. Moreover, albeit slightly reduced BoNT entry, BoNT intoxicated neurons are still capable of taking up further BoNT in the absence of depolarization again supporting a synaptic vesicle recycling-independent entry (Pellett et al. 2015).

The BoNT endocytosis machinery in neuronal cells via the synaptic vesicle recycling exploits the clathrin pathway involving dynamin, adaptor protein

complex-2 (AP2), amphiphysin, and sorting nexin 9 (Neale et al. 1999; Granseth et al. 2006; Shin et al. 2007; Couesnon et al. 2010; Harper et al. 2011; Meng et al. 2013; Pellett et al. 2015). But BoNTs can use different isoforms of these molecules according to the neuronal cell types. Indeed, in cerebellar granule neurons which mainly release glutamate from small clear synaptic vesicles, the recycling of these vesicles and subsequent BoNT entry are predominantly dynamin-1-dependent, whereas in sensory fibers like trigeminal ganglionic neurons, which mainly secrete neuropeptides from large dense-core vesicles, the endocytic machinery uses preferentially dynamin-2 and to a lower extent dynamin-3 (Meng et al. 2013). In contrast, the basal uptake of BoNT in both neuron types has been found to be dynamin-independent (Meng et al. 2013).

Differences in cell entry also exist between BoNT types and subtypes. BoNT/A requires a longer time than BoNT/E after internalization to cleave the intracellular substrate, which reflects a differential translocation process between the two toxinotypes (Keller et al. 2004). A faster intoxication process of BoNT/A2 versus BoNT/A1 has also been evidenced. But the entry or translocation mechanism difference between BoNT/A2 and BoNT/A1 has not been elucidated (Pier et al. 2011).

BoNT endocytosis in neuronal cells leads to an acidified compartment, which is required for the translocation of the L chain into the cytosol. Acidification of the endosomal lumen mediated by the H^+-vATPase pump triggers the insertion of the H_N domain into the membrane and unfolding of L chain. The H_N domain contains in its N-terminal part an unstructured region, called the belt region, and two unusually long twisted α-helices, which are reminiscent of α-helical hairpin of some colicins or viral fusion proteins (review in Poulain et al. 2008). The belt region is an unstructured loop which wraps LC. At the acidic pH of endosome (pH 5.3), H_C inserts into the endosomal membrane and forms small conductance channels (65 pS in 200 mM NaCl, and estimated inner diameter of 15–24 Å) (Montal 2010; Fisher 2013). It has been found that the pH dependence results from the cooperation between the receptor-binding domain (H_C) and the translocation domain (H_N), since H_N alone is able to form channel at acidic and neutral pH (Fischer and Montal 2013). The belt region seems to play an important role in the pH regulation. Indeed, the numerous negatively charged residues of the belt region limit the electrostatic interaction of H_N with the membrane. At acidic pH, the negative charges become protonated, notably the His residues which are protonated at pH 5, thus reducing the electrostatic repulsion (Galloux et al. 2008). The mode of passage of the L chain through the endosomal membrane remains unclear. The unfold L chain at acidic pH seems not to be able to pass through the small H_N channels, unless several translocation domains may cooperate to form larger channels. But, the H_N channels can be a dynamic structure cycling between various conformation states. The fact that during the L chain translocation the Na^+ conductance progressively increases supports a passage through the H_N channels. Another possibility is a chaperone activity between H_N and L chain including a partial structure rearrangement (molten globule state) facilitating the exposition of hydrophobic helices and their subsequent insertion into the membrane. However, no conformational change of isolated

H_N has been detected. The translocation of L chain requires a pH gradient, redox gradient and electrical transmembrane potential gradient between the endosome and cytosol. The pH gradient is necessary for H_N channel formation, L chain unfolding and provides, as well as the electrical transmembrane potential gradient, a driving force for the translocation. It was supposed that acidification of the vesicle lumen triggers a conformational change of the neurotoxin facilitating the subsequent translocation of the L chain into the cytosol. However, BoNT/A L chain does not show major conformational change upon acidic pH, but has the property to interact and permeabilize anionic lipid membrane at acidic pH. This property is prevented when L chain is linked to H_N. Thus H_N acts as chaperone protein which stabilizes the L chain (Galloux et al. 2008; Montal 2009; Araye et al. 2016). The disulfide bridge between L and H chains has to be maintained to initiate the translocation process, which supposes oxidizing conditions in the endosomal compartment. Then the L chain is released into the cytosol in reducing conditions, which dissociate the disulfide bound. The thioredoxin reductase–thioredoxin system has been found to be involved in this process (Galloux et al. 2008; Poulain et al. 2008; Montal 2010; Fischer and Montal 2013; Beard 2014; Pirazzini et al. 2015a, b).

7 Concluding Remarks

BoNTs and TeNT are highly potent neurotoxins which inhibit the evoked release of neurotransmitter mainly at neuromuscular junctions and central inhibitory interneurons, respectively. Their intracellular activity consists of a proteolytic activity towards specific proteins of the exocytose machinery of neurotransmitters. Enzymatic activity of BoNTs and TeNT is in the range of other metalloproteases (Schiavo et al. 1993). Therefore, the extreme potency of BoNTs and TeNT is not based on a super enzymatic activity but rather on an extreme efficient targeting to critical cells and specific substrates. Indeed, TeNT uses a sophisticated pathway from its local site of synthesis, a wound, to specific central interneurons via axonal retrograde transport. The highly specific binding to nerve endings contributes to concentrate the toxin molecules produced locally to neurons which are used as vehicle for delivery to target central neurons. Moreover, inside the target cells, the L chain specifically interacts with a unique substrate. The double receptor recognition on cell surface, gangliosides and membrane protein receptor, by the H chain C-terminal part H_C and the specific interaction of L chain with its intracellular substrate avoid the dispersion of toxin molecules in non-target cells, notably cells which could inactivate and remove the toxin, and ensure a strict toxin routing and concentration on the final substrate. Similarly, the extreme potency of BoNTs is based on an efficient trafficking to neuromuscular junctions which is still incompletely understood. BoNTs are most often acquired from the environment by oral or respiratory route and are able to cross epithelial and endothelial barriers by transcytosis, but with a low efficiency. Then, BoNTs efficiently enter neuromuscular junctions and specifically cleave SNARE proteins. Recognition of gangliosides

leads to BoNT molecule concentration on transport cells and binding to both gangliosides and specific protein receptor allows toxin uptake into target neuronal endings and subsequent interaction with the intracellular target. However, the precise pathway of BoNT dissemination by using transport cells like TeNT or through the extracellular compartment or blood circulation remains debatable.

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Receptors and Binding Structures for *Clostridium difficile* Toxins A and B

Ralf Gerhard

Abstract Two characteristics of toxins A and B from *C. difficile* (TcdA, TcdB) are important for the understanding of the pathogenic effect of these homologous toxins. First, these toxins are huge single-chain but multidomain proteins that display their action intracellularly within the cytosol of host cells. And second, albeit various cell types highly differ in their sensitivity toward these toxins, no toxin-resistant cell type has been described yet. Investigation of receptor-mediated uptake of these toxins is very ambitious. It demands discrimination between cell surface binding, interaction with more than one functional receptor responsible for uptake as well as other functional receptors that recognize bacterial pathogens and are not necessarily related with endocytosis. The current understanding of a complex uptake process is that TcdB interacts with at least two facultative receptors that mediate entry into host cells by redundant endocytotic pathways. Although both homologous toxins do obviously not share the same receptors, this principle of redundant binding domains found for TcdB does also account for TcdA.

Contents

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1 Receptor-Binding Domains of TcdA and TcdB

1.1 A/B-Type Structure of Toxins

TcdA and TcdB belong to the group of large clostridial glucosyltransferases. Homologous toxins are also expressed by *C. sordellii*, which produce the lethal toxin (TcsL) and the hemorrhagic toxin (TcsH), by *C. novyi*, which produces the alpha-toxin (TcnA), or by *C. perfringens*, producing the large enterotoxin TpeL. All these glucosyltransferases share the same principle of a large protein that is subdivided into two functional components: an enzymatically active pathogenic factor (A-subunit) and the binding component (B-subunit) that facilitates the translocation of the A-subunit into the cytosol of target cells (Just and Gerhard 2004). They also share the same molecular mode of action, which is glucosylation of small monomeric GTPases, predominantly of the Rho and Ras family (Just et al. 1995b). Each toxin has been evolved as variations of this A/B-type structure specialized for the needs to efficiently propagate the respective microbe. Most variations are found within the B-subunit that is in charge of binding to cell surface, interaction with one or more receptors to initiate endocytosis, pH-dependent conformational changes to insert into the vesicle membrane for pore formation, and also in charge of translocation and intracellular release of the A-subunit (Pruitt and Lacy 2012). The more subdivided ABCD model is a demonstrative description of the toxins that dissects functional elements for pathogenic enzyme activity (A), for toxin binding (B), for autoproteolytic cleavage (C), and for intracellular delivery (D) (Fig. 1).

A closer look at cell-binding properties of TcdA and TcdB, however, and the use of deletion mutants and chimeras of these toxins revealed that this model has to be improved to include further specific subdomains for the needs of the respective toxin. The complexity and dynamic of the binding and uptake process even increase when the required conformation, stability, and flexibility of the toxins in a deleterious milieu in the colonic lumen are additionally taken into account. This flexibility is in contrast to a stable conformation that protects the protein from inactivation by premature cleavage. In the following, the three functional elements of the B-subunit will each be presented in more detail.

1.2 Functional Characterization of the Receptor-Binding Domains

Historically, the very C-terminal part of TcdA was first characterized as a receptor-binding domain due to its homology to streptococcal glucosyltransferases (von Eichel-Streiber and Sauerborn 1990). This region consists of repetitive elements where long and short repeats are combined. These combined repetitive oligopeptides (CROPs) are hydrophilic and rather immunogenic. Since antibodies that recognize the CROPs could inhibit TcdA-induced haemeagglutination, this

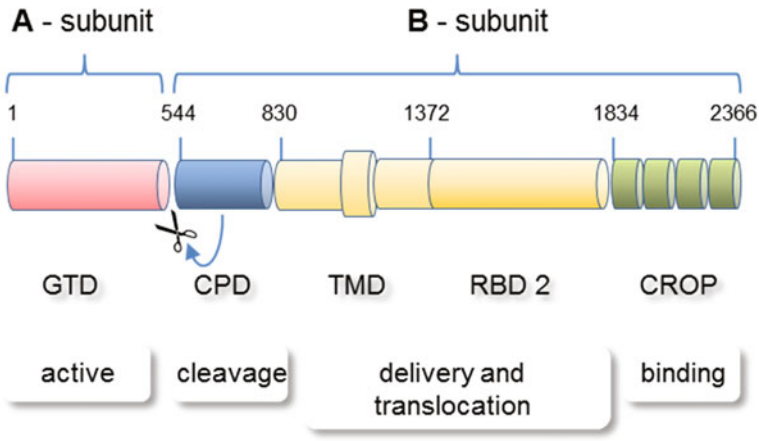


Fig. 1 TcdA and TcdB are toxins of an *A*/*B*-type structure commonly found for large protein toxins that act intracellularly. The *A-subunit* represents the pathogenic enzymatically active subunit, which is a glucosyltransferase in case of TcdA and TcdB. The *B-subunit* stands for "binding" and facilitates binding and uptake into host cells. The *B-subunit* can be subdivided into further elements that are in charge of cell surface binding and autoproteolytic *cleavage* after *delivery* of the *A-subunit* into the cytosol. This extended model is therefore named ABCD model. Beside the *A-subunit* which is represented by the glucosyltransferase domain (GTD), the *B-subunit* comprises defined functionally and structurally separated domains which are the cysteine protease domain (CPD), the transmembrane domain including the hydrophobic region (TMD), the intermediate domain harboring at least one receptor-binding domain (RBD2), and the C-terminal combined repetitive oligopeptides (CROP). Shown is the model of TcdB with numbers that roughly indicate amino acid position where the domains begin, except the exact cleavage site between aa 543/544 in TcdB

region was hypothesized to bind to cell surface carbohydrate structures and was considered as the receptor-binding domain (Lyerly et al. 1986). A couple of years later, competition assays with TcdA gave indirect proof that the region N-terminally adjacent to the CROPs supposedly also contributes to receptor binding (Frisch et al. 2003). Furthermore, the identification of TpeL from *C. perfringens* as a large clostridial glucosyltransferase that lacks the CROPs (Amimoto et al. 2007) proved that the CROPs are not essential for uptake of this particular toxin. Together, these findings raised the question whether the CROPs are dispensable for the function of TcdA and TcdB as well. In 2003, the *B. megaterium* expression system was introduced for large-scale expression of full length TcdA, which made the gene accessible for genetic engineering of point and deletion mutants of TcdA (Burger et al. 2003; Gerhard et al. 2005) and TcdB (Yang et al. 2008). Accordingly, recombinant deletion mutants of TcdA and TcdB without CROPs were generated and, in fact, found to be cytopathic in cell culture assays. These findings changed the paradigm of CROPs being the essential receptor-binding domain (Olling et al. 2011). Further truncations of TcdB revealed that the minimum N-terminal part that exhibits pathogenic function comprises aa 1–1495 (Genisyuerek et al. 2011; Manse and Baldwin 2015). Obviously, TcdA and TcdB have two distinct functional

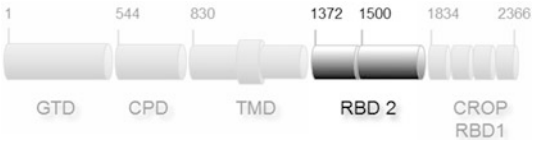
regions, i.e., the CROPs (aa 1847–2710 and 1852–2366, respectively) and the region N-terminally adjacent to the CROPs (approximately aa 1400–1850), which independently serve as receptor-binding domains.

1.2.1 The Combined Repetitive Oligopeptides

In 1986, Krivan and coworkers characterized the binding of TcdA to cell surface structures (Krivan et al. 1986). TcdA was found to bind to the carbohydrate structures Gal-α-(1,3)-Gal-β-(1,4)-GlcNAc (Krivan et al. 1986). This carbohydrate structure is highly abundant on calf thyroglobulin and rabbit erythrocytes but is not present in humans (Tucker and Wilkins 1991). Lectin-like binding to this structure on rabbit erythrocytes leads to haemagglutination as one characteristic feature of TcdA. The C-terminal CROPs were first characterized as the receptor-binding domain of TcdA since antibodies that were raised against the recombinantly expressed CROP domain blocked haemagglutination (Clark et al. 1987). This lectin-like binding to carbohydrate structures was also assumed for the CROPs of TcdB which have a highly homologue structure. However, only TcdA specifically binds to the glycoprotein thyroglobulin for which reason immobilized calf thyroglobulin is used for specific purification of TcdA from *C. difficile* supernatants (Krivan and Wilkins 1987). Lectin-like binding of TcdA is thermo-sensitive, meaning that toxin binds to thyroglobulin at 4 °C and elutes at 37 °C. The complete TcdA and TcdB genes were sequenced in 1990 (Dove et al. 1990; von Eichel-Streiber et al. 1990). Recently, Gly-1832 in TcdA and Gly-1834 in TcdB were suggested to be the first amino acid of a short repeat and, thus, the beginning of the CROP domain (Orth et al. 2014). The amino acid sequences revealed the precise architecture of TcdA and TcdB CROPs. They consist of 30–38 and 19–24 repeats, respectively, that are combined to repetitive oligopeptides (CROP). TcdA exhibits seven elements each comprising one long (30 aa) and 4–6 short (15–21 aa) repeats. The TcdB CROPs show four elements of one long and 4–6 short repeats. The crystal structure of the very C-terminal fragment of the TcdA CROPs revealed a β-solenoid consisting of 4–5 short repeats and one long repeat. The long repeat which is flanked by 2–3 short repeats on either side induces a kink (∼30°) of the formed β-solenoid by disrupting regular arrangement within the rod-like structure build by the short repeats (Ho et al. 2005; Greco et al. 2006; Pruitt and Lacy 2012). Each long repeat along with the adjacent short repeat represents an epitope for binding of one carbohydrate moiety (Greco et al. 2006; Pruitt and Lacy 2012).

Thus, it was assumed that the CROP of TcdA constitutes a multivalent-binding structure, which was indeed shown by Dingle and coworkers (Dingle et al. 2008). Likewise, the CROPs of TcdB show angles within the long repeats; however, the horseshoe-like TcdB CROP domain is shorter and more rigid than the S-shaped TcdA CROP domain. Furthermore, the conserved amino acids that contribute to glycan binding (Glu2623, Gln2638, Ser2660, and Lys2661) characterized by Greco and coworkers are not present in TcdB (Greco et al. 2006). Nevertheless, isolated repeats of TcdB CROP were found to bind to Gal-α-(1,3)-Gal-β-(1,4)-GlcNAc, showing that the principle of glycan binding is also true for TcdB (Dingle et al. 2008; El-Hawiet et al. 2011). At least in TcdA, the CROP has additional function in stabilizing conformation of the protein. By intramolecular association with the N-terminal part of the toxin, the CROPs prevent premature extracellular autoprocessing (Olling et al. 2014; Zhang et al. 2015; Chumbler et al. 2012). This feature has not been found for TcdB CROP and neither have carbohydrate structures been identified that show high affinity to this domain. Despite lack of a known domain-specific receptor, the CROP domain, nevertheless, increases potency of TcdB compared to TcdBΔCROP (Olling et al. 2011).

1.2.2 Additional Receptor-Binding Domains



Large clostridial glucosyltransferases that lack the C-terminal CROP are still pathogenic. The first naturally occurring large clostridial glucosyltransferase without CROP domain in its native form was the large enterotoxin from *Clostridium perfringens*, TpeL (Amimoto et al. 2007). *C. perfringens* produces several toxins and plays a major role in livestock infections where it induces necrotic enteritis, but also plays a role in epidemic gastrointestinal outbreaks in humans. TpeL was the latest toxin described for specific *C. perfringens* strains and shows high homology to the other large clostridial glucosyltransferases. Meanwhile, the LDL receptor-related protein 1 (LRP1) was identified as receptor for TpeL, and the finding was taken as proof of principle for a two receptor model for large clostridial glucosyltransferases. This revised model now included an independent receptor-binding domain N-terminally located of the conserved CROP within the amino acid region 1335–1779 (Schorch et al. 2014). The two receptor model was assumed for TcdA and TcdB long before TpeL was described. A first hint was given by the application of various C-terminally truncated TcdB mutants in cytotoxicity assays already in 1994 (Barroso et al. 1994). Since the standard *E. coli* expression system is not suitable for preparative toxin expression, this fundamental study was performed with *E. coli* lysates. Due to limited toxin yield,

the cytotoxic titers were used for evaluation. Later on, competition experiments using the CROP domain and the N-terminally extended CROP domain of TcdA suggested contribution of a CROP-independent region in TcdA in receptor binding (Frisch et al. 2003). These findings were substantiated in the following by application of CROP deletion mutants of TcdA and TcdB in cell culture assays (Olling et al. 2011). We found that hamster ovary cells (CHO) cells, unlike murine NIH3T3 fibroblasts or human HT-29 cells, were equally sensitive to TcdA and TcdA 1–1874, lacking the CROPs. This showed that CROP-dependent binding of TcdA is not essential for pathogenic effect and might not be relevant for binding to some cells.

The region located N-terminally to the CROP domain was further investigated by successive C-terminal truncation as it was done before by Barroso and coworkers, showing that the minimal cytopathic fragment of TcdB comprises the amino acids 1–1493 whereby the region 1372–1493 was essential for uptake (Manse and Baldwin 2015) (Fig. 2). Specific deletion of the 97 amino acids directly N-terminally adjacent to the CROPs, however, was also reported to completely inactivate TcdB (Zhang et al. 2013) and is therefore in contradiction with the

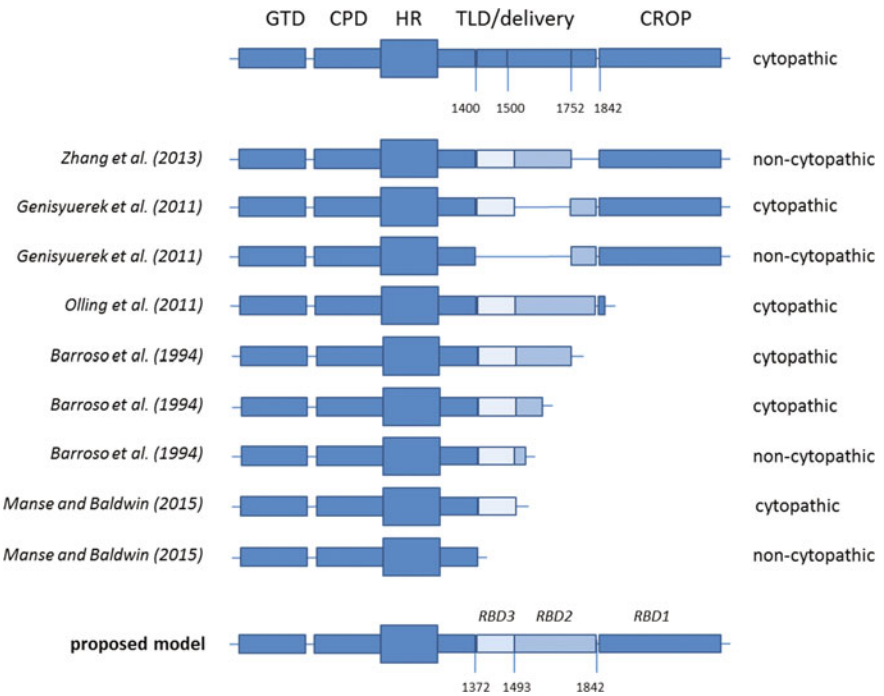


Fig. 2 Summary of C-terminal and intermediate deletion mutants of TcdB used in different studies. Note that experiments by Barroso et al. are based on cytotoxic effects of *E. coli* lysates with unknown but limited toxin concentrations. The modular binding motif postulated by Manse and Baldwin comprises three different receptor-binding regions (RBD1–3)

findings by Manse and Baldwin. Further C-terminal truncations leading to toxin fragments of less than about 1400 amino acids completely abrogated cytotoxicity, even when TcdB was applied at micromolar concentrations. By their systematic approach, Manse and Baldwin dissected three different receptor-binding regions in TcdB. The authors suggested an archaic binding domain in the region of 1372–1493. Interestingly, co-immunoprecipitation experiments showed that this region is essentially involved in binding to the poliovirus receptor-like protein-3 (PVRL3), identified by LaFrance and coworkers (LaFrance et al. 2015). TcdB with only this proposed binding domain showed identical low potency to three different cell types from three different species. A further binding domain was postulated for the region 1494–1848. A TcdB fragment additionally encompassing this domain showed higher potency than TcdB 1–1494, albeit with variations regarding origin of cells. The region 1500–2366 was reported to bind to the chondroitin sulfate proteoglycan-4 (CSPG4) identified by Yuan and coworkers (Yuan et al. 2015). The third module is the classical receptor-binding domain termed CROP (aa 1832–2366). Manse and Baldwin therefore proposed modular-binding motifs for TcdB. Binding to cells via different domains is assumed to happen independently. Deletion of either the CROPs or the region of amino acids 1500–1750 reduces the potency of TcdB, but does not lead to complete functional inactivation, since deletion mutants lacking either single domain are pathogenic. Even an intermediate deletion mutant showed that amino acids 1501–1753 are dispensable for cytopathic effects (Genisyuerek et al. 2011). Comparison of different studies dealing with domain function analyses reveals that there is not a general agreement where the CROP domain begins, leading to toxin fragments that end or begin somewhere in the amino acid region between 1832 and 1852. In addition, a unique restriction site in *tcdB orf* for Spe*I* endonuclease (base pair 5260; aa 1753) is also exploited for cloning of the C-terminal alterations or CROP domain (Zemljic et al. 2010; Genisyuerek et al. 2011). Considering a relevant epitope for receptor binding or protein folding, those studies have to be interpreted very carefully.

The delivery domain of TcdA has not been investigated in more detail. Deletion of the CROP domain still renders TcdA (aa 1-1847) cytopathic (Olling et al. 2014), whereas the fragment TcdA 1-1065 is completely inactive in cell culture experiments (Teichert et al. 2006). From what is known for TcdA, a two or even three receptor model that has been suggested for TcdB can be assumed for TcdA as well. Interestingly, the three-dimensional structure of TcdA was recently described and can supposedly be taken as a template for domain organization in TcdB (Chumbler et al. 2016). If this is true, a much closer proximity of different receptor-binding regions, as the two-dimensional scheme suggests, can be extrapolated. Even the cholesterol-binding region upstream of the hydrophobic region seems to be associated with the amino acid regions that were found to contribute to receptor binding in TcdB.

In summary, three different binding regions can be stated for TcdB: RBD1 (1852–2366) is historically represented by the CROPs. In contrast to the CROPs of TcdA, the complementary high-affinity glycan binding structure for TcdB CROPs on target cell surface has not been identified yet. The RBD2 (1372–1848) is

N-terminal of the CROPs and might be subdivided into RBD2 and RBD3 since PVRL3 binds to a different region (aa 1372–1495) than CSPG4 (Manse and Baldwin 2015). In contrast to what is known for TcdB, no cell surface receptor was shown to specifically interact with the corresponding region of TcdA. It has to be shown in future whether postulated RBD2 and RBD3 in TcdB are independent domains or if they act cooperatively. Although a dual-receptor model can be claimed for TcdA, it is unclear whether this postulated tripartite receptor binding is also true for TcdA.

2 Functional Receptors for Binding and Uptake

2.1 Carbohydrates and Lipid Structures

As mentioned above and based on several reports of recent years, it is common sense that TcdA and TcdB bind to more than only one specific receptor. Whereas glycans were identified as high-affinity binding structures for TcdA, specific protein receptors were described for TcdB. It is not clear whether glycans can function as sole receptors for either toxin, independent of the nature of protein or lipid that displays the specific glycan moiety. Likewise, it is vice versa not clear whether the protein receptors that were found for TcdB have to be glycosylated for high affinity. From what is known for TcdA and TcdB, one can assume that there is a kind of task sharing for CROPs and the receptor-binding region N-terminally adjacent to the CROPs: Whereas the interaction of CROPs and glycans might provide a reservoir of cell surface bound toxin, the interaction of further receptor-binding domain with specific receptor facilitates endocytosis and translocation into the cytosol of the target cell. Yet, a variety of glycans have been shown to bind to TcdA or part of the TcdA CROPs with high affinity. Twenty years after first description of Gal α1-3 Gal β1-4 GlcNAc functioning as binding structure for TcdA, the use of glycan arrays by the consortium for functional glycomics was employed for a systematic approach to define carbohydrate structures binding to either toxin. A list of glycans that bind to TcdA with high affinity is shown in Table 1. All these carbohydrate structures bear the core structure Gal β1-4 GlcNAc (N-acetyllactosamine) where GlcNAc moiety can be un-fucosylated or fucosylated (Gal β1-4 (Fuc α1-3) GlcNAc) as in the Lewis-x/Lex-3 structure. It is noteworthy that the high-affinity binding glycans identified in glycan arrays do not necessarily reflect physiological conditions or cell-binding assays.

For example, the erythrocytes blood group B structure Gal α-1,3 (Fuc α1-2) Gal β1-4 GlcNAc bound to TcdA in glycan arrays, whereas Krivan and coworkers showed that only the un-fucosylated glycan binds to TcdA (Krivan et al. 1986). Obviously, other parameters such as temperature or buffer are also important. It is striking that binding of TcdA to the proteoglycan thyroglobulin only happens at 4 °C and in phosphate buffer (Krivan and Wilkins 1987). Differences in temperature-dependent binding to rabbit erythrocytes and hamster brush border membranes

Table 1 Summary of glycans with high affinity to TcdA identified in glycan arrays (www.functionalglycomics.org)

Glycans binding to TcdA	
1	Gal α1-3 **Gal β1-4 GlcNAc**
2	Gal α1-3 **Gal β1-4(Fuc α1-3) GlcNAc**
3	Gal α1-3(Fuc α1-2) **Gal β1-4 GlcNAc**
4	GlcNAc α1-3 **Gal β1-4 GlcNAc**
5	GalNAc α1-3(Fuc α1-2) **Gal β 1-4 GlcNAc**
6	GalNAc α1-3(Fuc α1-2) **Gal β 1-4(Fuc α1-3) GlcNAc**
7	Gal β 1-3 GlcNAc β 1-3 **Gal β 1-4(Fuc α1-3) GlcNAc**
8	Gal β 1-4(Fuc α1-3) GlcNAc β 1-4 **Gal β 1-4(Fuc α1-3) GlcNAc**
9	NeuGc α2-6 **Gal β 1-4 GlcNAc**
10	NeuAc α2-3 Gal β 1-3 GlcNAc β 1-3 **Gal β 1-4 GlcNAc**
11	NeuAc α2-3 Gal β 1-3(Fuc α1-4) GlcNAc β 1-3 **Gal β 1-4(Fuc α1-3) GlcNAc**
12	Fuc α1-2(Gal β 1-3 GalNAc α1-3) **Gal β 1-4 GlcNAc**
13	Fuc α1-2(GalNAc α1-3) Gal β 1-4 GlcNAc β 1-3 **Gal β 1-4 GlcNAc**

The core binding structure found in all glycans is shown in bold

(Krivan et al. 1986) illustrate the challenge of detailed binding studies. Binding of TcdA to this specific glycan is not extraordinary, since the core structure Gal β1-4 GlcNAc is often found to be associated with binding to pathogenic factors or microbes (Gillece-Castro et al. 1991; Inoue et al. 2001; Grange et al. 2002; Thomas and Brooks 2004; Bavington and Page 2005).

Despite the proof that isolated stretches of the TcdB CROP are able to bind carbohydrates, published data of glycan arrays do not show relevant hits for the complete CROP domain. At least none of the glycans found for TcdA binds with high affinity to the C-terminal half of TcdB CROP that was used in the array (www.functionalglycomics.org, 03.29.2016).

Translocation of the GTD of TcdA and TcdB requires pore formation which is built by the hydrophobic region of the toxins (von Eichel-Streiber et al. 1992; Pruitt and Lacy 2012). Pore formation into the plasma membrane depends on cholesterol binding (Giesemann et al. 2006). Two glutamate residues (E970, E976) within the hydrophobic region were found to be essential for pore forming (Genisyuerek et al. 2011). It can be assumed that the region aa 830–990 which is in charge of pore formation harbors the cholesterol binding motif. In fact, very recently a mirror code for cholesterol–protein interaction was reported (Fantini et al. 2016). This mirror code L/V-X_{1-5}-(Y)-X_{1-5}-K/R and K/R-X_{1-5}-(Y/F)-X_{1-5}-L/V can be found in TcdB, e.g., $_{933}$**K**GTI**F**DT**V**$_{940}$ and $_{968}$**L**IE**Y**NSS**K**$_{975,}$ and can also be found in TcdA in the region 830–990. Although binding to cholesterol is essential for toxin effect, cholesterol itself is not a functional receptor, according to the proposed model shown in Fig. 2, showing that deletion mutants smaller than amino acids 1–1400 are not functional in cell-based assays.

Beside glycans and cholesterol, phospholipids have also been described as further nonprotein structures that bind to large glucosltransferases (Mesmin et al.

2004; Varela Chavez et al. 2015). Binding to phospholipids occurs via the very N-terminal part of toxins (aa 1–93). This feature is predominantly found in the lethal toxin (TcsL) from *C. sordellii*, as well as in TcdB, but is also found in TcdA and other large clostridial glucosyltransferases, although to a lesser extent. The negatively charged phospholipids phosphatidylserine (PS) and phosphatic acid are specifically bound by TcsL and TcdB. PS is highly abundant in the inner leaflet of plasma membranes. Association of toxins with PS directs the translocated and released GTD of toxins to the membrane compartment, where substrate GTPases are abundant. In addition to the localization of the GTD, binding to PS also facilitates the glucosylation reaction (Varela Chavez et al. 2015). Yet, binding to PS has only been recognized as contribution to intracellular glucosylation events. An interesting question is whether the affinity to PS also contributes to extracellular binding. Although PS is almost exclusively located at the cytoplasmic leaflet of plasma membranes, it can also be found in the exoplasmic leaflet of apoptotic cells, coagulating platelets, exosomes, and also on the surface of phagocytes (Reutelingsperger and van Heerde 1997; Krysko et al. 2008; Thery et al. 2009; Mapes et al. 2012). In particular, the latter ones—exosomes and phagocytic cells—are of interest and should be considered as putative vehicles for toxin uptake or as preferred target cell. Up to now, PS has not been considered as extracellular binding structure but should be discussed in search for further receptors of large clostridial glucosyltransferases. Since toxin fragments of TcdA and TcdB that are smaller than 1400 amino acids are not cytotoxic, PS cannot be considered as a sole functional receptor but might instead contribute to surface binding.

2.2 Receptors for TcdA

To date, two receptors were described for TcdA. Twenty years ago, sucrose-isomaltase (SI) of rabbit intestinal cells was found to function as protein receptor for TcdA (Pothoulakis et al. 1996). The appearance of SI correlated with the intestinal damage induced by TcdA in newborn and adult rabbits (Eglow et al. 1992). Newborn rabbits which lack SI for the first few days of lifetime were relative resistant to TcdA-induced mucosal damage in the small intestine. The fact that SI is not expressed in the human colonic mucosa weakened this finding and started a discussion about more than one specific receptor. It can be assumed that SI as a glycoprotein binds TcdA preferentially via glycans. This was indeed shown by Pothoulakis and coworkers by treatment of SI with alpha-galactosidase, which led to reduced binding of TcdA (Pothoulakis et al. 1996). In 2008, the same group reported about a further glycoprotein (gp96) serving as receptor for TcdA, which is present in the human colonic mucosa (Na et al. 2008). Again, it is unclear whether toxin binding to gp96 is mediated by CROP–glycan interaction or by specific protein–protein interaction. In vitro precipitation assay showed that binding of TcdA was not temperature-sensitive, being the same at 4 and 37 °C. Thus, gp96 showed different binding characteristics compared to SI. Specific knockdown of gp96 by siRNA

reduced sensitivity of cells by 60 % in cell rounding assay. Complete resistance of cells to TcdA could not be achieved, either due to technical limitations or due redundant receptors. Interestingly, none of the groups that identified protein receptors for TpeL or TcdB by using either a haploid cell line, shRNAmir library or the CRISPR/Cas9 system, respectively, reported about a (alternative) receptor for TcdA so far (Schorch et al. 2014; LaFrance et al. 2015; Yuan et al. 2015).

2.3 Receptors for TcdB

The unsuccessful search for specific TcdB receptors lasted more than two decades. Eventually, the use of shRNAmir library led to the identification of chondroitin sulfate proteoglycan-4 (CSPG4) as receptor candidate (Yuan et al. 2015). It was the most promising CRISPR/Cas9 technique that brought breakthrough by specific gene silencing to generate toxin-insensitive cells and also *NG2*$^{-/-}$ mice (Yuan et al. 2015). NG2 (neuronal/glial 2) is the rat homologue of human CSPG4. Silencing of the *CSPG4* gene reduced sensitivity of HeLa cells by 50 %. Yuan coworkers characterized the extracellular N-terminal part of CSPG4 (aa 30–640) as toxin-binding domain in precipitation experiments. CSPG4/NG2 was previously named HMW-MAA because of being a high molecular weight melanoma-associated antigen or *Melanoma Cell Surface Protein (*MCSP*)*. CSPG4/NG2 is expressed in tissues of brain, gastrointestinal tract, endocrine organs, and >50 cell types (Levine and Nishiyama 1996; Nicolosi et al. 2015). This cell surface protein is characteristic for progenitor cells of epithelial and mesodermal origin (Russell et al. 2013). The ectodomain of CSPG4 binds to type V and VI collagens, multi-PDZ domain proteins, and others (Stallcup et al. 1990; Nishiyama et al. 1996; Tillet et al. 1997).

The isolated soluble domain was found to compete with cell surface CSPG4 for TcdB binding and, thus, reduced TcdB effects. Precipitation experiments further revealed that CSPG4 binds to an epitope somewhere in the region of amino acids 1500–1852. Since this region also encompasses the first short repeat of the CROP domain, a precise interaction of CSPG4 with either RBD1 or RBD2 or both RBDs is not clear, yet.

The proposed CSPG4 binding region in TcdB is not the region that interacts with the poliovirus receptor-like 3 (PVRL3), also known as nectin-3. PVRL3 was independently reported to function as receptor for TcdB just a few months after the identification of CSPG4. Transfection of human intestinal CaCo-2 cells with retroviral gene trap vector for random gene disruption identified *PVRL3* as a gene that significantly contributes to sensitivity of cells toward TcdB (LaFrance et al. 2015). The structural and functional aspects of PVRL1-3 are summarized by Samanta and Almo: PVRL3 (and the homologue PVRL1, 2, and 3) belongs to the nectin family (nectin 1–4) and is a cell adhesion molecule that exhibits three Ig-like domains (one IgV and two IgC) as ectodomain and a cytoplasmic domain that interacts with afadin (Samanta and Almo 2015). Nectins function as immune modulators by interacting with T cells and are of importance as virus entry receptors

for Herpes simples (HSV-1, -2) or measles virus (Samanta and Almo 2015). Nectins interact with other nectins and cell adhesion molecules (Katsunuma et al. 2016) to form cell–cell contacts especially at adherens junctions (Takai et al. 2003).

Again, CRISPR/Cas9-mediated gene silencing in HeLa cells substantiated PVRL3 as one receptor. La France and coworkers showed that TcdB binds independently of the CROP to the extracellular domain of PVRL3. This binding is specific, since neither PVRL1 nor PVRL2 could compete with the receptor for TcdB. Manse and Baldwin located binding of PVRL3 to the region further upstream of the region where CSPG4 binds (aa 1373–1495) (Manse and Baldwin 2015). Both receptors, CSPG4 and PVRL3, are important since their tissue distribution and cell-specific expression differ. As reported by LaFrance and coworkers, CSPG4 was not found in CaCo-2 cells or colonic tissue. Likewise, CSPG4/NG2 seems not to be expressed in the colonic epithelium, but in the intestinal subepithelial myofibroblasts (Terada et al. 2006). Thus, a significant residual effect of TcdB in CSPG4- and PVRL3-deficient cells indicates further receptor(s). Interestingly, *PVRL3* gene disruption specifically reduced the cytotoxic effect of TcdB (early cell death), but not the cytopathic effect (cell rounding). The cytotoxic effect was first characterized by Chumbler and coworker and was found to be independent of Rho-glucosylation (Chumbler et al. 2012). Further reports elucidated the role of Rac1 and reactive oxygen species (Farrow et al. 2013) and described the morphotype of affected cells (Wohlan et al. 2014). PVRL3 mediates this cytotoxic effect of TcdB in a way that is not understood, yet. A mere ligand effect of TcdB does not seem likely, since we observed that a CROP-truncated version of TcdB did not induce cytotoxic effect (unpublished data). Interestingly, CSPG4/NG2 and PVRL/nectins both transduce signals via Cdc42 and Rac1 (Honda et al. 2003; Majumdar et al. 2003). Thus, toxin binding to these receptor molecules might initially activate these Rho GTPases which will be subsequently inactivated by toxin-catalyzed glucosylation (Just et al. 1995a).

3 Further Toxin Interacting Structures

3.1 Ligand Effect of TcdB

TcdB was repeatedly described to activate signal transduction within seconds to minutes after application. It is unclear whether these signals result from specific receptor activation or are generally associated with endocytosis. Upon application,

TcdB induces an immediate increase in intracellular free Ca^{2+} in NIH 3T3 fibroblasts, which can be measured before significant glucosylation of Rho GTPases (Gilbert et al. 1995). Toxin-catalyzed inhibition of Rho GTPases can affect signal transduction in an activating and inactivating manner. This bivalent behavior is emphasized in macrophages, e.g., murine Raw264.7 cells, where TcdB shows activating and inactivating qualities at the level of receptor-coupled heterotrimeric Gαi and Gαq proteins, respectively (Rebres et al. 2010). Since inhibition of Rho GTPases begins 10 min after addition of toxins (Farrow et al. 2013), the experimental setup must exclude intracellular glucosylation of substrate GTPases. Activation of cells by TcdA was reported by Warny and coworkers, showing that TcdA elevated CD11a and CD11b in monocytes but not in neutrophils after 30 min exposure (Warny et al. 1996). TcdA also induced activation of NF-κB in monocytes within 30-min treatment (Jefferson et al. 1999). The mechanism or receptor by which TcdA affects these cells was not identified. Beside the indirect evidence for receptor-activated signaling, one particular receptor was characterized, on which TcdB exhibits a direct ligand effect: TcdB and fragments of TcdB are agonists for the formyl peptide receptor (presumably FPR-1) present on human neutrophil granulocytes (Goy et al. 2015). FPR1 is also expressed in monocytes and macrophages. The abundance in monocytes, however, is rather weak and is increased in activated macrophages (Mandal et al. 2005; Gemperle et al. 2012). Interestingly, none of the known C-terminally located receptor-binding domains is responsible for FPR-1 activation. The most potent FPR-1 activating part of TcdB turned out to be the N-terminal fragment comprising the glucosyltransferase domain. The epitope or amino acid stretch that interacts with the FPR-1 has not been identified, yet. Although the relevance of this feature for pathogenesis of *C. difficile* associated diseases has not been investigated in animal studies up to now, it is important that even non-cytotoxic fragments of TcdB can provoke pro-inflammatory response of the native immune system. The effect of TcdB is the same as of the formylated peptide Met-Leu-Phe (fMLF), which is used as reference substance for FPR activation. The potency of TcdB to induce FPR-1-mediated rise in intracellular free Ca^{2+}, release of reactive oxygen species and degranulation of neutrophils, was comparable with that of fMLF. Thus, interaction of TcdB as ligand for specific receptor represents a new quality in pathogenesis that is different from receptor-mediated endocytosis and intracellular inhibition of Rho GTPases.

4 Conclusion

The pathogenic effect of TcdA and TcdB requires uptake of the enzymatic active domain, i.e., the GTD, into the cytosol of target cells. Toxin uptake is a complex process that involves binding to cell surface receptors, induction of endocytosis, association with the endosomal membrane, and integration of toxin domains into the vesicle membrane for pore formation and translocation of the GTD. Moreover, this process is not cell specific since TcdA and TcdB affect any cell type and

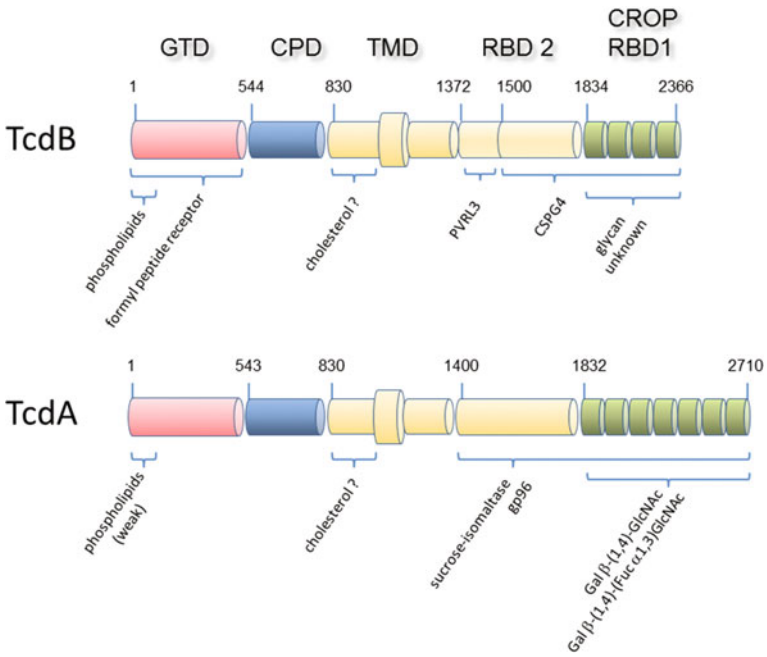


Fig. 3 Summary of domain-specific interacting partners for TcdA and TcdB. A postulated "unknown" receptor results from functional assays, indicating that the region significantly contributes to binding and potency of toxin. The glycans Gal β-(1,4)-GlcNAc and Gal β-(1,4)-(Fuc α-1,3)GlcNAc are only core structures that were found in various glycans that bind TcdA with high affinity. Where interacting partners cannot be allocated to a specific epitope or domain, the minimum toxin fragment that was shown to interact with or that can be deduced from studies is indicated by bracket

species, which only vary in their sensitivity toward these toxins. Structural and domain function analyses led to the current model of modular binding that includes at least two separate and structural independent domains of the toxins (RBD1 and RBD2) binding to at least two independent cell surface binding structures (Fig. 3). It is unclear whether RBD1 and RBD2 share tasks of cell surface binding for providing a reservoir on one hand and binding to the functional receptor that mediates uptake on the other hand. The redundant uptake processes make TcdA and TcdB as well as the other large clostridial glucosyltransferases highly efficient with respect to different species. It also makes development of therapies that specifically aims at only a single toxin receptor highly inefficient. Although tremendous progress have been made in identification of toxin receptors due to diverse techniques based on gene editing for functional inactivation, all recent reports point toward additional yet un-identified receptor(s) for TcdA and TcdB. The evolution of redundant binding domains either for TcdA or TcdB as well as the combination of these two homologous toxins that obviously show no overlap in receptor binding makes *C. difficile* most successful as pathogen for a variety of species.

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Cell Entry of C3 Exoenzyme from *Clostridium botulinum*

Astrid Rohrbeck and Ingo Just

Abstract *Clostridium botulinum* C3 is the prototype of C3-like ADP-ribosyltransferases that selectively ADP-ribosylate the small GTP-binding proteins RhoA/B/C and inhibit their downstream signaling pathways. It is used as pharmacological tool to study cellular Rho functions. In addition, C3bot harbors a transferase-independent activity on neurons to promote axonal and dendritic growth and branching. Many bacterial protein toxins interact specifically with proteins and/or other membrane components at the surface of target cells. Binding enables access to the appropriate cellular compartment so that the knowledge of the receptor allows essential insight into the mechanism of these toxins. Unlike other bacterial protein toxins (such as the clostridial C1 and C2 toxins from *C. botulinum*), C3 exoenzyme is devoid of a binding and translocation domain, with which toxins usually initiate receptor-mediated endocytosis followed by access to the intact cell. To date, no specific mechanism for internalization of C3 exoenzyme has been identified. Recently, vimentin was identified as membranous C3-binding partner involved in binding and uptake of C3. Although vimentin is not detected in neurons, vimentin is re-expressed after damage in regenerating neurons. Reappearance of vimentin allows C3 to get access to lesioned neurons/axons to exhibit axonotrophic and dentritotrophic effects.

Contents

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1 Introduction

C3 exoenzyme is an ADP-ribosyltransferase which was first described in *Clostridium botulinum* (Aktories et al. 1988; Rubin et al. 1988). Now, eight different C3 isoforms with similar molecular weights are identified, which are from *Clostridium limosum* (C3lim), *Bacillus cereus* (C3cer), *Staphylococcus aureus* (C3stau), and *Paenibacillus larvae* (C3larvin) (Aktories and Just 2005; Just et al. 1992, 1995; Wilde et al. 2001; Inoue et al. 1991; Krska et al. 2014).

The prototype of the C3-like ADP-ribosyltransferase family is C3 from *C. botulinum* (C3). It is produced as a single-chain protein harboring a signal sequence of 40 amino acids, which mediates secretion from the vegetative form of the clostridium. During this process, the signal peptide is cleaved off. C3 shows a strong basic isoelectric point (pI > 9) and exhibits a molecular weight of about 23.5 kDa. It is a mere exoenzyme, devoid of a receptor binding and translocation domain, with which classical protein toxins mediate their cell entry. So far, little is known about the binding of C3 to eukaryotic cells and its uptake. It was assumed that the exoenzyme is internalized via non-specific processes such as pinocytosis. The reported requirement of high concentrations of C3 in the μM range and extended incubation times seem to support this notion (Boquet et al. 1998).

C3 cleaves the intracellularly ubiquitous nicotinamide adenine dinucleotide (NAD^+) into nicotinamide and ADP-ribose and transfers the ADP-ribose moiety onto Rho GTPases (Sekine et al. 1989). The C3 transfers the ADP-ribose moiety *N*-glycosidically to the acceptor amino acid asparagine 41 (Asn41) of the GTPases RhoA/B and C (Aktories et al. 1989; Chardin et al. 1989; Vogelsgesang et al. 2007; Wilde et al. 2001). ADP-ribosylation renders Rho inactive by inhibition of GEF-mediated activation and by stabilizing the inactive Rho-GDI complex (Genth et al. 2003; Sehr et al. 1998). This leads to redistribution of the actin filaments and depolymerization of stress fibers in cells (Barth et al. 1999; Chardin et al. 1989; Paterson et al. 1990; Wiegers et al. 1991). In addition to the Rho-ADP-ribosylating activity of C3, also non-enzymatic functions are reported, namely a direct interaction with the GTP-binding protein RalA (Wilde et al. 2002). C3 forms a high affinity complex with Ral, however, without transferring ADP-ribose. This complex formation with RalA results in inhibition of the transferase activity of C3 like a GDI complex (Pautsch et al. 2005).

The delimited substrate specificity of C3 (only three GTPases out of about 170 GTPase of the Ras superfamily) is the basis of its use as tool to study cellular function of Rho GTPases.

2 C3 Exoenzyme from *Clostridium Botulinum*

2.1 C3 Exoenzyme as Virulence Factor

The role and importance of C3 as virulence factor is largely unknown. C3 exoenzyme has no translocation domain, compared to classical bacterial toxins, and could act as ligand. An interesting hypothesis to explain how C3 exoenzyme acts as virulence factor is the intracellular release of C3 by *S. aureus* bacteria that invaded host cells. During their intracellular habitation in the host cell cytosol (Menzies and Kourteva 1998), bacteria release the C3 (C3stau) which immediately ADP-ribosylates Rho. This mechanism is, to date, only found for *S. aureus* (Madden et al. 2001). Inhibition of RhoA/B/C results in actin depolymerization accompanied by significant morphological (Wiegers et al. 1991; Paterson et al. 1990; Rohrbeck et al. 2012) and functional changes. Indeed, treatment of macrophages with C3 results in inhibition of phagocytosis (Park et al. 2003) and migration (Rotsch et al. 2012). Therefore, macrophages are no longer capable of eliminating pathogens. Additionally, the functional inactivation of RhoA in human monocytes leads to a flattening shape and formation of neurite-like extensions (Aepfelbacher et al. 1997). C3 treatment of natural killer cells or cytotoxic T lymphocytes causes inhibition of their cytolytic function (Lang et al. 1992). Invasion of T lymphoma cells into fibroblast cells is inhibited by C3 (Stam et al. 1998). Furthermore, leukocyte adhesion is inhibited by C3 (Laudanna et al. 1996). Thus, it is conceivable that C3 disables immunological defense processes such as reduction of cytotoxicity of immune cells, inhibition of macrophage phagocytotic activity, and inhibition of migration of immune cells. Both the innate and the acquired immune system are affected. Conversely, C3 exoenzyme thereby enhanced the survival of C3-producing microbes in the host organism.

2.2 C3 Exoenzyme as Cell Biological Tool

Based on the selective inactivation of RhoA/B/C GTPases out of approximately 170 low molecular weight GTP-binding proteins (Aktories and Just 2005; Just and Boquet 2000), C3 is used as cell biological tool, even in the era of knockout techniques. C3 has a clear advantage over the usage of RhoA-siRNA. Application of RhoA-siRNA is always accompanied by massive expression and activation of RhoB, which at least partially replaces cellular RhoA functions (Ho et al. 2008). As

RhoA suppresses the *rhoB* promoter, RhoA inactivation results in deinhibition and thus in RhoB expression (Huelsenbeck et al. 2007). C3 also causes strong RhoB expression, but RhoB is immediately inactivated by C3-mediated ADP-ribosylation. Thus, application of C3 represents at this time the only approach to effectively inhibit RhoA without concomitant RhoB activation (Just et al. 2011). Although C3 lacks a cell binding and translocation domain, it is widely used as tool to study RhoA signal pathways. For two decades, C3 was thought to be internalized into cells through non-specific pinocytosis. But Fahrer et al. 2010 claimed a selective and specific uptake process of C3. The C3-induced morphological changes, which occurred significantly delayed after Rho-ADP-ribosylation, were used as readout for a successful uptake of C3. To accelerate C3 uptake and thus cytoskeletal reorganization, C3 has been introduced in eukaryotic cells by microinjection (Ridley and Hall 1992; Olson et al. 1998), electroporation (Tokman et al. 1997), or permeabilization with digitonin or streptolysin O (Mackay et al. 1997; Fensome et al. 1998; Koch et al. 1993). Moreover, chimeric fusion toxins, in which C3 was fused to the binding domain of the C2 toxin (Barth et al. 1998) or diphtheria toxin (Aullo et al. 1993), were used. Furthermore, a membrane-permeating form of C3 was created by fusing Tat (trans-activating transcription factor) transduction domain of human immunodeficiency virus (Frankel and Pabo 1988) to the C3 amino terminus (Tan et al. 2007). Tat domain is known to cross cell membrane even when fused with large heterologous protein (Fawell et al. 1994). The Tat-protein transduction domain improves the uptake of C3 into NIH3T3 fibroblasts and resulted in disruption of actin stress fibers after 16 h (Sahai and Olson 2006). However, in this study, no direct comparison between ADP-ribosylation of RhoA by C3 and Tat-C3 was performed. In our study, treatment of murine hippocampal HT22 cells and J774A.1 mouse macrophages with C3 or Tat-C3 caused a time-dependent ADP-ribosylation of RhoA finally resulting in degradation of ADP-ribosylated RhoA. In both cell lines, C3 and Tat-C3 induced a multinucleated phenotype and the disappearance of actin stress fibers after same time. Thus, no differences in uptake kinetics between C3 and Tat-C3 were detected in both cell lines. In this context, the mechanism responsible for Tat-dependent membrane translocation is controversially discussed. Some studies demonstrated that Tat-protein rapidly crosses the plasma membrane of cells (Frankel and Pabo 1988; Mann and Frankel 1991). Other data suggest that membrane translocation of Tat-protein is mediated by binding to cell surface-expressed heparan sulfate glycosaminoglycans (HPSGs) (Rusnati et al. 1999; Suzuki et al. 2002) and that Tat-protein promotes cellular uptake of cargo through glycosaminoglycan receptor-mediated endocytosis (Richard et al. 2003; Console et al. 2003). Perhaps more C3 enters the cells as C3-Tat-protein, but it seems that this does not increase the biological effect of C3. Meanwhile, it is accepted that C3 enters eukaryotic cells despite the absence of a binding and translocation domain. In addition, recent findings show that C3 causes ADP-ribosylation of RhoA in a short time and at nanomolar concentrations (Ahnert-Hilger et al. 2004; Fahrer et al. 2010; Rohrbeck et al. 2015). The development of alternative application techniques in fact prevented a detailed study of binding and uptake of C3.

2.3 C3 Exoenzyme and Enzyme-Independent Effects on Neuronal Function

A number of studies have demonstrated a role of RhoA in mediating neurite retraction (Wahl et al. 2000; Gallo 2006). Pharmacological inhibition of RhoA or expression of dominant negative RhoA resulted in neurite outgrowth of neuronal cell lines (Nishiki et al. 1990; Albertinazzi et al. 1998; Sebök et al. 1999). In neuronal cells, inhibition of Rho by C3 exoenzyme led to increased axonal and dendritic growth and branching (Kozma et al. 1997; Winton et al. 2002; Ahnert-Hilger et al. 2004). C3 promoted axonal elongation from chick DRG neurons (Jin and Strittmatter 1997). Additionally, C3 treatment of crushed optic nerves resulted in significant axonal growth passing the lesion site into white matter (Lehmann et al. 1999). Interestingly, this axonotrophic activity of C3 is independent from its enzymatic activity (Ahnert-Hilger et al. 2004). The enzymatically inactive mutant form C3-E174Q and an enzyme-deficient C-terminal peptide fragment covering amino acids 156–181 of C3 increased dendritic as well as axonal growth and synaptic connectivity of neurons in organotypic cultures in vitro (Höltje et al. 2009; Loske et al. 2012). Furthermore, treatment for acute spinal cord injuries in mice with enzyme-deficient C3-peptide significantly improved axonal and functional regeneration (Höltje et al. 2009). Furthermore, a single injection of the 26mer C3-peptide into the nerve repair sites (rat sciatic nerve lesion model) increased axonal elongation and maturation which finally resulted in better functional motor recovery than in NGF reference-treated animals (Huelsenbeck et al. 2012). Notably, the C3lim and C3stau from *C. limosum* and *S. aureus*, respectively, have no influence on axonal growth although they are homologues of C3bot (Ahnert-Hilger et al. 2004). Recently, a recombinant cell-permeable variant of C3 (VX-210® formerly Cethrin®) has been evaluated in a clinical trial for safety in the treatment of human acute spinal cord injury (Fehlings et al. 2011).

3 Binding and Uptake of C3 Exoenzyme

3.1 Effect of Posttranslational Modifications on the Binding of C3

Many bacterial protein toxins interact with proteins and/or other membranous structures at the surface of target cells. The binding to specific structures initiates the cellular uptake and mediates access to the appropriate cellular compartment, so that the knowledge of the receptor allows essential insight into the uptake mechanism. Posttranslational modifications such as glycosylation or phosphorylation can influence the interactions with other proteins and the localization of receptors within membrane domains (lipid rafts) (Dennis et al. 2009; Gu et al. 2012). The majority of plasma and membrane proteins of mammals are glycoproteins. Glycosylation is

the most common posttranslational modification of proteins in eukaryotic cells and in terms of the modified amino acids also the most diverse structures (Moremen et al. 2012). Several studies described that carbohydrates can affect ligand–receptor binding. Thus, a highly glycosylated form of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) resulted in a lower receptor affinity compared to less glycosylated or non-glycosylated form (Cebon et al. 1990). Another example of the influence of carbohydrates on ligand binding to receptors is the observation that an increased sialylation resulted in a reduced receptor binding of erythropoietin (EPO) (Darling et al. 2002). Also pathogens and bacterial toxins use glycosylated proteins at the cell surface as a receptor. However, despite some studies, so far no specific receptor for C3 has been identified.

C3 overlay binding assays, in which murine hippocampal HT22 cell lysates were immobilized by blotting onto nitrocellulose membrane, revealed that C3 bound within the 55-kDa protein band. This indicates that C3 recognizes a carbohydrate structure because denatured proteins are present in the overlay assay (i.e., proteins with altered secondary and tertiary structure due to the sample preparation with SDS and 95 °C boiling) and bound C3 can still be detected. And in fact, an elimination of carbohydrates with glycosidase F caused a reduced binding of C3 in overlay assay. Interestingly, this finding was not confirmed with intact cells. After cleavage of carbohydrate moieties with glycosidase F, the opposite was observed, namely an increased binding of C3 suggesting that removal of carbohydrates in the context of native proteins resulted in more binding sites (e.g., proteins or lipids) for C3.

In addition to glycosylation, the phosphorylation state of membrane proteins also influences binding of C3. Phosphorylation is the covalent attachment of a phosphate group to serine (S), threonine (T), or tyrosine (Y) residues of proteins and is catalyzed by kinases (Manning et al. 2002). However, the cleavage of phosphate moieties by phosphatase (CIP, calf intestinal phosphatase) resulted in a decreased binding of C3 within the 55-kDa protein band. By contrast, the stabilization of phosphate moieties by adding phosphatase inhibitors such as ortho-vanadate caused an increased binding of C3. There are few reports for extracellular phosphoproteins and their influence on binding of ligands. In the past five years, extracellular phosphoproteins were identified in the cerebrospinal fluid (Bahl et al. 2008), in serum (Zhou et al. 2009), and in the extracellular matrix (Yalak and Vogel 2012; Tagliabracci et al. 2015) by mass spectrometric approaches. Recently, extracellularly phosphorylated membrane proteins were identified in human umbilical vein endothelial cells (HUVECs) and K562 cells (Burghoff et al. 2015). The importance and influence of these identified membranous and secreted phosphoproteins on the interaction of bacteria or bacterial toxins with their target cells is not yet clear. Interestingly, phosphorylation commonly occurs at the same serine or threonine residues which are glycosylated. Thus, phosphorylation can compete with glycosylation at the same residues (Comer and Hart 2000). On the other hand, both modifications may be necessary for protein–protein interaction. For example, phosphorylation and *O*-glycosylation both affect binding of insulin-like growth

factor-binding protein-5 (IGFBP-5) to heparin (Graham et al. 2007). However, our binding assays demonstrate that C3 binds to an extracellular posttranslationally modified membrane structure. These findings argue against the hypothesis that C3 is taken up into cells by unspecific processes.

3.2 *Uptake of C3 Exoenzyme into Cells*

It was assumed for long time that C3 enters cells by non-specific processes or processes involving endosomal uptake (Fahrer et al. 2010) and that cell accessibility of C3 is generally low. Recently, it was shown that C3 enters different cells within few hours (Rotsch et al. 2012). In hippocampal cells (HT22), murine fibroblasts (NIH3T3), and human intestinal cells (HT29), C3 treatment resulted in morphological changes, which is a clear indicator of internalization of C3. Slight morphological changes occurred only after 24 h. Initially, some rounded cells with neurite-like extensions were observed increasing with longer incubation time (>24 h). In addition to these morphological changes (rounded cells with bipolar neurites), cells became large, amorphous, and multinucleated after 48 h (Rohrbeck et al. 2012; Rotsch et al. 2012). However, an appreciable ADP-ribosylation of RhoA was detected already after few hours. In the majority of reports, the morphological changes are viewed as a clear indicator of a successful cellular uptake of C3 (Chardin et al. 1989; Wiegers et al. 1991; Just et al. 1992; Miura et al. 1993; Krska et al. 2014; Slauson et al. 2015). Since morphological changes were detectable not before 12–24 h (depending on the cell type), the majority of cell lines were classified as relatively insensitive to C3 (leading to the above-mentioned diverse delivery techniques of C3). In contrast, macrophages have been postulated as C3 sensitive because C3 reached the cytosol of these cells within 2–3 h, and after 4–6 h, morphological changes were observed (Fahrer et al. 2010). But also in the hippocampal HT22 cells, a significant proportion of the cellular pool of RhoA is inactivated by ADP-ribosylation after 4 h. But significant morphological changes were only detectable after 48 h. This divergence is of particular importance because the morphological cell changes, e.g., cell rounding (as easily representable alteration of cytoskeleton), are regarded generally as an indicator of the C3 effect and thus as an indicator of the sensitivity of cells to C3. Since the morphological changes occur after 24–48 h, almost all cell lines were considered as insensitive to C3. However, the findings indicate rather the opposite; most cell lines are in fact sensitive. The detection system (cell rounding) is therefore inappropriate. The appropriate detection for internalization of C3 is measuring the rate of intracellular ADP-ribosylation of RhoA. Moreover, the relatively rapid and efficient internalization of C3 in J774A.1 cells (2 h) or HT22 cells (4 h) points to a specific internalization of C3.

3.3 Classical Endocytosis Mechanism and Internalization of C3 Exoenzyme

Different routes of internalization are reported for toxins such as cholera toxin (Torgersen et al. 2001). Involving clathrin- (Parton 1994) and caveolae-dependet (Schwitzer et al. 1996) pathways as well as endocytosis via lipid rafts (van Deurs et al. 1993). Although various chemical inhibitors of endocytosis as well as substances altering the cytoskeleton were used to clarify the uptake mechanism of C3, no distinct endocytotic mechanism has been identified.

Lipid rafts play an important role in uptake of numerous protein toxins. However, filipin and methyl-beta-cyclodextrin (MBCD) did not show any significant effect on uptake of C3, although MBCD was effective in inhibiting the uptake of *C. difficile* toxin B (Rohrbeck et al. 2015). Therefore, the uptake mechanism of C3 exoenzyme seems to be independent of cholesterol. The cytoskeleton has a prominent role in endocytosis and trafficking of endocytotic vesicles. Microtubules are also involved in uptake and are therefore disrupted by nocodazole (Hasegawa et al. 2001). The function of F-actin is inhibited by latrunculin B, which sequesters G-actin and prevented F-actin assembly (Helal et al. 2013). Disruption of both cytoskeletal structures (actin and microtubule filaments) did not result in any alteration of C3 uptake. C3 was internalized even if the formation of the clathrin-coated vesicles is blocked by acidification of the cytosol (Sandvig et al. 1987) or when clathrin assembly and disassembly are inhibited by chlorpromazine (Wang et al. 1993). This indicates that clathrin-coated pits did not play a role in the uptake of C3 (Rohrbeck et al. 2015). Also, bafilomycin A1, an inhibitor of the vacuolar proton ATPase, did not inhibit the uptake of C3. It should be noted that one contrary result was reported (Fahrer et al. 2010). This study revealed that bafilomycin A1 reduced the uptake of C3 into J774A.1 cells and human promyelocytic leukemia HL-60 cells but only partially. In addition, bafilomycin A1 only inhibited the uptake at low C3 concentration and showed no effect at high C3 concentration. However, in our studies, bafilomycin A1 did not significantly alter the uptake of C3 even at low concentrations, although it efficiently blocked the uptake of *C. difficile* toxin B (Rohrbeck et al. 2015). Toxin B is known to enter cells via receptor-mediated endocytosis involving acidic endosomes. Experiments with dynasore, which blocks dynamin-dependent endocytosis by inhibition of the dynamin GTPase activity, showed a clear-cut reduction in the uptake of C3 into HT22 cells. These results suggest that the dynamin-dependent pathway of endocytosis may participate but is not the exclusive route of internalization of C3. Dynamin was originally noted for its role in severing vesicles from the plasma membrane in clathrin-dependent and caveolae-mediated endocytosis (Guha et al. 2003). There is evidence for clathrin- and caveolae-independent but dynamin-dependent endocytosis (Benlimame et al. 1998; Damm et al. 2005; Mayor and Pagano 2007). Several studies reported the involvement of dynamin but not of caveolae in the formation of non-coated vesicles at the plasma membrane (Nabi and

Le 2003; Parton and Richards 2003). Indeed, for rotavirus, it was described that internalization of viruses is not mediated by either clathrin- or caveolae-dependent endocytosis, but it is dependent on the function of the large GTPase dynamin (Sánchez-San Martín et al. 2004). The coronavirus, feline infectious peritonitis virus, enters monocytes through a clathrin- and caveolae-independent pathway that strongly depends on dynamin (Van Hamme et al. 2008). This endocytosis mechanism (clathrin- and caveolae-independent but dynamin-dependent) has so far not been described for bacterial toxins. It is known that bacterial toxins can use different mechanisms of endocytosis. For example, the internalization of cholera toxin involves different entry routes such as clathrin-dependent (Nichols 2003), caveolae-mediated (Orlandi and Fishman 1998), Arf6-dependent (Jobling and Holmes 2000), and dynamin-independent (Massol et al. 2004). However, the findings for C3 indicate that the internalization of C3 does not strictly require either clathrin-coated vesicle or the caveolae-like pathways of endocytosis, but the endocytosis mechanism is dynamin-dependent.

4 Vimentin Mediates Binding and Uptake of C3

4.1 Identification of Vimentin as Binding Partner of C3

To identify the membrane interaction partner of C3, different binding assays with subsequent mass spectrometric analyses of the bound proteins were performed and the intermediate filament vimentin was identified (Rohrbeck et al. 2014). It is known that vimentin is not exclusively intracellularly localized, but it is also expressed at the cell surface (Podor et al. 2002; Mor-Vaknin et al. 2003; Mitra et al. 2015). Furthermore, it is involved in endocytosis (Kim and Coulombe 2007). Vimentin is a filament protein that can be both glycosylated (Rho et al. 2009) and phosphorylated (Eriksson et al. 2004). These posttranslational modifications reportedly affect the binding to C3. Indeed, vimentin has a glycogen synthase kinase 3 (GSK3)-regulated *O*-GlcNAcylation site at S54 (Wang et al. 2007). Moreover, phosphorylation of vimentin regulates stability, organization, and function of vimentin (Hyder et al. 2008). Phosphorylation of intermediate filaments typically occurs at the head and tail domains. Phosphorylation sites at the central helical rod domains have been predicted by mass spectrometry but not validated by other methods (Hornbeck et al. 2004). Therefore, vimentin has been studied in detail as a possible interaction partner of C3. The direct interaction of C3 with vimentin was confirmed by pulldown with recombinant His-tagged vimentin fragments in a cell-free system. Further evidence that vimentin interacts with C3 resulted from the immunoprecipitation and colocalization. C3 is not the only pathogen to bind as also *Pasteurella multicoda* toxin (Shime et al. 2002) as well as *Salmonella* virulence protein SptP (Salmonella protein tyrosine phosphatase) (Murli et al. 2001) binds directly to vimentin.

Biotinylation of cell surface proteins with subsequent mass spectrometric analysis and flow cytometry assay evidenced that the intermediate filament vimentin in fact appears at the cell surface. The observation that the type III intermediate filament vimentin, which is a cytoskeletal component, is present at cell surface is surprising. However, recent studies revealed that vimentin is also present at the cell surface (Steinmetz et al. 2011; Bryant et al. 2006; Satelli et al. 2015; Mitra et al. 2015) and it is even secreted into the extracellular milieu (Mor-Vaknin et al. 2003). Vimentin interacts when located at the cell surface with several pathogens (Table 1).

Recent data indicate that vimentin interacts directly with the severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein and serves as a putative coreceptor involved in the entry of this virus (Yu et al. 2016). Moreover, vimentin presented at endothelial cell surface binds specifically to both a peptide-25 (Leu132 to Arg152 in the latency-associated peptide molecule) (Nishida et al. 2009) and a 12 amino acid SP-peptide (SAHGTSTGVPWP) (Glaser-Gabay et al. 2011). The exact mechanism how vimentin reaches the plasma membrane and reenters the cytosol

Table 1 Pathogenic interaction with vimentin

Pathogen	Vimentin mediates	References
Human immunodeficiency virus type 1	Virus binding and infection	Thomas et al. (1996)
Vaccinia virus	Virus binding	Risco et al. (2002)
Cowpea mosaic virus	Virus binding and invasion	Koudelka et al. (2009)
Theiler's murine encephalomyelitis virus	Virus binding	Nedellec et al. (1998)
Enterovirus 71	Virus binding and invasion	Du et al. (2014)
Blue tongue virus	Virus binding and invasion	Bhattacharya et al. (2007)
Porcine reproductive and respiratory syndrome virus	Virus binding and invasion	Kim et al. (2006)
Japanese encephalitis virus	Virus binding	Das et al. (2011)
SARS-coronavirus	Virus binding and invasion	Yu et al. (2016)
Escherichia coli	Bacterial binding and invasion	Zou et al. (2006), Chi et al. (2010)
Shigella flexneri	Bacterial binding	Russo et al. (2016)
Salmonella enterica	Bacterial invasion	Murli et al. (2001)
Streptococcus pyogenes	Bacterial binding	Bryant et al. (2006), Icenogle et al. (2012)
Mycobacterium avium subspecies *hominissuis*	Bacterial binding	Babrak et al. (2015)
Trypanosoma cruzi	Bacterial binding and invasion	Teixeira et al. (2015)

after ligand binding remains unanswered. In this context, Bhattacharya et al. (2009) demonstrated that β3-integrin regulates recruitment and interaction of vimentin with the cell surface at the site of focal adhesions in several different cell types. Another hypothesis is that the surface recruitment of vimentin is carried out by phosphorylation-mediated disassembly of vimentin (Ise et al. 2012). Recently, it was shown that vimentin filaments are recomposed by filament severing and reannealing in a phosphorylation-independent way (Robert et al. 2015). Short filaments of vimentin such as squiggles and particles were found in the cell periphery (Yoon et al. 1998) and were actively transported bidirectionally along microtubules (Hookway et al. 2015). Additional analysis will be required to explore this mechanism in more detail.

Depletion of vimentin by use of vimentin siRNA led to increased binding of C3. This finding is amazing, but it is explained by immunohistochemical studies of siRNA-transfected cells. Although vimentin siRNA resulted in a significant reduction of intracellular vimentin, vimentin fragments were at the cell periphery and also at the cell surface to a greater extend, which resulted in an increased binding to C3. This observation is supported by Chou and Goldman (Chou 2000). They identified that small vimentin fragments moved in the cytoplasm and preferably accumulated at the cell periphery. This hypothesis was confirmed with the intermediate keratin filaments. Soluble keratin oligomers dissociate from keratin filaments and diffuse through the cytoplasm to the cell periphery (Robert et al. 2016).

The extracellular addition of recombinant vimentin caused a biphasic effect which is dependent on the vimentin concentration. Low extracellular concentrations of vimentin led to increased binding of C3, whereas high concentrations decreased binding of C3 (Rohrbeck et al. 2014). One explanation is that vimentin forms oligomers in the culture medium at high concentrations, which allows binding of C3 to vimentin oligomers. C3 was trapped and could not bind vimentin at the cell surface. At low concentrations, vimentin was monomeric; monomeric vimentin binds to the membrane and enhances the interaction with C3 at the cell membrane. Although it is not yet understood how vimentin arrives at the plasma membranes and gets to extracellular leaflet of the membranes, it clearly works as receptor for C3.

4.2 Role of Vimentin in the Uptake of C3

Vimentin is not only involved in the binding to C3 but also in its internalization. Compared to the scramble siRNA-transfected cells (control cells), a significantly stronger signal band of unmodified RhoA was detectable after vimentin depletion. In control cells, C3-mediated ADP-ribosylation of RhoA, which increased the susceptibility of ADP-ribosylated RhoA to proteasomal degradation, caused a faint signal band of RhoA in Western Blot. This difference of the signal intensity of RhoA indicates a delayed internalization of C3. Further evidence that vimentin is

involved in the uptake of C3 resulted from acrylamide experiments. Acrylamide at low concentrations caused a resolution of intermediary filament networks, whereas neither the actin nor microtubule cytoskeleton was influenced (Eckert 1985; Sager 1989; Aggeler and Seely 1990; Miller and Hertel 2009). Acrylamide-induced alteration of vimentin system in cells showed that vimentin is involved in both the binding and in the internalization of C3. The findings are reasonably supported by the cellular functions of the vimentin filament network in endocytosis, vesicular membrane traffic, and localization of cell organelles like Golgi apparatus (Faigle et al. 2000; Gao and Sztul 2001; Styers et al. 2004, 2005). The GTPases Rab7 and Rab9 directly interact with filamentous and soluble vimentin (Walter et al. 2009; Cogli et al. 2013). Both Rab-proteins regulate aggregation and fusion of late endocytic structures/lysosomes (Bucci et al. 2000; Ganley et al. 2004). Interaction with vimentin caused sequestration of Rab-GTPase positive endosomes and inhibition of endocytosis (Walter et al. 2009). So far, the involvement of vimentin in the binding and uptake of pathogens has been described several times (Table 1).

However, preincubation of cells with recombinant vimentin resulted in an increased internalization of C3, as evidenced by a more rapid ADP-ribosylation of RhoA. Preincubation of primary astrocytes with vimentin caused a significant acceleration of C3-induced morphological changes. Interestingly, the study of Bonfiglio supports this finding. Bonfiglio and coworkers reported that vimentin translocated to the membrane in HT22 cells and associated with dynamin by the internalization of the cortico-releasing hormone receptor-β-arrestin 2 complex (Bonfiglio et al. 2013). This report supports our finding that vimentin and dynamin are required for internalization of C3.

4.3 Significance of Vimentin as a Binding Partner of C3

The importance of vimentin in binding and uptake of C3 was supported by a proof of principle study at primary vimentin-knockout neurons. The vimentin knockout was verified by genotyping and Western Blot analysis. In primary vimentin-knockout hippocampal neurons, C3 induced only very slight axonal and dendritic elongation. Additionally, vimentin-knockout neurons showed a very weak cytosolic C3 signal in fluorescence microscopy and ADP-ribosylated RhoA was significantly reduced compared to the amount in the wild-type neurons (Adolf et al. 2016). Thus, vimentin-knockout neurons were almost insensitive to C3, whereas primary wild-type neurons were highly sensitive. These findings clearly demonstrate that vimentin is functionally involved in the uptake of C3.

Although the exact mechanism of C3 uptake and the functional impact on neurons are not yet clear, it mediates neuro-regenerative effects in primary hippocampal neurons and animal models. Thus, C3 is used in the treatment of spinal cord injuries, both in animals (Dergham et al. 2002; Ellezam et al. 2002) and in humans (clinical phase II trial, Fehlings et al. 2011; McKerracher and Anderson 2013). C3 induced axonotrophic effects in neurons although vimentin was not

detectable in neurons under physiological conditions. These findings raise the important question how C3 is taken up into the neurons. Vimentin is (re-)expressed after neuronal lesions so that the receptor of C3 is present at the cell surface under this condition. In fact, upregulation of vimentin was shown in mice after neural lesions (Takano et al. 2004; Perlson et al. 2005) and after optic nerve lesion (Martin et al. 2003). It was also discussed that the lack of vimentin caused significantly slowed progression of axonal regeneration after sciatic nerve lesion (Berg et al. 2013). Down regulation of vimentin expression in dibutyryl cAMP-differentiated neuroblastoma cells is accompanied by a slowing down axonal elongation and re-expression of vimentin promotes axonal growth again (Dubey et al. 2004). The findings indicate a participation of vimentin in neuro-regeneration. Neurons and axons in a regenerative context are thus much more sensitive to C3 than in a non-damaged state.

5 Conclusion

In the last twenty years, several molecular and cell biology standard methods have been applied to study the internalization and effects of C3 (Just et al. 1992; Aullo et al. 1993; Olson et al. 1998; Barth et al. 1998; Marvaud et al. 2002; Park et al. 2003; Fahrer et al. 2010; Lillich et al. 2012; Rohrbeck et al. 2015). Yet there are substantial gaps in our knowledge about uptake and molecular effects of C3. However, recently, it was clearly shown that intermediate filament protein vimentin is crucial for binding and uptake of C3 into neuronal cells (Rohrbeck et al. 2014; Adolf et al. 2016). The mechanism how vimentin reaches the cell surface is unknown so far. Additionally, it is not clear whether there are other binding partners which act as coreceptor together with vimentin. Therefore, further research is needed to evaluate whether further binding partner for C3 does exist and how C3 induces the observed enzyme-independent effects. The availability of alternative binding partners suggests further uptake mechanism of C3, if the receptor or receptors are widely distributed among tissues, allowing C3 to act on a variety of cell types. In the other case, when the receptors are specific for certain cell types such as neuronal cells, it is possible to administer C3 in a cell-type-specific manner.

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Receptor-Binding and Uptake of Binary Actin-ADP-Ribosylating Toxins

Panagiotis Papatheodorou and Klaus Aktories

Abstract Binary actin-ADP-ribosylating toxins (e.g., *Clostridium botulinum* C2 toxin or *Clostridium perfringens* iota toxin) consist of two separate proteins: An ADP-ribosyltransferase, which modifies actin thereby inhibiting actin polymerization, and a binding component that forms heptamers after proteolytic activation. While C2 toxin interacts with carbohydrate structures on host cells, the group of iota-like toxins binds to lipolysis-stimulated lipoprotein receptor (LSR). Here, we review LSR and discuss the role and function of LSR in interaction of iota-like toxins with host cells.

Contents

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1 Introduction

Bacterial protein toxins are usually characterized by high specificity, extraordinary potency, and extreme efficiency. These properties depend on selective interaction of toxins with target cells, on orchestrated toxin uptake and highly effective cellular actions. The high toxin efficiency is most often achieved by enzyme activity of the toxin. One of the best-studied enzyme activities of toxins is the ADP-ribosylation (Krueger and Barbieri 1995; Simon et al. 2014; Holbourn et al. 2006). ADP-ribosylating toxins transfer the ADP-ribose group of NAD^+ onto eukaryotic targets, which in most cases are proteins. Toxin-catalyzed attachment of ADP-ribose causes major functional changes of the eukaryotic target with the aim to manipulate cellular responses for the benefit of the toxin-producing pathogen.

All bacterial ADP-ribosylating toxins act inside of eukaryotic target cells. Because the toxins are often large proteins, they developed highly sophisticated machineries to cross the cell membrane and, eventually, to reach the cytoplasma. Usually, the uptake of toxins depends on several steps (Montecucco et al. 1994; Olsnes and Sandvig 1988; Sandvig and Olsnes 1984), including (1) binding of the toxins to the cell membrane of target cells. (2) This is followed by endocytosis of the receptor/toxin complex (Sandvig and Van Deurs 1996) and (3) the transport into a low pH endosomal compartment. The low pH of endosomes most likely induces separation of the toxin from the receptor and (4) forces conformational changes, which (5) favor the introduction of parts of the toxins into the vesicle membrane (London 1992; Olsnes et al. 2000; Sandvig and Olsnes 1980, 1982; Haug et al. 2003a, b; Barth et al. 2002). Membrane insertion of toxins is the prerequisite for (6) toxin translocation into the cytosol, where modification of eukaryotic targets occurs. Dependent on the toxin type, the last step of toxin uptake (translocation) appears to be controlled by interacting chaperons, which are involved in translocation and refolding of the toxins (for details see other chapters of this Volume) (Kaiser et al. 2011; Haug et al. 2003a, b; Kaiser et al. 2009). To fullfill all these functions and requirements, bacterial protein toxins have complex structures. The most simple toxin model is the AB model, which is characterized by the active domain A and the binding and translocation domain B. These domains can be part of a single chain toxin (e.g., diphtheria toxin) or they are organized as separated toxin components, which are non-covalently attached (cholera toxin, *Photorhabdus luminescence* Tc toxins).

Another type of the arrangement of an AB toxin is the binary toxin structure (Barth et al. 2004; Aktories and Barth 2004; Considine and Simpson 1991; Ohishi et al. 1980; Stiles and Wilkins 1986). Binary toxins consist of an enzyme component and a binding/translocation component, which are not associated with each other until the binding component is activated in the host. Examples of binary toxins are the actin-ADP-ribosylating binary toxins and also anthrax toxin. In the following, we will discuss in detail the binding, uptake of actin-ADP-ribosylating toxins and its receptors.

2 Binary Actin-ADP-Ribosylating Toxins

The family of binary actin-ADP-ribosylating toxins comprises several toxins produced by clostridia, including the prototypic *Clostridium botulinum* C2 toxin and various isoforms, *C. perfringens* iota toxin, *C. difficile* transferase CDT and *C. spiroforme* toxin CST (Barth et al. 2004; Aktories et al. 1990, 1992). Furthermore, *Bacillus sphaericus* (new name *Lysinibacillus sphaericus*) and *Bacillus thuringiensis* produce the binary vegetative insecticidal proteins (Vip1 and Vip2) that also appear in multiple isoforms (Chakroun et al. 2016).

2.1 Structure of Binary Toxins

All these toxins possess an enzyme component of ~45 kDa (Barth et al. 2004; Tsuge et al. 2003; Schleberger et al. 2006). Moreover, the enzyme component possesses two domains, which are largely related (Han et al. 1999). The C-terminal domain harbors the ADP-ribosyltransferase activity, while the N-terminal domain is involved in interaction with the binding components. Notably, also the N-terminal domain has a typical ADP-ribosyltransferase folding, however, this domain has lost specific amino acid residues crucial for ADP-ribosyltransferase activity and emerged to an adoptor domain (Han et al. 1999; Schleberger et al. 2006; Tsuge et al. 2003; Sundriyal et al. 2009). Thus, the enzyme components of the binary toxins evolved most likely by gene duplication (Han et al. 1999). The binding components of the binary toxins consist of 80–107 kDa proteins, which share similarities in sequence, structure, and function with the binding component of anthrax toxin, the protective antigen PA (Young and Collier 2007).

All binding components of binary toxins consist of four domains (Barth et al. 2004; Stiles et al. 2014). At the N-terminus, the activation domain I is located, which is followed by the membrane-insertion domain II, the oligomerization domain III, and the receptor-binding domain IV (Blöcker et al. 2000; Stiles et al. 2002; Hale et al. 2004). Domain I is activated by proteolytic cleavage, resulting in release of an ~20 kDa fragment and formation of heptamers (Ohishi 1987; Gibert et al. 2000; Barth et al. 2000). Heptamer formation may occur at the surface of target cells after monomer binding or already before cell surface binding. While the binary toxins share significant sequence similarity within domains I, II, and III, the sequence similarities in domain IV varies largely between binary toxin subfamilies indicating different receptor interactions (Barth et al. 2004). The structure and sequence similarities between binary toxins include anthrax toxin-binding component PA, which is highly homologous with the other binary toxins in domains I-III but not in domain IV (Collier and Young 2003).

Moreover, the family of binary actin-ADP-ribosylating toxins produced by clostridia is divided into two subfamilies, the C2 and the iota-like toxin family, respectively. The C2 toxin family comprises various C2 toxin isoforms from *C. botulinum*

(Ohishi and Hama 1992). The iota-like toxin family includes the *C. perfringens* iota-like toxin, *C. spiroforme* toxin (CST), and *C. difficile* transferase (CDT) (Stiles et al. 2011; Considine and Simpson 1991; Ohishi and Hama 1992. Binding and enzymatic components of members of the iota-like toxin family are mutually interchangeable to form fully active toxins (Stiles et al. 2011). However, such functional complementation has not been observed between C2 and iota-like toxins (Considine and Simpson 1991). Interestingly, the binding component of anthrax toxin PA has been shown to translocate C2I into target cells (Kronhardt et al. 2011). However, the efficiency is rather poor.

2.2 Functions of Binary Actin-ADP-Ribosylating Toxins

All binary actin-ADP-ribosylating toxins studied so far modify G-actin at arginine-177 (Aktories et al. 1986, 2011). ADP-ribosylation of this residue inhibits actin polymerization. Moreover, ADP-ribosylated actin acts as a capping protein at plus ends of growing actin filaments (Wegner and Aktories 1988). Thereby, further growth is inhibited, whereas the minus end is not affected and depolymerization of F-actin can occur. Thus, ADP-ribosylation increases the critical concentration of non-modified actin for polymerization. All these effects result in depolymerization of F-actin in toxin-targeted cells with major consequences for actin-dependent cellular processes including numerous motile functions, morphological features (Dominguez and Holmes 2011), and even actin-dependent regulation of transcription and translation (Pfaumann et al. 2015). Notably, toxin-induced inhibition of actin polymerization has major consequences for other cytoskeleton proteins, which dynamically interact with each other. For example, toxin-induced depolymerization of actin causes formation of microtubule dependent cell membrane protrusions (Schwan et al. 2009).

3 Cell Surface Receptor of *C. Botulinum* C2 Toxin

Identification of receptors involved in binding of *C. botulinum* C2 toxin to host cells started with the establishment of a toxin-resistant cell line (Fritz et al. 1995). To this end, Chinese hamster ovary (CHO-K1) cells were mutagenized by treatment with *N*-methyl-*N*-nitrosourea (MNU) and selected with C2 toxin for resistant cells. Binding studies with radiolabeled C2 toxin suggested that mutagenesis of a cell surface receptor for C2 toxin was the reason for toxin resistance. Moreover, the finding that C2 toxin-resistant cells were still sensitive for iota-like toxins indicated that both groups of toxins bind to different types of receptors. Using mutant CHO cell lines with defined glycosylation defects revealed that CHO mutant 15B, which lacks complex and hybrid N-linked carbohydrates, was C2 toxin resistant, while Lec8 and Lec2 CHO cells, which lack sialylated and galactosylated glycoproteins and

glycolipids, were even more sensitive than wildtype cells (Eckhardt et al. 2000). Further analysis of the MNU mutagenized CHO-RK14 cells revealed that these cells were deficient in alpha-1,3-mannosyl-glycoprotein 2-beta-*N*-acetylglucosaminyl-transferase I (EC 2.4.1.101, GlcNAc-TI). This defect prevents the synthesis of N-linked complex and hybrid carbohydrates. Accordingly, ectopic expression of GlcNAc-TI in these cells, reconstitutes C2 toxin sensitivity. The studies also show that galactose and sialic acid are not necessary for toxin binding. However, the findings cannot exclude that a specific protein is additionally essential for the action of the toxin. Because C2 toxin is toxic for all cells studied so far, a putative receptor protein must be expressed ubiquitously. The above findings clearly indicated that iota-like toxins use a membrane receptor different from C2 toxins. However, it took more than 10 years to identify the receptor of iota-like toxins (see below). The receptors of insecticidal *B. sphaericus* and *B. thuringiensis* binary Vip toxins are not known. Preliminary data suggest insect specific receptors (Sattar and Maiti 2011).

4 Cell Surface Receptor of Iota-like Toxins

4.1 Identification of LSR as Host Receptor for Iota-Like Toxins

Early binding studies of Stiles and colleagues showed that iota-like toxins use a proteinaceous receptor for cell entry (Stiles et al. 2000). To identify the host receptor for clostridial iota-like toxins, a novel genetic approach (haploid genetic screen), which is based on the human haploid cell line Hap1 (Carette et al. 2009, 2011a), was applied. The Hap1 cells are mutagenized with a retroviral gene-trap vector resulting in null mutations in non-essential genes (Carette et al. 2009, 2011a, b). Then, mutagenized Hap1 cells are incubated with CDT and cells that survive the toxin treatment are collected and expanded for identification of mutagenized genes that give rise to toxin resistance. The only gene that was significantly enriched in this screen coded for the lipolysis-stimulated lipoprotein receptor (LSR, LISCH7, ILDR3, angulin-1) (Papatheodorou et al. 2011).

LSR is essentially required for intoxication of Hap1 and HeLa cells with CDT (Papatheodorou et al. 2011). HeLa cells that do not express detectable amounts of LSR, as well as cells that are derived from a Hap1 LSR knockout clone, regain sensitivity towards CDT after ectopic expression of LSR. Pull-down assays with recombinant proteins show the direct interaction between the binding component of CDT and the ectodomain of LSR. In addition, the ectodomain of LSR competitively inhibits cell intoxication by CDT by acting as a decoy receptor. LSR is not only the host receptor for CDT but also for CST and iota toxin (Papatheodorou et al. 2011, 2012). As expected, studies showed that LSR is not a receptor for the more distantly related C2 toxin (Papatheodorou et al. 2011). A schematic overview describing receptor binding, uptake and mode-of-action of iota-like toxins is summarized in Fig. 1.

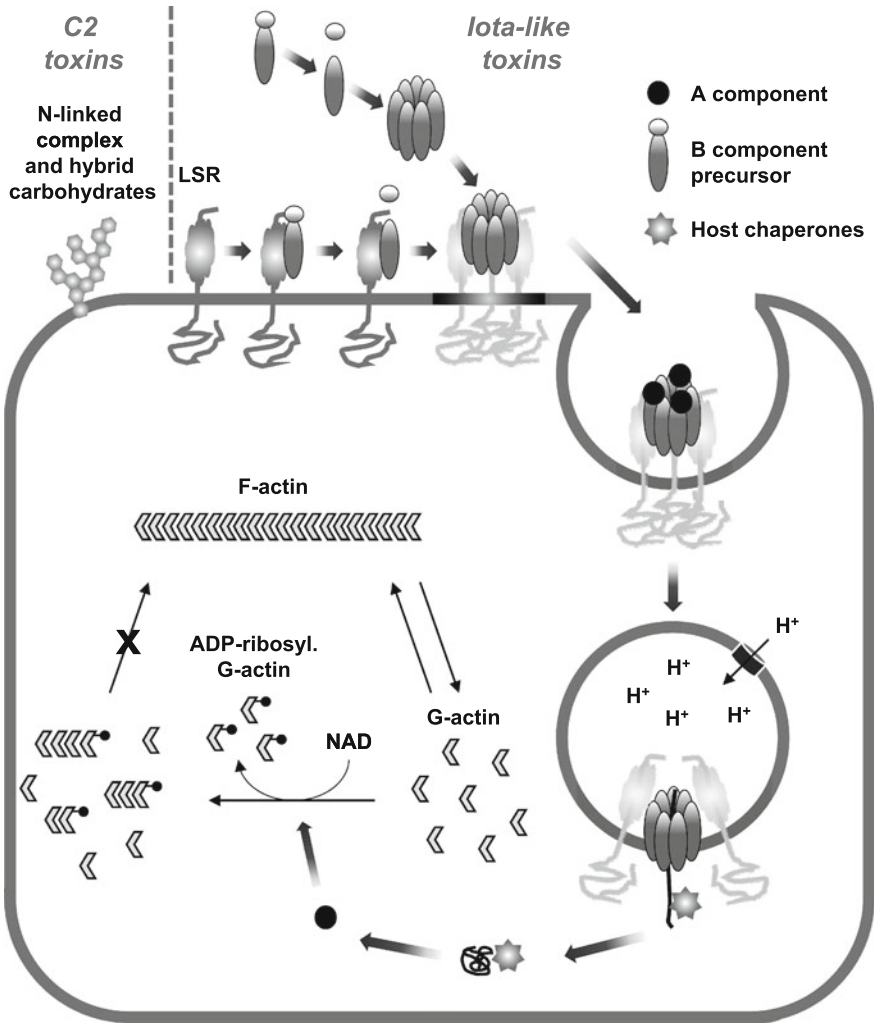

Fig. 1 Model of binding, uptake and mode-of-action of iota-like toxins. The precursor of the binding (B) component binds to LSR, is then proteolytically activated and triggers clustering of the receptor into lipid rafts, which finally leads to the oligomerization of the activated B components. Alternatively, processing and oligomerization of the B component may occur before binding to LSR. The enzyme (A) component binds to the B oligomer and triggers receptor-mediated endocytosis of the toxin-receptor complex. Acidification of the endosome leads to membrane-insertion and pore formation by the B oligomer and to the release of the A component into the cytosol. Host cytosolic chaperones assist during the translocation process and refolding of the A component. Eventually, the A component ADP-ribosylates monomeric G-actin, which in turn prevents polymerization of F-actin. As a consequence, continuous depolymerization of F-actin leads to the collapse of the actin cytoskeleton

4.2 Structures of LSR Interacting with Binary Toxins

LSR is a type-I single-pass transmembrane protein that consist of an extracellular domain, a membrane-spanning α-helix and a relatively large intracellular domain. The extracellular domain features an immunoglobulin-like, V-type domain which is preceded by an N-terminal, undefined sequence, which shows a variable length in different isoforms of LSR (Bihain and Yen 1998; Higashi et al. 2013; Hemmasi et al. 2015). A schematic representation of the domain architecture of LSR is depicted in Fig. 2. By a pull-down approach it was shown recently that the receptor-binding domain of CDTb interacts with the Ig-like domain of LSR (Hemmasi et al. 2015). Moreover, the binding kinetics obtained by surface plasmon resonance spectroscopy revealed a K_D value of ~110 nM for this interaction. A slightly lower dissociation constant (K_D = ~30–40 nM) was obtained when the binding of the receptor-binding domain (RBD) of CDTb to the surface of various cultured cells was studied by flow cytometry (FACS) analysis. A cysteine-rich region connects the transmembrane helix of LSR with its intracellular domain. As we could show recently, LSR constructs that lacked either the cysteine-rich region or the intracellular domain were still able to localize to the plasma membrane and to mediate uptake of the CDT toxin into host cells (Hemmasi et al. 2015). These findings may suggest that additional membrane proteins might form a complex with LSR upon binding of CDT to trigger endocytic uptake. One candidate would be CD44 (see below). Thus, the data available indicate that LSR is absolutely required for binding and uptake of CDT, however, one cannot exclude that other toxin-binding proteins participate.

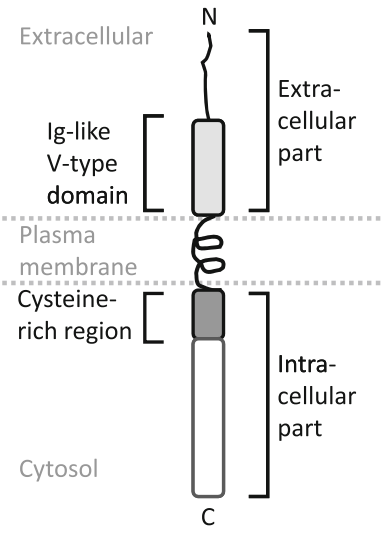

Fig. 2 Schematic representation of the domain architecture of LSR. LSR is a type-I, single-pass transmembrane protein. The N-terminal, extracellular part of LSR contains an Ig-like V-type domain preceded by an uncharacterized sequence, which varies in length in different isoforms. A transmembrane helix connects the extracellular part of LSR to the C-terminal, intracellular part of LSR. A cysteine-rich region followed by a large, uncharacterized C-terminal tail are the main features of the intracellular part of LSR

4.3 Toxin Structures Involved in Interaction with LSR

It is generally accepted that the receptor-binding domain (RBD) of iota-like toxins is located at the C-terminus of the binding component (Marvaud et al. 2001; Blöcker et al. 2001). Accordingly, a protein consisting only of the C-terminal part of the binding component of CDT (CDTb) is sufficient for interaction with LSR (Papatheodorou et al. 2013; Hemmasi et al. 2015). However, since no structural information is available for the binding component of iota-like toxins, the exact receptor-interacting epitopes of iota-like toxins are still unknown. With a series of N- and C-terminal truncations of the RBD of CDTb followed by pull-down approaches and competition assays, the LSR-interacting region of CDTb could be narrowed down to amino acids 757–866 (Hemmasi et al. 2015). Moreover, with a transposon-based mutagenesis approach epitopes within the RBD of CDT were identified that are crucial for interaction with LSR. Three out of a total of four identified epitopes are between amino acids 843–866 (Hemmasi et al. 2015). Strikingly, this region matches exactly to a linear epitope in the binding component of the iota toxin (Ib), which is recognized by a monoclonal antibody that is capable of inhibiting the iota toxin in vitro (Marvaud et al. 2001; Stiles et al. 2002).

4.4 LSR and Lipid Rafts

The binding component of iota-like toxins is not only important for binding to the host cell receptor but also for pore formation in endosomes to allow the translocation of the enzyme component into the cytosol. The endosomal pore is generated by oligomerization of the binding component after proteolytic activation and subsequent insertion into the endosomal membrane (Barth et al. 2000; Barth 2004; Aktories and Barth 2004). It is still not clear, if the proteolytic activation and prepore conversion of the binding component occurs before or after binding to the cell surface. Thus, it is a matter of controversial debate, whether the binding component of iota-like toxins binds as a monomer or already as an oligomeric prepore to the host receptor. Studies on iota toxin showed that the oligomer is associated with cholesterol-rich membrane domains (lipid rafts) and that cholesterol depletion prevented oligomerization of the binding component of the iota toxin (Nagahama et al. 2004; Hale et al. 2004). However, other studies suggest that LSR is not a protein constituent of lipid rafts and localizes in lipid rafts only upon binding of CDTb (Papatheodorou et al. 2013). Notably, the RBD of CDTb, which is not able to oligomerize by itself, was sufficient to induce LSR-clustering into lipid rafts (Papatheodorou et al. 2013). Thus, oligomerization of the binding component seems not to be the main driving force that leads to accumulation of LSR in lipid rafts. In contrast, it is more likely that LSR clustering into lipid rafts facilitates oligomer formation of the binding component, which is important for recruiting the enzyme component into the toxin-receptor complex for subsequent

endocytosis. Toxin-driven receptor clustering into lipid rafts is not unusual and has been shown to also occur when *Helicobacter pylori* VacA toxin (Nakayama et al. 2006) and the anthrax toxin bind to their specific receptors, receptor protein-tyrosine phosphatase (RPTP) beta and anthrax toxin receptor (ATR), respectively (Abrami et al. 2003). Recently, Wigelsworth and colleagues reported that the lipid rafts-protein CD44 (cluster of differentiation 44) is necessary for uptake of CDT into host cells (Wigelsworth et al. 2012). CD44 is also detected in isolated lipid rafts obtained from Vero cells that were treated with the binding component of the iota toxin (Blonder et al. 2005). Therefore, CD44 might be a suitable candidate that binds to clustered LSR-toxin complexes in lipid rafts and triggers their endocytic uptake by a not yet fully understood mechanism.

4.5 The Endocytic Pathway of Iota-like Toxins

The endocytic route of iota-like toxins is still unclear. A study from Gibert and colleagues suggests that dynamin, but not clathrin, is crucial for endocytic uptake of the iota toxin (Gibert et al. 2011). The authors also found that iota toxin colocalized with the interleukin-2 receptor in endocytic vesicles, indicating similar endocytic routes for the interleukin-2 receptor and iota toxin (Gibert et al. 2011). Like iota like toxins, the interleukin-2 receptor associates with lipid rafts and is taken up via a clathrin-independent uptake mechanism. Endocytosis of the interleukin-2 receptor is regulated by RhoGDP-dissociation inhibitor (RhoGDI) (Lamaze et al. 2001). Strikingly, overexpression of RhoGDI in the African green monkey kidney fibroblast-like cell line Cos-1 prevented entry of iota toxin, which substantiates a common cell entry pathway of the interleukin-2 receptor and iota-like toxins (Gibert et al. 2011).

4.6 (Patho)Physiological Functions of LSR

LSR expression has been proven in various tissues. However, LSR is mainly expressed in the liver where it acts as a lipoprotein receptor. In this context, it was suggested that LSR contributes to the clearance of chylomicron remnants from the blood (Yen et al. 1994, 1999; Mesli et al. 2004). Surprisingly, a later study uncovered a so far unknown and unexpected function of LSR in the formation of tricellular tight junctions (Masuda et al. 2011; Furuse et al. 2012). Two LSR-related proteins, the immunoglobulin-like domain containing receptor (ILDR) 1 and ILDR2, also localize in tricellular contacts and recruit the protein tricellulin to the epithelial cell corners. LSR, ILDR1, and ILDR2 were thus designated as members of the angulin family (Higashi et al. 2012, 2013). Recently, the knockout of LSR, which was obtained by CRISPR/Cas9 technology, demonstrated and confirmed that LSR is crucial for proper localization of tricellulin in tricellular contacts also in the

colon-carcinoma cell line CaCo-2 (Czulkies et al. 2016). Another recent study indicates that LSR is critical for proper blood-brain barrier formation during development (Sohet et al. 2015). Given these important functions of LSR, it is not surprising that a homozygous deletion of LSR in mice lead to early embryonic death (Mesli et al. 2004).

4.7 A Role of LSR in Cancer

Compelling evidence accumulated for a role of LSR in cancer progression and metastasis. In a mouse mammary tumor model, LSR is found to be one of most up-regulated genes related to the development of visible metastasis (Yang et al. 2004). In addition, studies show that LSR is involved in both invasion and cellular movement of bladder cancer cells (Herbsleb et al. 2008). Moreover, LSR expression levels appear to be correlated with poor prognosis in human colon cancer (Garcia et al. 2007). Thus, these authors concluded that LSR might be a good tumor marker for colon cancer in humans. More recent studies demonstrate that LSR promotes aggressive breast cancer behavior (Reaves et al. 2014). Furthermore, knockdown of LSR in endometrial cancer cells induces increased cell migration, invasion, and proliferation (Shimada et al. 2016). Our laboratory attempted to study the effects of LSR knockout on the tumorigenic potential of CaCo-2 cells. We found in a mouse xenograft model that tumors originating from LSR-deficient CaCo-2 cells were not able to grow properly when compared to tumors originating from wildtype CaCo-2 cells. Histological analysis revealed increased apoptotic and necrotic cell death in tumors formed by CaCo-2 LSR knockout cells, as well as less prominent stromal component and less blood vessels (Czulkies et al. 2016). Thus, it seems that LSR is required for proper angiogenesis in tumors. Moreover, LSR knockout in CaCo-2 cells renders the cells unable to form a tight, impermeable epithelial barrier and microscopic analysis uncovered an aberrant epithelial sheet geometry (Czulkies et al. 2016). Alternative splicing generates several LSR isoforms that can be grouped into two categories based on the presence or absence of a transmembrane segment present in the middle part of the protein (Hemmasi et al. 2015). We could show for one such LSR isoform lacking a transmembrane helix that it resides within the endoplasmic reticulum (ER) after ectopic expression and that it does not integrate into the plasma membrane (Hemmasi et al. 2015). The function and fate of ER-localizing LSR isoforms is unknown so far and thus it will be of high interest to analyze how the different isoforms contribute to the various (patho)physiological functions of LSR and to cancer progression.

5 Conclusions

Knowledge about the uptake mechanisms of bacterial protein toxins not only enables the development of anti-toxin strategies but permits also the purposeful use of specific toxins as biological therapeutics. For instance, binary actin-ADP-ribosylating toxins were optimized for their use as molecular Trojan horses for drug delivery into target cells (Barth and Stiles 2008). However, identification of the toxins' receptors permits a more specific targeting of target cells. Fagan-Solis and colleagues emphasized the potential of toxin-LSR interaction for tumor therapy. They proposed that iota toxin might be useful for an effective, targeted therapy against LSR-overexpressing breast cancer cells (Reaves et al. 2014). Nonetheless, the potential role of LSR should not be restricted to breast cancer cells. Any type of cancer that exhibits overexpression of LSR might be targeted by iota-like toxins with higher potency and efficiency than healthy tissue.

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Clostridial Binary Toxins: Basic Understandings that Include Cell Surface Binding and an Internal "Coup de Grâce"

Bradley G. Stiles

Abstract *Clostridium* species can make a remarkable number of different protein toxins, causing many diverse diseases in humans and animals. The binary toxins of *Clostridium botulinum*, *C. difficile*, *C. perfringens,* and *C. spiroforme* are one group of enteric-acting toxins that attack the actin cytoskeleton of various cell types. These enterotoxins consist of A (enzymatic) and B (cell binding/membrane translocation) components that assemble on the targeted cell surface or in solution, forming a multimeric complex. Once translocated into the cytosol via endosomal trafficking and acidification, the A component dismantles the filamentous actin-based cytoskeleton via mono-ADP-ribosylation of globular actin. Knowledge of cell surface receptors and how these usurped, host-derived molecules facilitate intoxication can lead to novel ways of defending against these clostridial binary toxins. A molecular-based understanding of the various steps involved in toxin internalization can also unveil therapeutic intervention points that stop the intoxication process. Furthermore, using these bacterial proteins as medicinal shuttle systems into cells provides intriguing possibilities in the future. The pertinent past and state-of-the-art present, regarding clostridial binary toxins, will be evident in this chapter.

Contents

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1 Some Basics on Clostridia and Their Binary Toxins

1.1 Clostridium botulinum *C2 Toxin*

C. botulinum, like all clostridia, is a Gram-positive spore-forming bacterium found throughout the biosphere. Classic disease attributed to this clostridial species includes neurotoxin-based botulism, a flaccid paralysis that is potentially lethal and obtained via contaminated foods or wounds (Table 1) (Poulain et al. 2006). Like *C. perfringens*, *C. botulinum* also consists of multiple toxin types (A-G) neutralized by specific antisera in classic mouse lethal, or more recent gene probe-based, assays (Aras and Hadimli 2015; Fenicia et al. 2011; Holdeman et al. 1977). Although the botulinum neurotoxins are one of the most toxic toxins known and of nefarious interest (Roxas-Duncan and Smith 2015), these proteins are used medicinally and serve as a classic "sword to plowshare" template for other bacterial protein toxins (Dolly and Wang 2015; Poulain et al. 2015).

Quite distinct from the botulinum neurotoxins of *C. botulinum*, in structure and function, is the chromosome-based C2 toxin (Ohishi et al. 1980; Schleberger et al. 2006). This binary toxin is not implicated in human disease, but associated with *C. botulinum* types C and D intoxication of fowl, domesticated and wild (Anza et al. 2014; Ohishi and Sakaguchi 1982). The C2, and other, clostridial binary toxins portrayed in this chapter are enterotoxins naturally produced within the gastrointestinal tract. Furthermore, C2 toxin was the first clostridial binary toxin characterized as two, non-linked proteins in solution (Iwasaki et al. 1980; Ohishi et al. 1980). Excellent pioneering work was done by the group of Ohishi in Japan, which

Table 1 Clostridia-associated diseases and binary toxins

Microorganism	Diseases[a]	Binary toxin (AB components)
Clostridium botulinum	Botulism (infant, injected, food and wound), diarrhea	C2 (C2I and C2II)
Clostridium difficile	Colitis, diarrhea	CDT (CDTa and CDTb)
Clostridium perfringens	Gangrene, diarrhea, food poisoning, multiple sclerosis?	Iota (Ia and Ib), BEC (BECa and BECb), and CPILE (CPILE-a and CPILE-b)
Clostridium spiroforme	Diarrhea	CST (CSTa and CSTb)

[a]Noted diseases are not all linked to binary toxins, and in fact, many of these bacteria produce multiple toxins implicated in different diseases of humans and/or animals

in turn helped spawn discovery and characterization of other clostridial binary toxins around the world.

The C2 toxin components, produced during sporulation and not vegetative growth like many of the other clostridial binary toxins (Nakamura et al. 1978), consist of enzymatic A [C2I (49 kD)] and cell-binding B [C2II (81 kD protoxin/60 kD proteolytically activated)] proteins devoid of a leader sequence (Fujii et al. 1996; Kimura et al. 1998; Ohishi et al. 1980). The C2 toxin intoxicates all known cell types in vitro, and various animal organs in vivo, exploiting a ubiquitous asparagine-linked proteoglycan as a cell surface receptor for C2II (Barth et al. 2004; Blocker et al. 2001; Eckhardt et al. 2000; Fritz et al. 1995; Sugii and Kozaki 1990). All B components of the clostridial binary toxins are proteolytically activated within their N-terminus, resulting in loss of an ~15–25 kD peptide, promotion of homo-oligomerization (consisting of a hexameric or heptameric ring), and subsequent docking with up to three molecules of A (Barth et al. 2000; Blocker et al. 2001; Gibert et al. 2000; Kaiser et al. 2006; Nagahama et al. 2002, 2009; Ohishi 1987; Stiles 1987; Stiles et al. 2002a, b). For proteolytically activated C2II (C2IIa), the ensuing SDS-stable/heat-labile protein ring is ~420 kD with an outside/inside diameter of ~13 nm/ ~3 nm, respectively (Barth et al. 2000; Schleberger et al. 2006). The inner diameter of the C2IIa homo-oligomer is similar to that formed by heptameric protective antigen (PA; ~2 nm) from *Bacillus anthracis* binary toxins (edema and lethal) (Petosa et al. 1997). Assembly of a holotoxin occurs outside of the bacterium, either in solution or on the targeted cell surface (Barth et al. 2000; Blocker et al. 2001; Kaiser et al. 2006). The clostridial binary toxins thus differ from many other AB toxins, such as cholera or shiga, that are secreted from the bacterium as a biologically functional holotoxin (Heggelund et al. 2015; Sandvig et al. 2015).

The B components of clostridial binary toxins possess four domains, similar to *B. anthracis* PA (Petosa et al. 1997), consisting of the following: (1) domain I (N-terminal region) used for docking A component; (2) domain II (membrane insertion); (3) domain III (homo-oligomerization); and (4) domain IV (C-terminal region) involved in binding to cell surface receptor (Barth et al. 2004; Blocker et al. 2000; Marvaud et al. 2001). Variants of C2 toxin do exist depending upon the bacterial strain; for instance, domain IV can be longer than normal which in turn increases biological effects on cells (i.e., they round up more quickly following toxin exposure) (Sterthoff et al. 2010). It had been noted years prior that structural heterogeneity exists for both C2I and C2II components produced by different strains of *C. botulinum* (Ohishi and Hama 1992; Ohishi and Okada 1986), but similar studies have curiously not been reported for other clostridial binary toxins. This might be a fertile area of exploration in the future, which could gauge evolutionary drift of these toxins within a clostridial species.

Once inside the cytosol of an intoxicated cell following release from an acidified endosome (Barth et al. 2000; Blocker et al. 2001), the A components of clostridial binary toxins transfer ADP-ribose (from nicotinamide adenine dinucleotide, or NAD) onto a specific, surface-exposed arginine (Arg-177) found on various forms of globular (G) actin (Aktories et al. 1986, 2011, 2012; Belyy et al. 2015;

Ohishi and Tsuyama 1986; Popoff et al. 1989; Schering et al. 1988; Tsuge et al. 2008; Vandekerckhove et al. 1987). This latter event disrupts filamentous (F) actin formation and the cytoskeleton of a targeted cell, upsetting various aspects of homeostasis that then ultimately lead to death. Intracellular actin represents a common eukaryotic target exploited by various protein toxins (effectors) of different pathogenic genera (i.e., *Aeromonas*, *Photorhabdus*, *Salmonella*, *Shigella*, and *Vibrio*), using rather diverse mechanisms (Aktories et al. 2011, 2012; Barth et al. 2015). Prokaryotes also possess actin-like molecules (i.e., MreB) that maintain a bacterium's shape and facilitate growth, reproduction, and motility (Celler et al. 2013). Clearly, actin and related analogs are quite conserved and important for various life-forms found throughout our biosphere.

1.2 Clostridium perfringens *Iota Toxin and Binary Enterotoxin (BEC)/Iota-Like Enterotoxin (CPILE)*

Clostridium perfringens is one of the most "toxic" bacteria known, with over sixteen different protein toxins produced by types A-E that are involved in myriad diseases of humans and animals (Uzal et al. 2014). *C. perfringens* can be found throughout our biosphere that includes the soil, various foods, and feces. One of the four "major" typing toxins is iota, with alpha, beta, and epsilon representing the others classically tested in mouse lethal or guinea pig dermonecrotic assays using type-specific antisera (Holdeman et al. 1977; Oakley and Warrack 1953). The plasmid-based iota toxin is produced during vegetative growth of *C. perfringens* type E (Stiles 1987) and not made by *C. perfringens* types A-D (Holdeman et al. 1977; Oakley and Warrack 1953; Wilsdon 1931). *C. perfringens* type E iota toxin is associated with sporadic enterotoxemias in cattle, dogs, goats, and sheep (Billington et al. 1998; Bosworth 1943; Redondo et al. 2015; Ross et al. 1949; Songer and Miskimmins 2004). To date, there is no evidence of *C. perfringens* type E iota toxin causing disease in humans; however, other structurally related toxins produced by *C. perfringens* and *C. difficile* are strongly implicated, as described below.

C. perfringens type E iota toxin was the first clostridial binary toxin: (1) presented in the literature, over seventy years ago (Bosworth 1943), and (2) found to require proteolysis for activation (Ross et al. 1949). However, iota toxin's binary nature was elucidated decades later and after that described first for C2 toxin (Ohish et al. 1980; Stiles and Wilkins 1986a, b). The breakthrough in iota's binary composition came from coupling cross-reacting antiserum against *Clostridium spiroforme* with crossed-immunoelectrophoresis of *C. perfringens* type E culture filtrate. Results revealed two immunoprecipitin arcs that represent the slower-migrating iota a (Ia, 45 kD enzyme component) and faster-migrating iota b (Ib, 94 kD protoxin/81 kD proteolytically activated cell binding and translocation forms) (Perelle et al. 1993; Stiles and Wilkins 1986a, b). Like C2, once inside a cell the iota toxin (Ia component) attacks the cytoskeleton via mono-ADP-ribosylating G-actin (Vandekerckhove et al. 1987).

Unlike C2, the iota family of toxins uses another surface receptor(s) for binding to a cell (Hemmasi et al. 2015; Papatheodorou et al. 2011, 2012, 2013), as described in detail below.

Recent discoveries of a novel, clostridial binary toxin(s) from *C. perfringens,* involved in human cases of food poisoning, provide unique insight into human susceptibility to clostridial binary toxins (Irikura et al. 2015; Yonogi et al. 2014). These toxins, called binary enterotoxin of *C. perfringens* (or BEC) and *C. perfringens* iota-like enterotoxin (CPILE), are described by different groups after investigating four different food-borne outbreaks (dating back to 1997) throughout Japan (Irikura et al. 2015; Monma et al. 2015; Yonogi et al. 2014). The isolates from each outbreak, typed as *C. perfringens* A and not carrying the *C. perfringens* enterotoxin (CPE) gene (*cpe*) commonly associated with human cases of *C. perfringens* food poisoning (Uzal et al. 2014), are genetically different as per pulsed-field gel electrophoresis (Monma et al. 2015). Patient's symptoms included diarrhea and abdominal pain occurring within 10–15 h after ingestion of different food types, including meats (Monma et al. 2015). Although not reported, it might be interesting to investigate the prevalence of CPILE/BEC genes in *C. perfringens* isolated from animals used for meat. A limited, gene-based study of *C. perfringens* from human feces ($n = 26$ isolates, not linked to aforementioned outbreaks) detected one CPE-negative, BEC-positive strain from a healthy patient that suggests (for now) a low prevalence rate of BEC in nature (Yonogi et al. 2014).

Varying amounts of characterization have occurred with the A and B components of BEC and CPILE, which are based on a plasmid like *C. perfringens* iota toxin. The sequence identity of CPILE-a and CPILE-b components, with other clostridial binary toxins, respectively, ranges between 29–44 % (C2I—Ia) and 37–39 % (C2II—*C. difficile* toxin B, or CDTb) (Irikura et al. 2015). Results for BECa and BECb are quite similar, respectively, revealing 29–44 % (C2I—Ia) and 41–43 % (C2II—Ib) sequence identity (Yonogi et al. 2014). BECb (not BECa) alone is enterotoxic in suckling mice, and this enterotoxicity is synergistically increased with BECa (Yonogi et al. 2014). Interestingly, both research groups respectively reveal the A and B components to be 419 and 799 amino acids, as well as devoid of signal peptides (Irikura et al. 2015; Yonogi et al. 2014). The A component possesses NADase and ADP-ribosyl transferase activities for G-actin (Belyy et al. 2015; Gulke et al. 2001; Irikura et al. 2015; Tsuge et al. 2008). A comparison of these collective results suggests that BEC and CPILE are the same toxins.

A lack of signal peptides for BEC and CPILE components is quite curious, as all clostridial binary toxins described to date (except C2) possess a signal peptide for each component (Popoff 2000). Production of this novel toxin by these different strains occurs in spore-forming media (i.e., Duncan-Strong), but not others that do not induce sporulation of *C. perfringens* (Monma et al. 2015). Furthermore, as CPE is often linked to food poisoning in humans and produced during sporulation within the small intestines, it is noteworthy that these Japanese isolates lack *cpe* (Monma et al. 2015). However, *C. perfringens* type E strains isolated from retail meats in Japan can evidently carry a functional *cpe* and thus represent a novel group of bacteria (Miyamoto et al. 2011). It was noted earlier by Billington et al. that type E isolates from scouring

neonatal calves might have been type A, converted by horizontal transfer of an episome containing the iota, plus silent CPE, toxin genes (Billington et al. 1998). Irikura et al. interestingly propose a modification of the *C. perfringens* typing scheme, thus splitting type E into E1 (strains that produce iota toxin and are pathogenic to animals) and E2 (strains that produce CPILE/BEC and are pathogenic to humans) (Irikura et al. 2015). This is a revolutionary thought for an originally adopted, rarely challenged typing scheme involving toxin–antitoxin reactions introduced in 1931 (types A-D; Wilsdon 1931), with the addition of type E in 1943 after iota toxin's discovery (Bosworth 1943). As an iota or iota-like toxin has never been associated with human disease before, this modified classification following recent discovery of CPILE/BEC might warrant further discussion. Additionally, these collective findings could also stimulate ongoing debate about *Clostridium difficile* binary toxin and its impact upon human disease (i.e., colitis), succinctly presented below (Androga et al. 2015; Eckert et al. 2015; Gerding et al. 2014).

1.3 Clostridium spiroforme *Toxin (CST)*

C. spiroforme is a distinctly coiled bacillus associated with rabbit enterotoxemias, spontaneous and antibiotic-induced (Borriello and Carman 1983; Borriello et al. 1986). The organism produces a chromosome-based CST discovered during the same time period as *C. perfringens* iota toxin (Borriello et al. 1986; Popoff and Boquet 1988; Popoff et al. 1989; Simpson et al. 1989; Stiles 1987). CST consists of enzymatic CSTa (44 kD) and cell-binding CSTb (92 kD protoxin/76 kD proteolytically activated) components (Gibert et al. 1997; Popoff et al. 1989; Simpson et al. 1989; Stiles 1987). Mixing of heterologous A and B components of CST and iota yields biologically active hybrid toxins (Perelle et al. 1997b; Stiles 1987), which is not the case with *C. botulinum* C2 toxin components (Popoff and Boquet 1988; Simpson et al. 1987, 1989). C2 toxin components are also immunologically distinct from CST and iota (Popoff and Boquet 1988; Popoff et al. 1989). It is still unknown if the C2 components form hybrid binary toxins with the other sporulation-linked BEC/CPILE components of *C. perfringens* (Irikura et al. 2015; Monma et al. 2015; Yonogi et al. 2014).

Furthermore, antibodies against CSTa and CSTb neutralize CST, as well as *C. perfringens* iota toxin (Perelle et al. 1997b; Popoff et al. 1989; Stiles 1987). In fact, the converse scenario occurs and early investigations of some rabbit enterotoxemias mistakenly incriminated *C. perfringens* type E as the causative agent, albeit this bacterium was never isolated from diseased rabbits (Baskerville et al. 1980; Borriello and Carman 1983; Borriello et al. 1986; Eaton and Fernie 1980). By using a clever, ethanol-based selection process on fecal samples that target clostridial spores, an English group was the first to link *C. spiroforme* to fatal diarrheal disease in rabbits (Borriello and Carman 1983). Similar antiserum-based leads (misleads?) linked to *C. difficile* toxins A and B (large molecular weight toxins not to be misconstrued as binary toxins), and cross-reacting/cross-neutralizing antisera

developed against *Clostridium sordellii* toxins is evident in the literature (Chang et al. 1978). Although clearly useful in many ways, results of antibody-based studies used to identify pathogens must be interpreted carefully as successful paradigms (i.e., protein toxins) are often copied by various species. Clearly, success breeds success.

These stories of the past suggest genetic sharing among bacteria of common virulence factors that are evolutionarily successful. Many diagnostic laboratories are now using gene-probe-based methods for detecting pathogens and virulence factors, which includes *C. spiroforme* and its toxin genes (Drigo et al. 2008). Given such technology, it might be interesting to do a "global" study on various bacterial genera to see whether clostridial binary toxin-like genes are dispersed elsewhere in nature.

1.4 Clostridium difficile *Toxin (CDT)*

CDT is the third, most recently recognized member of the iota family of toxins, as per amino acid sequence identity between A and B components (~80 %) from other clostridial species, antibody cross-reactivity and neutralization, production during vegetative growth, as well as chimeric toxin formation with *C. perfringens* iota and *C. spiroforme* CST components (Perelle et al. 1997a, b; Popoff and Boquet 1988; Popoff et al. 1989; Popoff 2000; Stiles 1987). Neither the *C. botulinum* C2 toxin nor *C. perfringens* BEC/CPILE are part of the iota family. Increasingly, "hypervirulent" strains of *C. difficile* producing CDT are being isolated from various outbreaks around the world (Carman et al. 2011; Gerding et al. 2014). It is still questionable though the role CDT plays during *C. difficile* colitis, pending more study (Gerding et al. 2014; Geric et al. 2006; Kuehne et al. 2014); however, strains lacking large molecular weight toxins A and B (not binary toxins), but possessing CDT, have been recently associated with human colitis (Androga et al. 2015; Eckert et al. 2015). Such strains (*tox A*-, *tox B*-, *cdt*+) are likely under reported as many diagnostic laboratories historically focus upon toxins A and B, not CDT.

The chromosome-based CDT components are structurally like other clostridial binary toxins, consisting of enzymatic CDTa (48 kD) and cell-binding CDTb (99 kD protoxin/75 kD proteolytically activated) (Perelle et al. 1997a). All of the clostridial binary toxins have similar enzymatic properties (i.e., mono-ADP-ribosylate G-actin), and not surprisingly, it has been recently discovered that the iota family of toxins also shares a common cell surface receptor(s) described below (Papatheodorou et al. 2011, 2012, 2013; Wigelsworth et al. 2012). Clostridial binary toxins (CDT, iota, C2, and perhaps others yet untested?) interestingly promote in vitro (cell culture) and in vivo (mouse large intestine) bacterial adherence via cytoskeleton rearrangements that include microtubule-based protrusions from the cell's membrane (Schwan et al. 2009, 2014). This process involves rerouting of fibronectin to the apical (not basolateral, extracellular matrix) surface, perhaps slowing bacterial (and toxin) expulsion from the gastrointestinal tract and exacerbating disease. These toxin-induced protrusions, and how rapidly they form plus

their overall length, are dependent upon toxin dose and degree of disruption upon the actin filaments.

In addition to humans, *C. difficile* is now known to colonize various animals subsequently consumed as meat by humans (Lund and Peck 2015). It is still unknown if contaminated food (also to include vegetables and shellfish) can lead to *C. difficile* colitis in humans, but the possibility (supported by strain ribotype identity) is intriguing and likely addressed more thoroughly in the near future. The disease aspects of *C. difficile* colonization upon the human intestinal tract can be mild (asymptomatic) to quite serious (death), involving various virulence factors characterized to date that include antibiotic resistance, spore formation, and various protein toxins (Awad et al. 2014; Gerding et al. 2014). Patients that are Caucasian, female, and over 65 years of age are more likely to suffer from *C. difficile* colitis, which in the USA during 2011 affected ~450,000 patients (killing ~29,000 or 6 %) (Lessa et al. 2015).

Early studies ironically suggested this bacterium (known then as *Bacillus difficilis*) to be part of the healthy flora in new-born infants (Hall and O'Toole 1935), difficult to grow (probably because of its anaerobic requirements), and non-pathogenic for adults (Smith and King 1962). It was not until the 1970s, and specific antibiotic use (i.e., clindamycin), that *C. difficile* was recognized as a toxin-producing gastrointestinal pathogen of human adults (Bartlett et al. 1978; Fekety et al. 1979; Larson et al. 1978). It is now known that *C. difficile* causes a major form of nosocomial infection, commonly associated with antibiotic use, and is also a community-acquired pathogen costing the USA over $3 billion/year for patient treatment (Awad et al. 2014; Lessa 2013). Besides forming long-surviving spores, strains of *C. difficile* have also evolved increased: (1) fluoroquinolone resistance; (2) production of toxins A and B; and (3) prevalence of binary toxin (Awad et al. 2014; Gerding et al. 2014). Many laboratories have rightly focused upon toxins A and B for many years, which share >60 % sequence similarity and glucosylation (inactivate) various Rho family of proteins controlling cytoskeleton formation and cell function (Jank and Aktories 2008). Cells are ultimately killed by toxins A and B, leading to decreased intestinal absorption and increased secretion of fluids into the intestinal lumen witnessed as classic, profuse (in some cases) diarrhea. Furthermore, toxins A and B also provoke inflammation, and this can explain the formation of a "pseudomembrane" (consisting of bacteria, fibrin, cell debris, and white blood cells) in advanced cases of *C. difficile* colitis (Ng et al. 2010; Shen 2012). Both adaptive and innate immunity play a role in host responses toward *C. difficile* and its toxins, surface layer proteins, flagellin, etc. (Sun and Hirota 2015). Other than select antibiotics (which can also initiate *C. difficile* colitis) and fecal flora transplants, there are no alternative therapies or prophylactics (i.e., vaccine) available for clinical use to date (Waltz and Zuckerbraun 2016). Recurring bouts of colitis can be common for some unfortunate patients.

The various clostridial binary toxins, and their bacterial producers, have now been introduced as per a very broad perspective involving physical attributes and disease. Much more could be, and has been, written regarding the many aspects of these clostridia and their binary toxins (for reviews, see Barth et al. 2004, 2015;

Gerding et al. 2014; Popoff 2000; Sakurai et al. 2009; Stiles et al. 2011). Let us focus upon the initial stages of intoxication via host-provided, cell surface receptors exploited by these toxins.

2 Cell Surface Receptor(s) for Clostridial Binary Toxins

Like many bacterial protein toxins, to be "toxic," the clostridial binary toxins must first specifically engage a "receptive" cell surface molecule (Horiguchi and Mekada 2006; Schmidt et al. 2015). This usurped, host-provided receptor (i.e., a specific protein, glycoprotein, glycolipid, and/or carbohydrate) enables the intoxication process that is quite complex, ultimately leading to a bad ending for the cell (and macro-organism!). One of the first molecules characterized as a receptor for any bacterial toxin (cholera) was a ganglioside, GM1 (Cuatrecasas 1973). Besides soluble proteins (toxins) released into the environment by various pathogenic bacteria, the use of secretion systems (basically a protein-based syringe, or "nanomachine") to inject toxins (effectors) into targeted cells represents another way of "subjugating" host cells but requires intimate contact between bacterium and targeted cell (Filloux and Sagfors 2015). To date as per the discoveries of many laboratories, various Gram-negative genera employ at least nine different secretion systems to transport effector molecules through the bacterium's complex cell wall/membrane and then through targeted, eukaryotic (or prokaryotic) membranes into the cytosol. An advantage of a soluble factor (i.e., toxin) released by a bacterium into the environment is a "global" or "carpet bombing" effect, which involves toxin dissemination throughout the host via the normal workings of the circulatory system, gastrointestinal peristalsis, interstitial fluids via lymphatic system, etc. However, a bacterium might "invest" more energy into producing many copies of a soluble protein toxin that by chance meets a receptor, versus relatively small amounts of an injected protein effector that goes directly into a targeted cell. Clostridial binary toxins require a relatively large investment by the bacterium, to produce many copies of A and B components. Might this seemingly random encounter of protein components, outside of the bacterium but on the cell surface or in the environment, represent an archaic method of intoxication? Clostridia and aerobic, spore-forming cousins (*Bacillus* species, such as *B. anthracis*) are considered early forms of life on earth (Fox et al. 1980), and both genera produce structurally similar binary toxins described in this chapter.

For the clostridial binary toxins, receptor-based studies were first reported with the C2 toxin. The binding of C2 toxin was initially explored in Japan using brush border membranes isolated from mouse intestines (Ohishi and Miyake 1985). Immunofluorescence-based results established C2IIa as the cell-binding component necessary for subsequent docking of C2I to oligomeric C2IIa and the cell surface. Subsequent pioneering work by the same group revealed similar results in Vero (African Green Monkey kidney) cells (Ohishi and Yanagimoto 1992), a cell type used by many groups for studying various aspects of clostridial binary toxins.

The first effort to further characterize the C2II receptor was via hemagglutination of animal and human erythrocytes (Sugii and Kozaki 1990). Results reveal that pronase pretreatment of cells, or use of various sugars (i.e., fucose, mannose, and N-acetylglucosamine) as competitive inhibitors of C2II, inhibit binding of the C2 toxin to erythrocytes. The authors accurately surmised the receptor to be a glycoprotein, leading to the next round of discoveries emanating a few years later from Germany.

Fritz et al. next employed chemical mutagenesis of CHO cells to generate C2-toxin-resistant mutants that do not bind C2II on their cell surface, in contrast to wild type (Fritz et al. 1995). Results show that one particular mutant still retained wild-type cytopathic effects when incubated with other bacterial toxins, including *C. perfringens* iota toxin. This latter fact plays a role in this same group discovering, with different cells and technology, the cell surface receptor for the iota family of toxins that is not shared by C2 (Papatheodorou et al. 2011, 2012, 2013). The actin cytoskeleton and internalization machinery in these mutant CHO cells are fully functional, as per the respective effects of a fungal alkaloid (cytochalasin D, which inhibits F-actin formation) and cytotoxicity of other internalized, actin-targeting bacterial toxins (i.e., *C. botulinum* C3, *C. difficile* toxin B, and *C. novyi* alpha). The authors also note that incubation of increasing fetal calf serum concentrations with wild-type CHO cells decreases C2 toxin cytotoxicity, perhaps suggesting linkage of the C2 toxin receptor to growth regulation (Fritz et al. 1995). Additional experiments in this study show the protective serum factor to be a heat-activated/heat-resistant, heparin-binding, pronase-sensitive protein of 50–100 kD size.

Five years later, this same group reported that binding of C2 toxin to cells is dependent upon asparagine-linked carbohydrates (Eckhardt et al. 2000). The aforementioned CHO cells, chemically mutagenized, are deficient in N-acetylglucosaminyltransferase I (GlcNAc-TI) which covalently links carbohydrates to asparagine-containing proteins. Glycolipid formation is not affected by this enzyme, thus ruling out a glycolipid receptor for C2 toxin. In particular, transfection of this C2-toxin-resistant cell line with GlcNAc-TI cDNA restores a wild-type (C2-toxin susceptible) phenotype. Whether C2 toxin binding is dependent upon carbohydrate alone, or requires protein interactions too, is still not resolved to date. Identity of the specific protein acting as an anchor for carbohydrates important in C2 toxin binding to cells also remains a mystery. Clearly, identification of a cell surface receptor and knowing (via existing literature) what "services" this molecule naturally provides for a eukaryotic cell during non-toxic, healthy-homeostatic circumstances can yield powerfully novel insight into the intoxication process and how to perhaps intervene therapeutically.

In contrast to the C2 toxin receptor story, receptor studies for the iota family of toxins have taken a different path toward some very definitive discoveries. Initial studies with *C. perfringens* iota toxin were reported from the USA using flow cytometry and various cell lines (Stiles et al. 2000). Flow-cytometry-based studies were also reported for binding of the C2 toxin components to Vero and CHO cells, further demonstrating the utility of this basic technique for studying clostridial

binary toxins (Stiles et al. 2002a). The first study unveiled a correlation between different cell types, their relative susceptibility to iota toxin, and binding of Ib to the cell surface (Stiles et al. 2000). Preincubation of Vero cells with either a lectin (n = 20 tested) or glycosidase (n = 8 tested) of varying specificities did not prevent iota cytotoxicity, thus suggesting carbohydrates as unimportant in cell binding by Ib. However, pronase pretreatment of cells effectively prevents Ib binding and suggests a rather robust protein receptor that is however resistant to ten other proteases (Stiles et al. 2000). This same study reveals that Ia docking is dependent upon trypsin-activated Ib, while the protoxin form of Ib (Ibp) binds to cells but does not interact with Ia. Ia, bound to Ib, readily disappears from the cell surface at 37 °C (i.e., 90 % loss within 30 min). The next level of receptor-based studies for the iota toxin, and family, came from Germany, and the results have definitively identified a common receptor: lipolysis-stimulated lipoprotein receptor (LSR) (Hemmasi et al. 2015; Papatheodorou et al. 2011, 2012, 2013). Identification of LSR represents a major breakthrough in this field.

LSR is a transmembrane, single-pass protein found in multimeric isoforms and tissues (especially liver). Normally, LSR facilitates cell uptake of low-density, triglyceride-rich lipoproteins but also plays a role in atherosclerosis as well as breast cancer (Reaves et al. 2014; Yen et al. 1999, 2008). The multifunctional LSR serves an integral role in epithelial barriers (i.e., intestines), particularly tight junctions, in which the latter have been exploited via other gut-acting, protein toxins such as CPE that bind to select claudins (Kimura et al. 2010; Masuda et al. 2011; Uzal et al. 2014). Leptin, an adipocyte-based hormone, can regulate expression of LSR in liver which in turn controls lipid storage and thus obesity (Stenger et al. 2010).

The revolutionary report that CDT and iota toxin exploit LSR as a cell surface receptor has opened up various opportunities in this field (Papatheodorou et al. 2011). The technology employed was mutagenesis (retroviral gene-trap versus chemical, as described above for C2 toxin) and inverse PCR of a leukemia cell line (KBM7 versus CHO, as described above). Pull-down assays of CDTb with the extracellular domain of LSR reveal direct binding. In contrast to CDT and iota, LSR-negative cells are still susceptible to C2 toxin which further supports the previous studies showing that the latter exploits a different cell surface receptor (Fritz et al. 1995; Papatheodorou et al. 2011). C2 toxin also does not colocalize with LSR during trafficking through the endosome (Papatheodorou et al. 2012). Furthermore, the cell-binding C-terminal domain (IV) of C2II and CDTb shares only 12 % sequence similarity that in turn structurally suggests recognition of different receptors (Papatheodorou et al. 2011). Not surprising though, the iota family member CST also binds to LSR (Papatheodorou et al. 2012).

Very recent studies show that the C-terminal region of CDTb (domain IV) binds to the C-terminal, immunoglobulin-like, surface-exposed region of LSR with nanomolar affinity via surface-plasmon resonance spectroscopy (Hemmasi et al. 2015). Flow cytometry studies also reveal saturable, nanomolar Kd binding of CDTb to LSR on two different human-colon cell lines. It still remains mysterious as to how LSR might further promote intoxication by the iota family of toxins. For instance, does LSR somehow facilitate internalization and pore formation? Perhaps

CD44 (size ranging from ~85–250 kD), a multifunctional transmembrane glycoprotein found in many cell types and isoforms (Jordan et al. 2015), plays a role in internalization and/or pore formation initiated by B component–LSR interactions (Wigelsworth et al. 2012). CD44 naturally binds to various ligands (i.e., hyaluronic acid that promotes wound healing) as well as facilitates adhesion, migration, and proliferation of eukaryotic cells; however, CD44 is also implicated in various diseases that include cancer (metastasis plus growth) and arthritis (Jordan et al. 2015). CD44 has also been exploited by infecting prokaryotes such as *Escherichia*, *Listeria*, *Shigella*, and *Streptococcus* species (Cywes and Wessels 2001; Jung et al. 2009; Lafont et al. 2002; Rouschop et al. 2006), as well as poliovirus (Shepley and Racaniello 1994). CD44, like clostridial binary toxins, is endocytosed into cells via a clathrin-independent pathway that does not merge with transferrin-containing endosomes (Eyster et al. 2009; Gibert et al. 2011). Interestingly, CD44 is associated with cell signaling and the actin cytoskeleton via a complex of proteins called ERM (ezrin–radixin–moesin) (Mori et al. 2008).

Why a potential role for CD44 during intoxication of eukaryotic cells by the iota family of toxins? Proteomic-based results with clostridial binary toxins (but not C2) show that CD44, increasingly found in lipid rafts (described below) following incubation of Vero cells with Ib, might be a coreceptor facilitating iota intoxication in vitro (cell cultures) and in vivo (mouse lethality) (Blonder et al. 2005; Wigelsworth et al. 2012). It should be noted that a physical link between LSR and CD44 has not been established via standard pull-down experiments. LSR and CD44 are type-I transmembrane proteins such as TEM8 and CMG2, in which the latter two molecules differ by a 1000-fold in binding affinity for the related *B. anthracis* B component, PA (van der Goot and Young 2009). So, how might CD44 facilitate entry of iota family toxins into cells? Clearly, there is work to be done to help clarify surface interactions and subsequent endocytosis, of clostridial binary toxins. LSR and CD44 provide very specific, membrane-based targets for some exciting studies in the near future.

It is known that CDTb, or just the domain IV fragment of CDTb, triggers clustering of LSR into lipid rafts (Papatheodorou et al. 2013). Lipid rafts are normal, cell membrane microdomains (cholesterol concentrated, detergent-insoluble at 4 °C) exploited by some bacteria (their toxins too) and viruses to gain entry into targeted cells (Simons and Ehehalt 2002). Interestingly, protein oligomerization such as that seen with B components, of clostridial binary toxins, can lead to higher affinity for lipid rafts. CDTb-induced clustering of LSR into rafts supports earlier studies showing the importance of lipid rafts in both iota and C2 intoxication (Blonder et al. 2005; Hale et al. 2004; Nagahama et al. 2004, 2009). With iota toxin, the Ib complex forms very rapidly (within 15 s) on Vero cells at 37 °C and remains for an extended time, thus acting as a docking platform waiting for a chance encounter with Ia (Nagahama et al. 2002; Stiles et al. 2000, 2002b). This too is seemingly demonstrated by earlier, in vivo studies showing that Ib effectively docks with Ia when intravenously injected 2 h before Ia (mouse lethality), or when Ia and Ib are injected into guinea pigs at distal sites (i.e., intraperitoneal and intradermal, respectively) (Ohishi 1983; Sakurai and Kobayashi 1995). Furthermore, only monomeric forms of

Ib are evident on cells at 4 °C in vitro, suggesting the importance of membrane movement for oligomerization (Nagahama et al. 2002; Stiles et al. 2002b). Once Ib binds to the cell, subsequent-formed homo-oligomers (but not monomers) become resistant to pronase which suggests embedment of this complex into the cell membrane (Nagahama et al. 2002). It is also evident that Ibp, when bound to cells, remains as a monomer and does not form an oligomer even after trypsin or chymotrypsin treatment of these cells (Stiles et al. 2002b). Ib can be transported in polarized CaCo-2 cells, from one surface to another (i.e., apical to basolateral), in a functional (i.e., docks with Ia) sense (Richard et al. 2002). Cell surface receptor for Ib is evidently more abundant on the basolateral surface of polarized CaCo-2 cells (Blocker et al. 2001; Richard et al. 2002). Upon internalization of Ib, which occurs regardless of Ia docking, lysosome-degraded forms of Ib can recycle back to the plasma membrane and perhaps extend the time period for Ia docking and intoxication (Nagahama et al. 2012, 2014; Richard et al. 2002).

Overall, many groups have provided very revealing insight into the cell surface interactions of clostridial binary toxins. Such studies could also logically lead to novel, receptor-based therapies useful (and necessary!) against these bacteria (and toxins), particularly hypervirulent strains of *C. difficile* (Waltz and Zuckerbraun 2016). From a therapeutic perspective, disruption of a bacterial toxin before it passes through the outer membrane of a targeted cell is easier than trying to chase down an intracellularly located toxin via drug delivery into a cell and hoping that a sufficient, localized concentration stops the intoxication process.

3 Internalization and Translocation: Watch Out Actin-Based Cytoskeleton!

Upon binding to a cell, clostridial binary toxins must be internalized to elicit an effect within the cell (Fig. 1). Enzymatic A component enters the cytosol, the source of substrate (NAD) and target (G-actin; ~42 kD), ultimately resulting in cytoskeletal destruction involving decreased F-actin formation. However, this classic paradigm has been challenged. For instance, there is a report that B component alone (i.e., Ib) can cause rapid necrosis in certain cell types, without forming a holotoxin with Ia (Nagahama et al. 2011). It was noted earlier that Ib only can cause dermonecrosis in guinea pigs, but at relatively high amounts (Stiles 1987). It was thought then that undetectable amounts of Ia might contaminate the Ib preparation (Stiles 1987), as iota toxin was isolated from crude, culture filtrate of *C. perfringens* type E versus being an *E. coli* (or *Bacillus* species) recombinant product commonly used today (Nagahama et al. 2011). There is obviously no possibility of Ia contamination of Ib, when the latter is produced as a recombinant product. The cell-based studies with Ib only show mitochondrial disturbance and rapid loss of ATP (~90 % in 120 min), which evidently play a role in Ib-induced necrosis of cells (Nagahama et al. 2011).

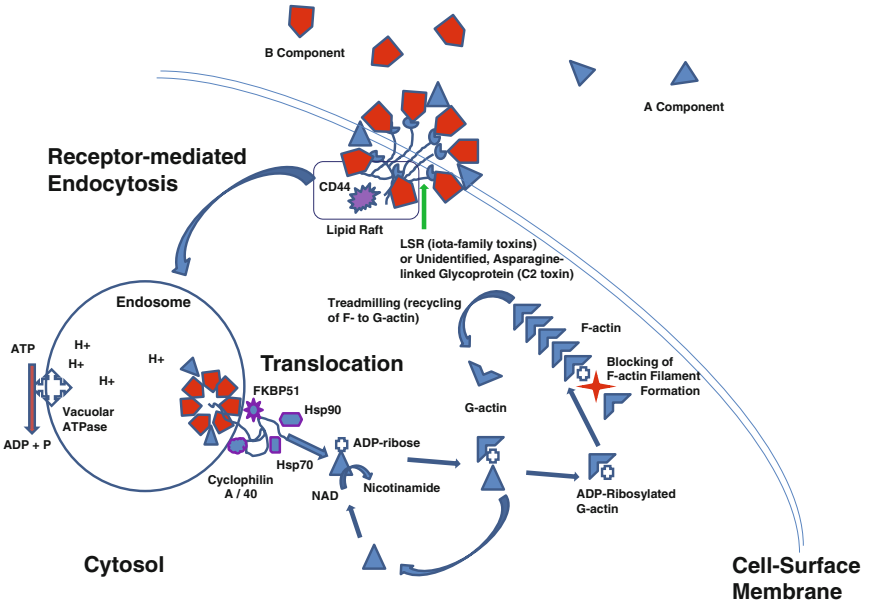

Fig. 1 Intoxication of eukaryotic cells by clostridial binary toxins: current, basic depictions

It is hypothesized that cells capable of internalizing Ib avoid necrosis, perhaps processing (degrading/inactivating?) Ib in a timely fashion. These results might also link to those showing that Ib alone decreases transepithelial resistance of cell monolayers or artificial lipid bilayers, forming sodium-/potassium-permeable pores that are closed in the presence of Ia (Knapp et al. 2002; Nagahama et al. 2002; Richard et al. 2002; Stiles et al. 2002b). Ibp, which does not form an oligomer, does not form ion-permeable pores (Knapp et al. 2002; Stiles et al. 2002b). Also, heat-treated Ib (60 °C/15 min) still binds to cells but will not form a biologically active iota toxin because there is no homo-oligomer formation or docking to Ia (Stiles 1987; Stiles et al. 2000, 2002b). Furthermore, pore formation by Ib (and other B components) depends upon proteolytic activation, oligomerization, and regarding translocation of A components into the cytosol, there must be acidic conditions (Barth et al. 2000; Blocker et al. 2001, 2003a, b; Knapp et al. 2015; Schmid et al. 1994). In addition to iota toxin and Ib, recent studies with *C. perfringens* BEC in suckling mice show that recombinant BECb alone ($\geq$1 μg) causes a dose-dependent fluid accumulation; however, this biological effect is enhanced (like other clostridial binary toxins) with complementary A component, BECa, which has no inherent enterotoxicity by itself (Yonogi et al. 2014).

Regarding receptor-mediated endocytosis of clostridial binary toxins (Simpson 1989), various groups have explored this aspect of intoxication as evidenced by a French group reporting that dynamin, not clathrin or Golgi, is important in a Rho-GDI governed endocytosis of both iota and C2 toxins (Gibert et al. 2007, 2011). This clathrin-independent endocytosis, by Vero cells, is akin to that

employed by the interleukin-2 receptor (Lamaze et al. 2001). In contrast though, another group has described endocytosis of C2 toxin as being partially clathrin-dependent in HeLa cells (Pust et al. 2010). A common note between these studies is the use of dynamin and Rho during receptor-mediated endocytosis of C2 toxin. It is however unknown why there are different results linked to clathrin involvement in C2 toxin internalization, but one obvious point involves experiments with different cells and methodology. Additionally, for endocytosis of C2 toxin, the phosphatidylinositol 3-kinase and protein kinase B/Akt pathways become activated and facilitate toxin entry after C2I docking to oligomeric C2IIa on lipid rafts (Nagahama et al. 2009). This pathway, important for normal vesicular trafficking and mitogenesis in eukaryotic cells, has been exploited by other pathogens for their own entry into targeted cells (Kierbel et al. 2005). Ironically, the actin cytoskeleton but not microtubules is important for internalization of C2 toxin, that in turn destroys the former (Barth et al. 2000). This perhaps is a governing mechanism preventing further toxin internalization into an already cytoskeletal-destroyed cell, thus conserving toxin for other less-intoxicated cells.

Direct cytosolic entry of clostridial binary toxins from the outside of a cell, bypassing endosomal trafficking, is possible and occurs in an acidic environment requiring both A and B components (Barth et al. 2000; Blocker et al. 2001, 2003b). An acidic pulse of the medium (as short as a 30 s exposure) leads to faster cytotoxic effects versus endosomal trafficking of toxin (Barth et al. 2000; Kaiser et al. 2011). There are though specific pH requirements that differ between toxins, as C2 and iota toxins require a respective pH minimum of 5.4 and 5.0 for direct entry mimicking early versus late endosomal release of C2I versus Ia (Gibert et al. 2007; Nagahama et al. 2014). Oligomeric B-induced pores of clostridial binary toxins naturally form a conduit for A molecules from an acidified endocytic vesicle, into the cytosol. These pores represent a promising target for therapeutics that include cationic (not neutral) beta-cyclodextrin derivatives (Bezrukov et al. 2012; Nestorovich et al. 2011) and heterocyclic azolopyridinium salts (Bronnhuber et al. 2014), which physically block B-induced pores when applied to the cis surface. Select phenylalanines within the lumen of these negatively charged pores, representing part of the psi-clamp, are seemingly important for binding of inhibitor (Bezrukov et al. 2012; Neumeyer et al. 2008). Interestingly, these same inhibitors block cytosolic entry of other binary toxins (edema and lethal) produced by *B. anthracis*, affording protection in vitro and in vivo (Bezrukov et al. 2012; Nestorovich et al. 2011). Chloroquine (a cationic molecule) is also effective against C2IIa pores (Bachmeyer et al. 2001), but not those generated by oligomeric Ib (Knapp et al. 2002), thus suggesting channel differences among the clostridial binary toxins. There are other evident differences in translocation of A components among these toxins. For instance, iota toxin requires a membrane potential (not C2 toxin), and Ia is released between early and late endosomes (Gibert et al. 2007). The C2I component enters the cytosol during early endosomes (Nagahama et al. 2014). Another way to prevent A entry into the cytosol is to use bafilomycin A1, a *Streptomyces*-derived, low molecular weight compound, which inhibits vacuolar ATPases that acidify endosomes (Barth et al. 2000; Blocker et al. 2001). Bafilomycin A1 prevents

intoxication by all clostridial binary toxins, tested to date. This inhibitor also causes accumulation of Ib in endosomes (Nagahama et al. 2012).

It is likely that clostridial binary toxin A components "thread" through the B-induced pore (~3 nm inner diameter for C2IIa: Barth et al. 2000; Schleberger et al. 2006) via reversible melting of the A component conformation (Haug et al. 2003b). This is further accomplished with the help of host-provided chaperones identified as heat-shock protein (Hsp) 90 and cyclophilin A, which facilitate translocation into the cytosol (Barth et al. 2015; Haug et al. 2003a, 2004; Kaiser et al. 2009, 2011). Hsp90 and cyclophilin A, the latter being a peptidyl-prolyl cis/trans isomerase, play important roles in various facets of cell life that pertinently include protein refolding and stabilization into a native, biologically active state (Barth 2011; Chen et al. 2006). Hsp90 is ubiquitously conserved in five subfamilies among diverse eukaryotic and prokaryotic life-forms, but not Archae (Chen et al. 2006). Like other heat-shock proteins, Hsp90 is multifunctional and affords protection against thermal stress, contains an N-terminal ATP-binding site targeted by drugs, and binds other proteins marked for translocation, proper refolding, degradation, etc., via its C-terminal region. The key to discovering Hsp90's role in translocating clostridial binary toxins was the use of *Streptomyces* geldanamycin and *Monosporium* radicicol, small molecular weight antifungals that individually inhibit Hsp90 activity by binding to the same ATP-binding site within the N-terminus (Schulte et al. 1998). Besides inhibiting endosome-trafficked C2 toxin, these same inhibitors also prevent direct cytosolic entry of C2I from the outer membrane via an acidic pulse (Haug et al. 2003a). Inhibition of Hsp90 affects neither C2I docking to C2IIa oligomers nor pore formation by C2IIa, as evidenced by rubidium release from preloaded cells incubated with C2IIa (not C2I) (Haug et al. 2003a).

Cyclophilins are naturally found in the cytosol, composed of various isoforms, and inhibited by another fungal (*Tolypocladium*), small molecular weight cyclic called cyclosporin A that is commonly used as an immunosuppressant (Handschumacher et al. 1984). A non-immunosuppressant derivative (VK112) of cyclosporin A also protects cells against various clostridial binary toxins (Ernst et al. 2015), perhaps representing a lead therapeutic compound against these toxins. Furthermore, it has been shown that A components of the *B. anthracis* binary toxins are transported into the cytosol through a PA-based pore in an unfolded (melted) state from acidified, late endosomes (Abrami et al. 2004; Dal Molin et al. 2006; Wesche et al. 1998). However, the same chaperone molecules that facilitate clostridial binary toxins evidently do not translocate *B. anthracis* lethal toxin as Hsp90 inhibition in different cells (murine macrophage-like, J774.A1) has no effects upon intoxication (Haug et al. 2003a). Additionally, *C. difficile* toxin B is also not affected by these same inhibitors in C2 toxin-sensitive cells (i.e., Vero) (Haug et al. 2003a).

In addition to Hsp90 and cyclophilin A, another Hsp (70) has just been described as another important chaperone for Ia entry into the cytosol, as evidenced by cytotoxicity and direct binding assays (Ernst et al. 2016). Similar work remains for C2 toxin, yet Hsp70 does directly interface with C2I. The breakthrough of Hsp70 involvement came via screening of a chemical library using an in vitro luciferase assay and discovery of a specific (acridizinium-based) inhibitor targeting Hsp70.

Hsp70 is a rather conserved protein (i.e., *E. coli* equivalent is DnaK) with ATPase as well as peptide-binding properties. Hsp70 is involved in various cellular functions that include cycling, signaling, apoptosis, and protein translocation through membranes. The ATPase activity of Hsp70 is important in translocating Ia into the cytosol (Ernst et al. 2016).

Regarding the clostridial binary toxins, experiments also show direct binding of C2I, Ia, and CDTa to Hsp90 and cyclophilin A via dot blots or pull-down experiments in solution (Ernst et al. 2015; Kaiser et al. 2009, 2011, 2012). Some of these experiments reveal specific binding of the N-terminal region of C2I to cyclophilin A, and cytosolic entry of C2I is prevented by cyclosporin A in which C2I is likely stuck in the C2IIa-generated pore (Kaiser et al. 2009). In contrast, the *C. difficile* toxin A (devoid of ADP-ribosyltransferase activity), which also uses endosomes for cytosolic entry, is not affected by cyclosporin A (Kaiser et al. 2009). Much of the current knowledge involving translocation of A components from clostridial binary toxins into the cytosol is derived from experiments with the C2 toxin, in which C2I also binds directly to FK506-binding protein 51 (FKBP51) but not closely related FKBP52 (Kaiser et al. 2012). FKBP51 also binds directly to Hsp90 (Nair et al. 1997). Binding of C2I to FKBP51, one of many FKBPs expressed by mammalian cells and possessing peptidyl-prolyl cis/trans isomerase activity, is stronger with guanidine-denatured versus biologically-functional C2I (Kaiser et al. 2012). This further highlights FKBP51 binding preferentially to a denatured version of this enzyme (i.e., C2I threading through the C2IIa pore), which is also the case with a cyclophilin isomer (cyclophilin 40) (Ernst et al. 2015). Similar results were also recorded with Hsp70 and native, versus denatured, Ia (Ernst et al. 2016). Like cyclosporin A with cyclophilin A, inhibition of FKBP51 occurs with the immunosuppressant FK506 (also known as tacrolimus or fujimycin), derived from *Streptomyces*. FK506 not only blocks entry of C2I into the cytosol, but it also affords protection for cells toward iota toxin and CDT (Kaiser et al. 2012). Furthermore, various ADP-ribosyltransferases also employ Hsp90 or cyclophilin A for cytosolic entry when delivered as a fusion protein, using the N-terminal domain of C2I and oligomeric C2IIa (Kaiser et al. 2012).

It is clear that a host-provided complex of proteins aids the entry of clostridial binary toxins into the cytosol from the endosome (Fig. 1). How this is accomplished remains a mystery, as these host-provided proteins have overlapping functions. Why do ADP-ribosyltransferases like the A components of clostridial binary toxins employ the aforementioned eukaryotic proteins for cytosolic entry, yet other endosome-delivered bacterial toxins do not (Dmochewitz et al. 2011; Kaiser et al. 2012)? There is much more work to be done towards unraveling the many intimate interactions of eukaryotic proteins with these prokaryotic toxins, at the endosome-cytosol interface. The growing list of proteins involved in translocating A components of clostridial binary toxins ensures that this aspect of intoxication will become even more interesting well into the future (Ernst et al. 2016).

Once in the cytosol, A components of clostridial binary toxins remain enzymatically active for at least 48 h and in turn cause caspase-dependent apoptosis of some cells (Heine et al. 2008; Hilger et al. 2009). Excellent work has been done by

various groups related to how A components recognize and enzymatically engage G-actin via the conserved motifs STS (serine–threonine–serine; stabilizes NAD binding) and EXE (glutamic acid-X-glutamic acid; facilitates NAD cleavage and transfer of ADP-ribose to Arg-177 on G-actin) (Aktories et al. 2011; Belyy et al. 2015; Gulke et al. 2001; Nagahama et al. 2000; Tsuge et al. 2003, 2008; Tsurumura et al. 2013). The latter motif is found on the ADP-ribosylating turn-turn (ARTT) loop that evidently plays a role in substrate specificity. Cytotoxic effects (cell rounding) caused by clostridial binary toxins are non-reversible and rapidly occur within a few hours, with caspase activation ~15 h after toxin exposure. The ADP-ribosylated G-actin evidently accumulates and is not readily digested in an intoxicated cell, which in turn disturbs the G- : F-actin ratio as more G-actin (unmodified) is not necessarily produced by the targeted, intoxicated cell (Heine et al. 2008; Hilger et al. 2009).

The overt target of clostridial binary toxins is the actin cytoskeleton, which plays critical roles for different cell types (Aktories et al. 2012; Barth et al. 2015). For instance, immune cells (i.e., macrophages) use the cytoskeleton for movement and phagocytosis of microbial prey. Other functions of a cell that employ the actin-based cytoskeleton include maintenance of shape, cytokinesis, endo-/exo-cytosis, transport of intracellular vesicles, and signal transduction. Loss of the cytoskeleton is a big deal for a eukaryotic cell, and the clostridial binary toxins very effectively attack it. Dead and dying cells represent an opportunity for a microbe that includes the following: (1) release of intracellular-based nutrients for adjacent microbes; (2) destruction of a cell-based barrier (i.e., intestinal epithelium, including the intercellular tight and adherens junctions), enabling decreased transepithelial resistance, increased permeability of large molecular weight molecules into the intestinal lumen, and potential spread of microbe and toxin(s) throughout the host's body and to other potential hosts (i.e., fecal-oral route of infection); and (3) paralysis of immune cells and overall immune response, thus enabling a microbe to survive and thrive in an infected host. It is evidently a good thing for select, enteric clostridia to possess a binary toxin as a virulence factor. Although clostridial binary toxins target the actin-based component of the eukaryotic cytoskeleton, the latter is more complex than actin alone. For instance, there are other monomeric proteins such as tubulin and lamin that dynamically form distinct filaments and tubules (Barth et al. 2015). Interconnectivity exists between microfilaments, microtubules, and intermediate filaments so that if one component (i.e., actin-based filaments) becomes disrupted, then the cytoskeleton as a whole breaks down into a non-homeostatic state.

4 Future Directions

Clostridial binary toxins are clearly an evolutionary "success," as per their synthesis by various species that intestinally affect different animals and now evidently humans. These toxins target a critical aspect of eukaryotic life, the actin-based

cytoskeleton, necessary for normal functioning of cells. Common modes of action and high amino acid sequence identities between toxin components suggest genetic-based roots, and sharing of DNA between microbial communities. Over the past forty years, there has been much work focused upon the clostridial binary toxins by various international groups. Fascinating physical and enzymatic characterization studies have led to many fundamental understandings of how clostridial binary toxins work on, and in, a cell. Such knowledge logically promotes futuristic concepts that include employing these toxins as medicinal shuttles, as well as understanding where therapeutics and vaccines might be useful for the near and distant future (Aktories and Schmidt 2015; Barth et al. 2002; Barth and Stiles 2008; Fahrer et al. 2011; Marvaud et al. 2002; Shreya et al. 2016).

Recent discoveries excitingly reveal the specific cell surface receptor(s) for the iota family of toxins, which might lead to adjunct receptor-based therapies for those suffering from colitis induced by hypervirulent, CDT-producing strains of *C. difficile*. It is still hotly debated as to whether CDT plays a role in *C. difficile* colitis; however, given the role other iota family of toxins evidently play in enteric diseases of various animals, it makes logical sense that CDT might impact negatively the human intestinal tract. Recent reports revealing *C. difficile* strains devoid of large molecular weight toxins A and B, yet possessing CDT and causing colitis, are further suggesting the importance of CDT in disease (Androga et al. 2015; Eckert et al. 2015). Discoveries from Japan of a novel *C. perfringens* binary toxin (BEC/CPILE) involved in human food poisoning outbreaks are quite exciting, and further promotes the concept of clostridial binary toxins playing a role in human disease (Irikura et al. 2015; Monma et al. 2015; Yonogi et al. 2014). There are many more studies to be done with BEC/CPILE, that in turn will shed further light upon the other clostridial binary toxins.

Finally, there are many questions that remain regarding clostridial binary toxins. For instance, are there toxin variants harbored by other clostridial species and perhaps other bacterial genera? It is quite possible that genetically related, ADP-ribosylating binary toxins targeting the actin cytoskeleton are out there, making this field quite fertile for future discovery. Methodical, gene probe studies could be an efficient way of studying "what is out there." As seen throughout all forms of life, success breeds further success that then lives on and further evolves. For this author, clostridial binary toxins are clearly a preserved, evolutionary success that will provide more and more stories in the future.

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Host Cell Chaperones Hsp70/Hsp90 and Peptidyl-Prolyl *Cis/Trans* Isomerases Are Required for the Membrane Translocation of Bacterial ADP-Ribosylating Toxins

Katharina Ernst, Leonie Schnell and Holger Barth

Abstract Bacterial ADP-ribosylating toxins are the causative agents for several severe human and animal diseases such as diphtheria, cholera, or enteric diseases. They display an AB-type structure: The enzymatically active A-domain attaches to the binding/translocation B-domain which then binds to a receptor on the cell surface. After receptor-mediated endocytosis, the B-domain facilitates the membrane translocation of the unfolded A-domain into the host cell cytosol. Here, the A-domain transfers an ADP-ribose moiety onto its specific substrate which leads to characteristic cellular effects and thus to severe clinical symptoms. Since the A-domain has to reach the cytosol to achieve a cytotoxic effect, the membrane translocation represents a crucial step during toxin uptake. Host cell chaperones including Hsp90 and protein-folding helper enzymes of the peptidyl-prolyl *cis/trans* isomerase (PPIase) type facilitate this membrane translocation of the unfolded A-domain for ADP-ribosylating toxins but not for toxins with a different enzyme activity. This review summarizes the uptake mechanisms of the ADP-ribosylating clostridial binary toxins, diphtheria toxin (DT) and cholera toxin (CT), with a special focus on the interaction of these toxins with the chaperones Hsp90 and Hsp70 and PPIases of the cyclophilin and FK506-binding protein families during the membrane translocation of their ADP-ribosyltransferase domains into the host

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cell cytosol. Moreover, the medical implications of host cell chaperones and PPIases as new drug targets for the development of novel therapeutic strategies against diseases caused by bacterial ADP-ribosylating toxins are discussed.

List of Abbreviations (Optional)

ADP-RT	ADP-ribosyltransferase
CsA	Cyclosporine A, inhibitor of cyclophilins
CT	Cholera toxin
Cyp	Cyclophilin
DT	Diphtheria toxin
FK506	Inhibitor of FK506-binding proteins
FKBP	FK506-binding protein
GA	Geldanamycin, inhibitor of Hsp90
PPIase	Peptidyl-prolyl *cis/trans* isomerase
Rad	Radicicol, inhibitor of Hsp90

Contents

1 Bacterial ADP-Ribosylating Toxins

Several severe diseases such as diphtheria, cholera, or enteric diseases are caused by bacterial ADP-ribosylating toxins. As these diseases still pose a severe threat to human health and survival, especially in developing countries, these toxins require extensive research to provide new starting points for the development of novel

therapeutic strategies. In this article, the uptake of the ADP-ribosylating clostridial binary toxins, the single-chain diphtheria toxin (DT), and diphtheria-based fusion toxins as well as the AB_5-type cholera toxin (CT) is discussed.

1.1 Clostridial Binary Toxins

The clostridial binary toxins display enterotoxicity and comprise the *Clostridium (C.) botulinum* C2 toxin, the *C. perfringens* iota toxin, and the *C. difficile* binary toxin CDT. The prototype of this family, the C2 toxin, causes hemorrhagic lesions as well as necrosis in the intestinal mucosa of mice when administered as isolated protein components (Ohishi 1983a, b; Ohishi et al. 1980; Simpson 1982) and also affects the lung and the intestinal loop of pheasants and chickens due to fluid accumulations (Kurazono et al. 1987). Moreover, it has been shown to be cytotoxic to many different cell lines (Ohishi et al. 1984). Comparable to the C2 toxin, the *C. perfringens* iota toxin causes enterotoxic symptoms mostly in lambs and calves posing a problem in veterinary medicine and causing financial losses in livestock breeding (Billington et al. 1998; Songer 1996). In recent years, CDT has been identified as an important virulence factor that contributes to the *C. difficile*-associated enteric diseases (CDAD) comprising antibiotic-associated diarrhea which can result in the potentially life-threatening pseudomembranous colitis (Carroll and Bartlett 2011; Schwan et al. 2009, 2011). In Europe and North America, these diseases are currently on the rise mostly in patients treated with antibiotics during a hospital stay resulting in an altered gut flora which favors the growth of *C. difficile*. In 2011, half a million *C. difficile* infections were observed and approximately 29,000 deaths were associated with *C. difficile* infection in the USA (Lessa et al. 2015). The main causative agents of *C. difficile* are the large single-chain AB-type toxins A (TcdA, 308 kDa) and B (TcdB, 270 kDa). The glycosylation of Rho, Rac, and Cdc42 GTPases by TcdA and TcdB leads to an inhibition of the regular function of these important molecular switches. The impaired Rho-signaling results in a rearrangement of actin filaments and eventually in the destruction of the actin cytoskeleton, which then leads to rounding of the target cells. In the gut, this rounding up of enterocytes results in the loss of the important barrier function of the intestine and leads to the severe symptoms of CDAD (for review, see (Carroll and Bartlett 2011)). Interestingly, hypervirulent strains of *C. difficile* produce and secrete the binary ADP-ribosylating CDT toxin additionally to the glycosylating TcdA and TcdB toxins. It was demonstrated that the presence of CDT during *C. difficile* infection worsens the course of disease and increases morbidity and mortality due to inducing formation of microtubule-based protrusions of the intoxicated cell. In these long protrusions, further *C. difficile* bacteria are "caught," which leads to an improved colonization of the gut by *C. difficile* (Schwan et al. 2009, 2011). Therapy of *C. difficile* infection is challenging because an effective use of antibiotics is limited mainly to the broad-spectrum antibiotics metronidazole and vancomycin, which leads to further perturbation of the gut flora strongly increasing

the risk of recurrence of *C. difficile* infection. Therefore, novel therapeutic strategies against *C. difficile* infection and the toxin-induced symptoms are demanded.

1.2 Diphtheria Toxin

Diphtheria toxin (DT), the causative agent of the severe disease diphtheria, is synthesized and secreted by toxigenic strains of *Corynebacterium (C.) diphtheriae* (Pappenheimer 1977). The toxin was first described in 1888 in the culture medium of *C. diphtheriae* (Roux and Yersin 1888). The gene for DT, *tox*, is encoded by a family of closely related corynebacteriophages (Greenfield et al. 1983; Uchida et al. 1971) and expressed only under conditions of iron deprivation (Pappenheimer 1977). The expression is regulated by the *C. diphtheriae* genome-encoded iron-activated DT repressor, which prevents the transcription of DT in the presence of iron and other transition metal ions (Love and Murphy 2006). The colonization of the respiratory tract by *C. diphtheriae* and the subsequent release of DT result in the severe respiratory tract disease diphtheria. It affects the throat and the tonsils, thereby causing fever, swollen glands, and sore throat. An adherent membrane, the so-called pseudomembrane, is built upon the throat which impairs breathing and swallowing. In severe cases, upon toxin distribution via the bloodstream, this can lead to myocarditis and neuropathy (Atkinson et al. 2007). Diphtheria is still of increasing prevalence in several countries. In 2000, the World Health Organization reported 30,000 cases including 3000 deaths worldwide (WHO position paper 2006). Currently, the treatment for diphtheria is a combination of antibiotics against *C. diphtheriae* and neutralizing antibodies that are active against circulating DT. Thus, under the current therapy, the portion of the toxin already internalized into the target cells is unaffected.

1.3 Cholera Toxin

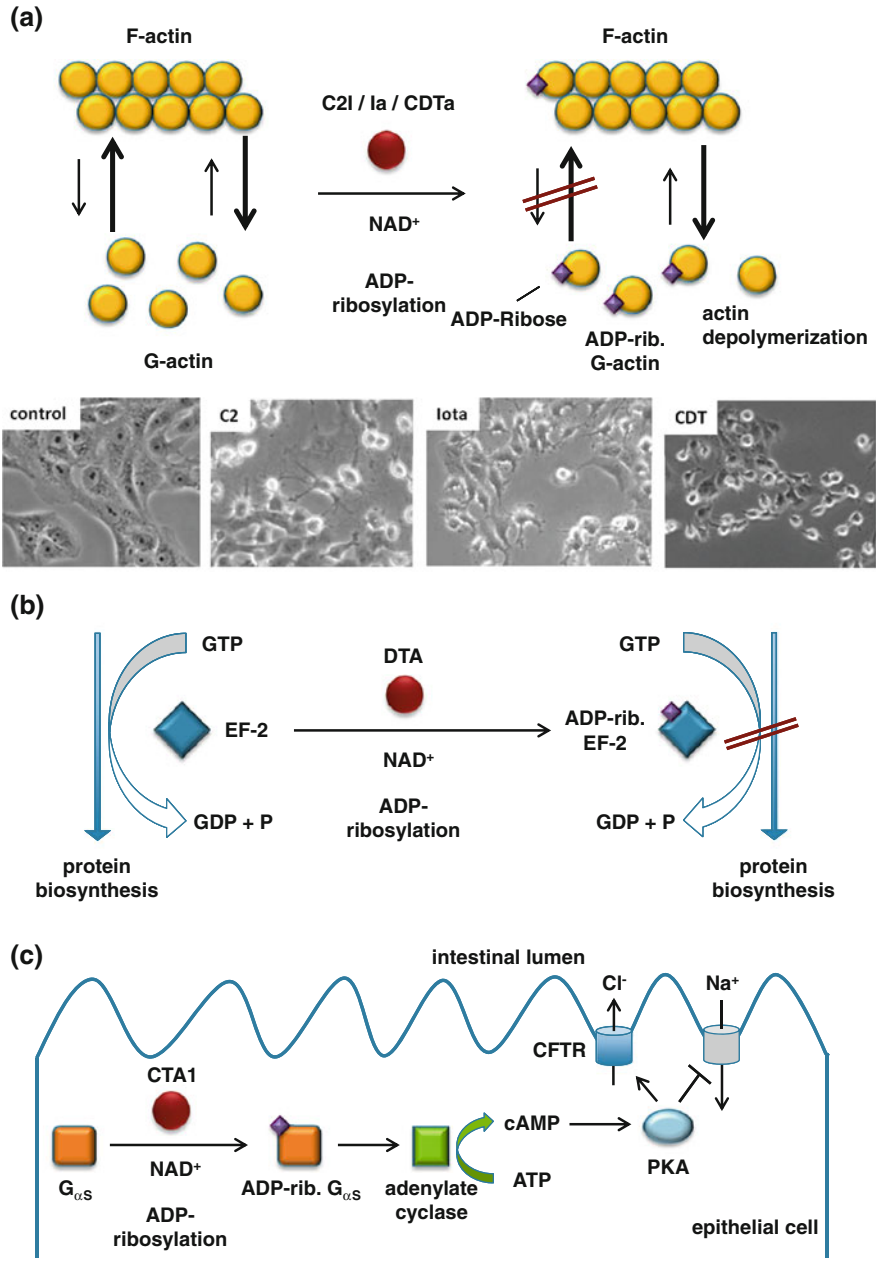
Another ADP-ribosylating toxin, the cholera toxin (CT), is one of the main virulence factors produced by *Vibrio (V.) cholerae* (De Haan and Hirst 2004; Sánchez and Holmgren 2008) and is the only causative agent for the severe watery diarrhea characteristic of cholera (De Haan and Hirst 2004; Sack et al. 2004). The pathology of cholera results from the colonization of the small intestine by the Gram-negative *V. cholerae*, which is taken up with fecally contaminated drinking water, followed by the secretion of CT into the intestinal lumen. After its uptake into enterocytes, CT catalyzes the ADP-ribosylation of a stimulatory G protein. This modification results in a permanent activation of the adenylyl cyclase, and the increased levels of cAMP trigger a series of intracellular events that finally lead to an electrolyte imbalance. There is rapid efflux of chloride ions and decreased influx of sodium ions, resulting in massive water efflux through the intestinal cells, causing severe diarrhea, which is the

characteristic clinical symptom of cholera. Thereby, the water loss by diarrhea can be life-threatening, up to 1 L/h, which untreated could result in death within hours after the first symptoms (Harris et al. 2012). According to the WHO report in 2010, there are currently 3–5 million cases of cholera and 100,000–130,000 deaths per year. Thereby, the sources of infection are contaminated food or water. Treatment of cholera is currently facilitated by replacing the lost fluids and application of antibiotics against the bacteria. Due to increasing antibiotic resistance as well as bacterial strains with enhanced virulence, especially in countries such as Asia and Africa, ongoing danger to society is caused by this disease (WHO position paper 2010).

1.4 ADP-Ribosylation of Intracellular Proteins by Bacterial Toxins and Its Consequences

The binary clostridial toxins, besides DT and CT, belong to the group of ADP-ribosylating AB-type toxins (i.e., the uptake of their enzymatic active A-domains is mediated by the specific binding/transport B-domains). After binding of the toxin to a receptor on the cell surface via the B-domain, the toxins are taken up into the cell by receptor-mediated endocytosis. Finally, the A-domain translocates into the host cell cytosol where it mono-ADP-ribosylates its specific substrate. ADP-ribosyltransferases (ADP-RTs) covalently transfer an ADP-ribose moiety from the cosubstrate NAD^+ onto their specific substrate molecule. This leads to the characteristic cellular effects and further to the typical clinical symptoms induced by each respective toxin. The C2, iota, and CDT toxins mono-ADP-ribosylate G-actin at Arg177 resulting in the inhibition of F-actin polymerization (Fig. 1a) (Aktories et al. 1986; Popoff et al. 1988; Schering et al. 1988; Stiles et al. 2011). For F-actin polymerization, the hydrolysis of ATP by G-actin is required so that a further G-actin can attach to the growing end of the filament. ADP-ribosylated G-actin forfeits the ability of ATP hydrolysis, resulting in the inhibition of polymerization (Geipel et al. 1989; Wegner and Aktories 1988). However, actin filaments are dynamic, meaning that at the other end of the filament, depolymerization continues, which finally results in a complete disassembly of the actin cytoskeleton. Finally, intoxicated target cells round up due to the loss of their actin cytoskeleton and this leads to an impaired barrier function when epithelial or endothelial cells of the gut are affected, resulting in the typical enterotoxic symptoms associated with these toxins and their producing bacteria (Aktories and Wegner 1992; Geipel et al. 1989; Wegner and Aktories 1988).

The enzymatically active domain of DT, DTA, catalyzes the NAD^+-dependent mono-ADP-ribosylation of the cytosolic elongation factor 2 (EF-2), a soluble translocase involved in the protein synthesis of eukaryotic cells (Pappenheimer 1977). Thereby, an ADP-ribose residue of NAD^+ is covalently transferred to diphthamide, which is a post-translationally modified histidine residue in EF-2, resulting in the inactivation of this factor. This in turn leads to the arrest of chain


(a)
F-actin
C2I / Ia / CDTa
NAD+
ADP-ribosylation
F-actin
ADP-Ribose
ADP-rib. G-actin
actin depolymerization
G-actin
control
C2
Iota
CDT
(b)
GTP
EF-2
DTA
NAD+
ADP-ribosylation
GDP + P
protein biosynthesis
GTP
ADP-rib. EF-2
GDP + P
protein biosynthesis
(c)
intestinal lumen
Cl-
Na+
CFTR
CTA1
NAD+
ADP-ribosylation
Gαs
ADP-rib. Gαs
adenylate cyclase
cAMP
ATP
PKA
epithelial cell

◂**Fig. 1** ADP-ribosylation of intracellular proteins by bacterial toxins and its consequences. **a** The clostridial enzyme components C2I, Ia, and CDTa of the C2, iota, and CDT toxins mono-ADP-ribosylate G-actin leading to the molecular and cellular effects that are discussed in the text in more detail. The pictures show Vero cells that have been incubated with C2, iota, or CDT toxins (or left untreated for control) for 3 h. Toxin-treated cells display the typical toxin-induced cell rounding (derived from Aktories et al. 2011). **b** The enzymatic active domain of the diphtheria toxin (DTA) mono-ADP-ribosylates the elongation factor 2 (EF-2). Consequences evoked by the ADP-ribosylated EF-2 for the target cell are explained in the text in more detail (derived from Collier 2001). **c** CTA1, the enzymatic active subunit of the cholera toxin, covalently transfers an ADP-ribose moiety onto the α-subunit of the heterotrimeric stimulatory G protein ($G_{\alpha s}$) leading to a constitutive active adenylate cyclase. Details are given in the text (derived from Clemens et al. 2011). (*ADP-rib.* ADP-ribosylated, *CFTR* cystic fibrosis transmembrane regulator, *PKA* protein kinase A)

elongation during protein translation, which is irreversible and therefore leads to the death of the DT-treated target cells by apoptosis (Fig. 1b) (Collier and Cole 1969).

Within the cytosol, the enzymatically active domain of CT, CTA1, transfers an ADP-ribose residue from NAD^+ to the α-subunit of the heterotrimeric stimulatory G protein ($G_{\alpha s}$), thereby activating $G_{\alpha s}$. This in turn stimulates adenylate cyclase (AC) at the cytoplasmic membrane, leading to the massive increase of intracellular cAMP (Fig. 1c). The high cAMP level triggers signaling events via protein kinase A (PKA), which opens chloride channels, also known as the cystic fibrosis transmembrane regulator (CFTR). The chloride release into the intestinal lumen and the inhibition of Na^+ absorption in intestinal epithelial cells cause water secretion and diarrhea (De Haan and Hirst 2004; Sánchez and Holmgren 2008).

2 Structure and Uptake of ADP-Ribosylating Toxins

The ADP-ribosylating toxins discussed in this article belong to the family of AB-type toxins and can be further distinguished by their structure and organization (see Fig. 2). DT is a single-chain toxin, i.e., the A-domain and the B-domain are located on one protein (Collier 1975). The functional domains of the clostridial binary toxins are located on two individual proteins called components. Only if both components are combined and act together, cytotoxic effects and clinical symptoms are caused (Ohishi 1983a, b; Perelle et al. 1997; Stiles and Wilkins 1986). CT is described as an AB_5-toxin consisting of an enzymatically active A-subunit and five B-subunits organized as a pentamer, which together form the cholera holotoxin.

2.1 Structure and Uptake of Clostridial Binary Toxins

The C2, iota, and CDT toxins display a binary structure, meaning that the enzymatically active A-component and the binding/transport B-component are

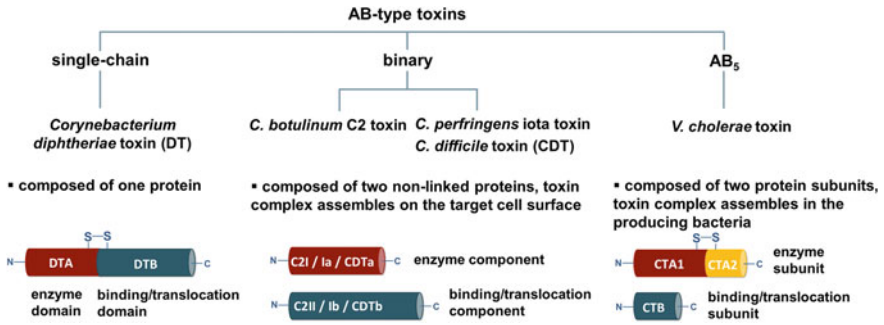


Fig. 2 Structure of different bacterial ADP-ribosylating toxins. The A- and B-domains of AB-type toxins can be organized in several ways: Single-chain toxins comprise both domains on one single protein. In contrast, binary toxins are composed of two non-linked proteins, one harboring the ADP-RT activity and the other responsible for binding and translocation. Both components are separately secreted by the bacteria, and the toxin complex is assembled on the target cell. The A- and B-subunits of AB_5 toxins are also located on different proteins, but the toxin complex is already assembled in the bacteria and therefore secreted by the bacteria as a holotoxin [summarized from data from Choe et al. (1992), Clemens et al. (2011), Collier and Cole (1969), Stiles et al. (2011)]

represented in two separate proteins. Both proteins are separately secreted by the clostridia into the surrounding environment (Stiles et al. 2011). The A-component C2I of the prototypic C2 toxin consists of an N-terminal part called C2IN that facilitates the binding to the activated B-component C2IIa. It has been demonstrated that C2IN can also serve as an adaptor for foreign cargo proteins that are fused to C2IN. In the recombinant fusion toxin C2IN-C3lim, the ADP-RT C3 of *C. limosum* has been fused to C2IN and is therefore very efficiently transported into the cytosol of all tested mammalian cell types via C2IIa. Noteworthy, C3lim is not able to enter these cell types on its own right (Barth and Stiles 2008; Barth et al. 1998a, 2002). The C-terminal portion of C2I harbors the ADP-RT activity and comprises several motifs that are highly conserved in ADP-RTs like the$_{387}$EXE$_{389}$ motif which encompasses the two "catalytic" glutamates essential for the ADP-ribosylation activity. This motif can also be found in the enzyme domains of the iota and CDT toxins, as well as in DT and CT (Aktories et al. 2011; Barth et al. 1998b; Carroll and Collier 1984; Gülke et al. 2001; Laing et al. 2011; Sakurai et al. 2003; Tsuge et al. 2008). The binding/translocation component C2II consists of four domains D1–D4. After proteolytic cleavage of a ~20 kDa fragment of D1, the activated C2IIa is able to bind C2I via the D1 domain. Moreover, the activated C2IIa forms heptamers facilitated by the D3 domain (Barth and Stiles 2008; Barth et al. 2000; Blöcker et al. 2000; Kaiser et al. 2006). The D2 domain is responsible for pore formation, and the D4 domain mediates binding of the toxin complex to a receptor on the cell surface. This receptor shows an asparagine-linked carbohydrate structure and is very likely expressed among all vertebrate cells, because all tested cell types respond to C2 toxin (Blöcker et al. 2000; Eckhardt et al. 2000; Ohishi et al. 1984). The binding/translocation components Ib and CDTb of the respective iota and CDT

toxins display homology with C2II, but the D4 domain differs, resulting in the binding of the toxin complexes to a different receptor, namely the lipolysis-stimulated lipoprotein receptor (LSR) which accumulates in lipid rafts (Hale et al. 2004; Nagahama et al. 2004; Papatheodorou et al. 2011, 2013). Moreover, CD44 is important for the uptake of iota and CDT toxins because CD44-deficient cells show a less efficient uptake of these toxins and CD44 knockout mice are not intoxicated by the iota toxin (Wigelsworth et al. 2012). The uptake mechanism of the C2 and iota toxins is widely comparable (Stiles et al. 2011). The uptake of the CDT toxin is not studied in detail so far, but due to the strong homology with the iota toxin, a similar uptake mechanism is assumed. Interestingly, it is possible to interchange the A- and B-components of the iota and CDT toxins, so that the B-component of the iota toxin can transport the A-component of the CDT toxin into the host cell cytosol and vice versa (Barth et al. 2004). After the proteolytic activation, C2IIa forms ring-shaped heptamers which bind to their receptor on the cell surface (see Fig. 3). C2I can bind to these heptamers and receptor-mediated endocytosis, which is probably clathrin- and Rho-dependent (Nagahama et al. 2009; Pust et al. 2010) and is triggered so that the AB_7-toxin complex is located in the lumen of early endosomes. The endosomes get

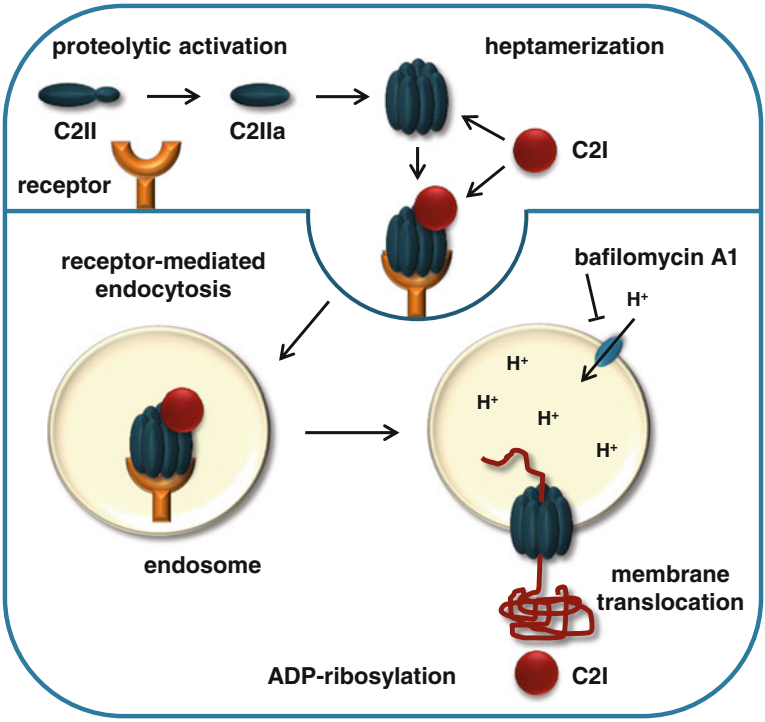


Fig. 3 Uptake mechanism of the *C. botulinum* C2 toxin. Details are given in the text (derived from Barth and Aktories 2011)

acidified by a vesicular ATPase (v-ATPase), which leads to the conformational changes of both components. The C2IIa heptamer inserts as a pore with an inner diameter of ~27 Å into the endosomal membrane (Schleberger et al. 2006), and C2I is at least partially unfolded and translocates through the narrow C2IIa-pore across the endosomal membranes into the cytosol of the host cell (Haug et al. 2003a). In the cytosol, C2I ADP-ribosylates G-actin, leading to the destruction of the actin cytoskeleton and cell rounding. In contrast to the C2 toxin, the iota toxin also requires a membrane potential additionally to the pH gradient for translocation of Ia into the cytosol (Gibert et al. 2007).

2.2 *Structure and Uptake of Diphtheria Toxin*

DT is expressed as a precursor containing a 26 amino acid signal peptide and is cotranslationally secreted as a single polypeptide chain. Upon cleavage of the signal sequence, the toxin is released into the culture medium in its mature form as a 535 (58 kDa) amino acid residue protein (Greenfield et al. 1983; Kaczorek et al. 1983; Smith et al. 1980). As such, the toxin is composed of three structural parts: The N-terminal part of DT represents the enzymatically active A-domain DTA (21 kDa) (Collier and Kandel 1971), which mediates specific substrate modification within the target cell cytosol. The C-terminal part consists of the receptor-binding (R) and transmembrane or translocation (T) domain, which are summarized as the transport B-domain DTB (37 kDa) (Choe et al. 1992). DTB mediates the transport of DTA into the target cell cytosol. The T-domain of DTB is thereby composed of nine α-helices which are arranged in three layers (Choe et al. 1992): Helices 1–3 form the first amphipathic layer, helices 5–7 the second hydrophobic layer, and helices 8 and 9 the third hydrophobic layer. Pore formation is performed by the insertion of the third helical layer into the endosomal membrane, stabilized by the insertion of the second layer. DTA and DTB are connected via a 14 amino acid, protease-sensitive loop, and a disulfide bond between Cys186 and Cys201 (Gill and Pappenheimer 1971). For proper intoxication of eukaryotic cells, furin-mediated cleavage within this loop and retention of the disulfide bond are necessities (Ariansen et al. 1993; Tsuneoka et al. 1993).

Like C2, iota, and CDT toxins, DT also belongs to the group of short-trip toxins, meaning that after receptor-mediated endocytosis, the enzyme domain translocates from early acidified endosomes into the host cell cytosol. Therefore, the first step in the intoxication process of sensitive eukaryotic cells by DT is the binding of the R-domain (residues 432–535) to its cell surface receptor, the heparin-binding epidermal growth factor-like growth factor precursor (HB-EGF) (Choe et al. 1992; Naglich et al. 1992). Thereby, the sensitivity of target cells is roughly related to the number of receptors that are present on the cell surface and is further enhanced by the DT receptor-associated protein 27 (DTRAP), the primate homologue of human

CD9, which is associated with the DT receptor but not DT itself (Brown et al. 1993; Iwamoto et al. 1994; Mitamura et al. 1992). Receptor-binding triggers receptor-mediated endocytosis of cell-bound DT and internalization of DT into endosomal vesicles. Thereby, receptor-bound DT is concentrated in clathrin-coated pits and internalized into clathrin-coated vesicles, which are then converted into early endosomes (Moya et al. 1985). After internalization into endosomal vesicles, the DTA and DTB domains are proteolytically separated by the protease furin (Tsuneoka et al. 1993) but remain linked via the interchain disulfide bond between Cys186 in DTA and Cys201 in DTB (Gill and Pappenheimer 1971). The clathrin triskelion is replaced on the vesicle membrane with a new set of protein components, including Arf-1, COPI complex, Rab-5, early endosomal antigen, and v-ATPase. The activity of this v-ATPase lowers the luminal pH of the endosome which triggers the dynamic unfolding of the T-domain (Boquet et al. 1976), leading to its insertion into the endosomal membrane and formation of a cation-selective 18–22 Å membrane pore (Donovan et al. 1981; Kagan et al. 1981). Through this pore, DTA translocates from early acidified endosomes into the host cell cytosol (Lemichez et al. 1997; Papini et al. 1993a, b), which was shown to occur from C- to N-terminus of DTA (Falnes and Olsnes 1995). Thereby, crossing the membrane requires at least partial unfolding of DTA (Falnes and Olsnes 1995; Falnes et al. 1994).

For DT action, reduction of the interchain disulfide bond between DTA and DTB is the rate-limiting step (Papini et al. 1993b). This step is likely facilitated by the thioredoxin/thioredoxin reductase system during or after translocation of DTA (Madshus 1994; Moskaug et al. 1987; Papini et al. 1993b; Schnell et al. 2015). In this respect, there is also evidence for the involvement of the protein disulfide isomerase (PDI) (Mandel et al. 1993).

2.3 *Structure and Uptake of Cholera Toxin*

Apart from the AB_7-toxins, there is also the group of AB_5-toxins, including *V. cholerae* CT, *Escherichia* (*E.*) *coli* shiga toxin, *E. coli* subtilase cytotoxin, and *Bordetella pertussis* toxin. Their B-subunits form a pentameric ring structure, which is required for receptor-binding.

CT is a member of this group of AB_5-toxins. Here, the two subunits A and B are organized from A and B monomers into an intact AB_5-holotoxin in the bacterial periplasm (Hirst and Holmgren 1987). The enzymatic active A-subunit is first synthesized as a 27 kDa protein, which is then post-translationally nicked by proteolysis, thereby generating a disulfide bond-linked CTA1/CTA2 heterodimer. The 22 kDa CTA1 polypeptide harbors the ADP-RT activity, and the 5 kDa CTA2 polypeptide acts as a linker between CTA1 and the B-subunit CTB. The homopentameric CTB is a highly stable ringlike structure, built from five 11 kDa

monomers, with a central pore that non-covalently interacts with CTA2 and contains binding sites for the cellular receptor, which are GM1 gangliosides, on the plasma membrane of target cells (De Haan and Hirst 2004; Sánchez and Holmgren 2008; Zhang et al. 1995). Receptor-binding triggers toxin endocytosis and subsequent delivery of the intact holotoxin to the endoplasmic reticulum (ER) via retrograde vesicular transport (Wernick et al. 2010). Within the ER, reduction of the disulfide bond linking CTA1 and CTA2 occurs and reduced CTA1 is then released from its non-covalent association with the holotoxin by protein disulfide isomerase (PDI) (Majoul et al. 1997; Orlandi 1997; Taylor et al. 2011a, 2014; Tsai et al. 2001). In its isolated form, the CTA1 polypeptide is a thermally unstable protein that spontaneously unfolds at the physiological temperature of 37 °C after PDI-mediated release from the holotoxin in the ER (Pande et al. 2007; Taylor et al. 2011a). Unfolded CTA1 is then exported through the ER-associated degradation (ERAD) pathway to the cytosol (Banerjee et al. 2010; Massey et al. 2009; Taylor et al. 2011b; Teter and Holmes 2002; Teter et al. 2003). The transport of proteins via the ERAD pathway is a normal cellular process to prevent the accumulation of protein aggregates in the ER. The transported proteins move through protein-conducting channels in the membrane of the ER and are then targeted for cytosolic degradation by the 26S proteasome in an ubiquitin-dependent manner (Bagola et al. 2011; Nakatsukasa and Brodsky 2008). The CTA1 polypeptide contains an arginine-over-lysine bias in its amino acid sequence, and the lack of lysine residues results in a limited number of ubiquitination sites, which in turn inhibits the degradation of CTA1 by the 26S proteasome (Hazes and Read 1997; Rodighiero et al. 2002). Instead, cytosolic CTA1 is degraded by an ubiquitin-independent proteasomal mechanism (Orlowski and Wilk 2003; Pande et al. 2007). CTA1 crosses the ER membrane in an unfolded state and must regain an active conformation in the cytosol, a process, which requires the action of certain host cell factors (Ampapathi et al. 2008; Burress et al. 2014; Ray et al. 2012). Enzymatically active CTA1 then interacts with host ADP-ribosylation factors (ARFs), which in their GTP-bound form act as an allosteric activator enhancing its activity, and CTA1 then catalyzes its enzymatic activity by the ADP-ribosylation of $G_{\alpha s}$ (Banerjee et al. 2014; Kahn and Gilman 1984; O'Neal et al. 2005; Welsh et al. 1994).

Despite all the differences regarding structure, uptake mechanism, substrate specificity, and resulting cellular effects and clinical symptoms, the clostridial binary toxins, DT and CT share the same enzyme activity: ADP-ribosylation of intracellular proteins. Therefore, this shared ADP-RT activity most likely explains why all these toxins require the same host cell chaperone Hsp90 for their effective membrane translocation as well as Hsp70 and protein-folding helper enzymes of the peptidyl-prolyl *cis/trans* isomerases (PPIases). The role of heat-shock proteins and PPIases during membrane translocation of ADP-ribosylating toxins is discussed in the following paragraph.

3 The Role of Host Cell Chaperones Hsp70/Hsp90 and PPIases During the Uptake of ADP-Ribosylating Toxins into the Host Cell Cytosol

ADP-ribosylating protein toxins act in the cytosol of mammalian host cells where they modify their specific substrates causing typical cellular reactions and clinical symptoms. To reach the cytosol, they employ very elaborate uptake mechanisms involving the exploitation of cellular protein-trafficking pathways. During the last 20 years and ongoing, it was investigated how ADP-ribosylating toxins take further advantage of the host cell for efficient delivery of their enzyme subunits in the cytosol by employing services of host cell chaperones and protein-folding helper enzymes of the PPIase family. The involvement of Hsp90 during toxin uptake was first shown for C2 and DT in 2003 by Haug et al. and Ratts et al., respectively, and was later demonstrated for the iota and the CDT toxins (Haug et al. 2004; Kaiser et al. 2011). Investigation of the role of Hsp90 during toxin uptake was possible by using the specific cell-permeable pharmacological inhibitors radicicol (Rad) and geldanamycin (GA) of Hsp90 activity (see Fig. 4a). Rad and GA significantly delay the intoxication of mammalian cells with C2, iota, and CDT toxins when cells are preincubated with the inhibitors, implying a crucial role of Hsp90. The toxin-induced changes in cell morphology were used as a very specific and sensitive endpoint of intoxication (see Fig. 4b), revealing that at the same time points of intoxication less rounded (i.e., intoxicated) cells were observed in the presence of Hsp90 inhibitors (Haug et al. 2003b, 2004; Kaiser et al. 2011). Rad belongs to the group of macrolactones, and GA is a natural benzoquinone ansamycin (Pratt and Toft 2003). They both bind with high affinity to the ATP-binding pocket of Hsp90, although to different sites, which prevents its chaperone activity leading to the degradation of the client protein (Arndt et al. 2007; Grenert et al. 1997; Prodromou et al. 1997; Roe et al. 1999). More precisely, the hydrolysis of ATP results in conformational changes in Hsp90 and therefore in the client protein and also is required for the release of the proper-folded client protein of Hsp90 (Li et al. 2012). Besides the delaying effect of Rad and GA, it was demonstrated that in the presence of Rad and GA, the amount of translocated enzyme component in the cytosol is reduced (Haug et al. 2003b; Kaiser et al. 2011). The enzyme components of the C2, iota, and CDT toxins translocate from early acidified endosomes into the host cell cytosol. Therefore, unfolding of the enzyme components is required and it seemed logical to assume that protein-folding helper enzymes like Hsp90 are involved in this membrane translocation step of toxin uptake. Evidence for the requirement of Hsp90 during membrane translocation was given by a well-established artificial toxin translocation assay. Here, the acidic endosomal conditions can be mimicked directly at the cytoplasmic membrane of cultured cells, which enable the detailed investigation of the membrane translocation step in an isolated manner independent of endocytosis pathways. In brief, cells are pretreated with Rad or GA and cooled to 4 °C so that the added toxin components only bind to the receptor but are not internalized. Subsequently, cells are challenged with warm acidic medium resulting

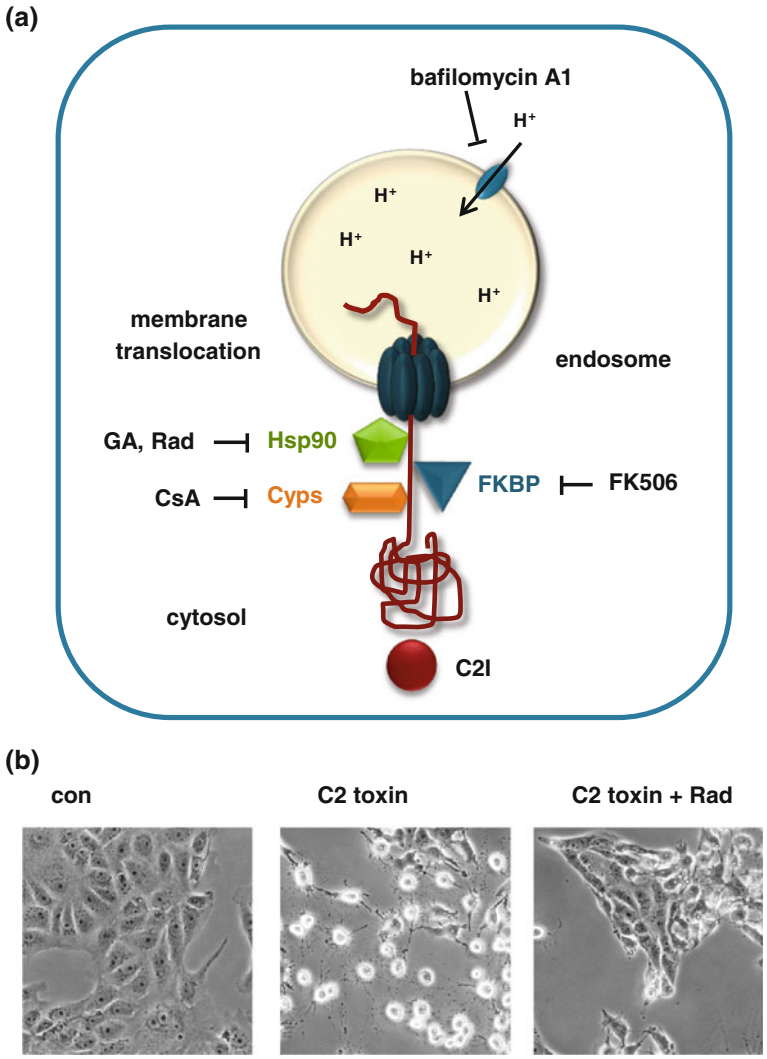


Fig. 4 **a** The membrane translocation of the enzyme component C2I of the *C. botulinum* C2 toxin is facilitated by the host cell chaperone Hsp90 and peptidyl-prolyl *cis/trans* isomerases of the Cyp and FKBP families. Details are given in the text [derived from data from Ernst et al. (2015), Haug et al. (2003b), Kaiser et al. (2009, 2012)]. **b** The Hsp90 inhibitor Rad prevents the intoxication of Vero cells with the C2 toxin. Vero cells were preincubated with 20 μM Rad for 30 min at 37 °C, and then, C2 toxin was added. Cells were further incubated at 37 °C, and cell morphology was monitored. The depicted pictures were taken after 2.5 h of toxin incubation. Cells treated with Rad show a significantly reduced amount of rounded, i.e., intoxicated, cells compared to cells treated with C2 toxin only. This demonstrates the protective effect of Rad for target cells. *GA* geldanamycin, *Rad* radicicol, *CsA* cyclosporine A, *Cyps* cyclophilins, *FKBP* FK506-binding protein

in the insertion and pore formation of the B-components into the cytoplasmic membrane and in the translocation of the A-components through these pores into the host cell cytosol. The "normal" uptake route of the toxins via endocytosis and their translocation from acidified endosomes are inhibited by bafilomycin A1 (BafA1), which prevents the acidification of endosomes by the v-ATPase. In this assay, both Rad and GA inhibit the pH-dependent membrane translocation of C2I, Ia, and CDTa, clearly indicating that Hsp90 facilitates this step of toxin uptake. Furthermore, other steps such as receptor-binding, endocytosis, pore formation, or enzyme activity are not impaired by the inhibitors of Hsp90 (Haug et al. 2003b; Kaiser et al. 2011). Therefore, an inhibition of Hsp90 activity leads to the accumulation of enzyme component in endosomes which was also demonstrated by fluorescence microscopy showing an enhanced colocalization of C2I with endosomal markers in the presence of Rad or GA (Haug et al. 2003b).

In recent years, it was demonstrated that the inhibition of Hsp90 activity also affects tumor growth (Li and Buchner 2013). As Hsp90 is 2- to 10-fold higher expressed in tumor cells, inhibition of Hsp90 by Rad or GA has a strong antitumor effect. However, adverse effects of Rad and GA due to high toxicity, low biologic stability, and poor solubility have been observed. Therefore, novel improved derivatives were developed based on Rad and GA structures, such as the GA derivative 17-AAG or radamide, which is a chimeric compound derived from Rad and GA. These novel compounds show less adverse effect but still potently inhibit Hsp90 activity resulting in antitumor effects. Furthermore, 17-AAG already revealed promising results in preclinical and clinical trials (Li and Buchner 2013). The prospect of improving pharmacological Hsp90 inhibitors regarding adverse effects suggests the possibility to use these inhibitors to prevent the membrane translocation of ADP-ribosylating toxins and therefore possibly protect or relieve patients from severe toxin-induced clinical symptoms.

In the eukaryotic cell, Hsp90 represents an essential, abundant, and highly conserved cytosolic protein. It features prominently in various fundamental processes such as stress response, hormone signaling, cell survival, and cell cycle control. More precisely, Hsp90 displays protein-folding helper activities and ensures that proteins remain in their correctly folded conformations. Therefore, it is important for protein folding and also refolding, preventing the aggregation of proteins and transport of proteins from the ER to the Golgi apparatus (Li and Buchner 2013). More than 200 client proteins are known that interact with, and are processed by, Hsp90. Hsp90 forms homodimers via its C-terminal dimerization site which is flanked by a middle domain and the N-terminal nucleotide-binding domain (NTD) (Li et al. 2012). The NTD binds ATP which leads to a closed conformation of the homodimer. Intrinsic ATPase activity leads to the conformational changes in the client protein and finally release of the folded client protein (Pratt and Toft 2003). Interestingly, Hsp90 is known to cooperate with various cochaperones which assist with the folding process, coordinate which client is processed, and regulate ATPase activity (Prodromou et al. 1999). The interaction is facilitated via specific motifs of the interaction partners. Hsp90 contains the MEEVD motif to which proteins encompassing a tetratricopeptide repeat (TPR), such as Hsp70 or

PPIases of the cyclophilin and FK506-binding protein families, can bind (Ratajczak and Carrello 1996). How these Hsp90 cochaperones act in a concerted manner is well characterized for the activation of steroid hormone receptor complexes in the cell (Li et al. 2012). Here, Hsp70 is one of the first host cell factors interacting with the client protein during this activation cycle. Hsp70, together with Hsp40 and the adaptor protein Hop, binds the unfolded client protein and transfers it to the Hsp90 dimer. Just recently, a role of Hsp70 during membrane translocation of the iota toxin was revealed (Ernst et al. 2016). Comparable to the investigation of Hsp90 involvement, specific pharmacological inhibitors of Hsp70 were employed. VER-155008 (VER) binds to the ATP-binding site of Hsp70 and Hsc70, the constitutive form of Hsp70. Intoxication of cells with iota toxin and the membrane translocation of the enzyme component Ia are inhibited by VER. However, VER has no inhibitory effect on receptor-binding and enzyme activity, suggesting that Hsp70 and Hsc70 facilitate, like Hsp90, the membrane translocation of Ia into the host cell cytosol. Due to the development of a novel inhibitor, it was possible to characterize the interaction between Ia and the heat-shock protein in more detail. The novel inhibitor is specific for Hsp70 and binds to its substrate binding domain, not to the ATP-binding site like VER. However, this inhibitor also prevents intoxication with the iota toxin, suggesting that not only the ATP-binding site, but also the substrate binding domain is required for an efficient uptake of Ia into the host cell cytosol (Ernst et al. 2016). In vitro, the enzyme components of not only the iota toxin but also the C2 and CDT toxins directly interact with Hsp70 and Hsc70, suggesting that Hsp/c70 might also play a role during the uptake of other ADP-ribosylating toxins (Ernst et al. 2016). Like Hsp90, Hsp70 is an abundant, highly conserved protein which participates in various processes in the cell-like de novo folding or organizing the assembly and disassembly of protein complexes. Interestingly, Hsp70 has been shown to facilitate the membrane translocation of proteins that include the pulling of polypeptides out of ribosomes or transport of proteins into mitochondria and the ER. Thereby, the current model of how Hsp70 assists the progress of membrane translocation postulates that Hsp70 binds the translocating protein on the *trans* site very close to the translocation pore, thereby limiting the Brownian movements that could impair directed translocation. This process is enforced by the entropic pulling force conducted by Hsp70 (Clerico et al. 2015; Finka et al. 2015). A comparable mechanism could be imaginable for the involvement of Hsp70 in membrane translocation of ADP-ribosylating toxins.

Further known cochaperones of Hsp90 are the Cyps and FKBPs. In recent years, it was demonstrated that in addition to Hsp90 and Hsp70, Cyps and FKBPs are also required for membrane translocation of ADP-ribosylating toxins. This was first shown for the C2 toxin and subsequently for the iota and CDT toxins (Ernst et al. 2015; Kaiser et al. 2009, 2011, 2012). PPIases catalyze the *cis/trans* isomerization of proline bonds in peptides which is considered a rate-limiting step during protein folding (Göthel and Marahiel 1999). The activity of Cyps and FKBPs can also be inhibited by specific pharmacological inhibitors, namely cyclosporine A (CsA) for inhibition of Cyps and FK506 for inhibition of FKBPs (Handschumacher et al. 1984; Harding et al. 1989). CsA and FK506 protect host cells from intoxication

with C2, iota, and CDT toxins and significantly reduce the amount of enzyme components that reaches the cytosol. Furthermore, less G-actin is ADP-ribosylated in the presence of the inhibitors compared to cells treated with toxin only. Comparable to Rad and GA, CsA and FK506 do not inhibit receptor-binding, endocytosis, or enzyme activity but instead impair membrane translocation of the enzyme components into the host cell cytosol as demonstrated by the toxin translocation assay (Ernst et al. 2015; Kaiser et al. 2009, 2011, 2012). The impaired membrane translocation due to PPIase inhibition could also be observed when the enzyme component C2I translocates across the membranes of isolated toxin-loaded endosomes in vitro. To this end, cells are incubated with C2 toxin in the presence of BafA1 to trap the toxin in early endosomes, which are then accumulated and purified by gradient centrifugation. Addition of fresh cytosol to the isolated, toxin-loaded endosomes leads to membrane translocation of C2I, as detected by measuring ADP-ribosylation of G-actin. Pretreatment of the fresh cytosol with CsA or alternatively with an antibody against CypA, one of the 18 known different Cyp isoforms, prevents the translocation of C2I from endosomes (Kaiser et al. 2009).

Interestingly, a synergistic inhibitory effect could be observed when combining Hsp90 inhibition by Rad with Cyp inhibition by CsA, suggesting that Cyps might interact in an Hsp90-containing multichaperone complex comparable to that facilitating the activation of steroid hormone receptors (Kaiser et al. 2009, 2011). Within the highly conserved Cyp and FKBP families, several isoforms are known which are divided into single- and multidomain enzymes (Schiene-Fischer 2014). Unfortunately, CsA and also FK506 are non-isoform specific inhibitors. However, coprecipitation, dot blot analysis, and isothermal titration calorimetry revealed a direct interaction between the enzyme components C2I, Ia, and CDTa with the single-domain isoform CypA and also with the multidomain isoforms Cyp40 and FKBP51 (Ernst et al. 2015; Kaiser et al. 2009, 2011, 2012). CypA comprises a single PPIase domain and represents an abundant cytosolic protein with many cellular functions that include signal transduction, oxidative stress response, and cell cycle regulation (Nigro et al. 2013). The PPIase domain is also the binding site for CsA, the specific inhibitor of PPIase activity (Fruman et al. 1994). In addition to the cytosolic Cyps, extracellular CypA and CypB are involved in cell-to-cell communication in inflammatory pathways. The activity of extracellular Cyps can be inhibited by the non-cell-permeable inhibitor MM284, a specifically designed CsA derivative (Hoffmann and Schiene-Fischer 2014; Malesevic et al. 2013). Pretreatment of cells with MM284 does not protect cells from intoxication with ADP-ribosylating C2, iota, and CDT toxins, clearly excluding a role of extracellular Cyps during toxin uptake (Ernst et al. 2015). The cytosolic Cyp40 belongs to the group of multidomain Cyps and comprises in addition to the PPIase domain three TPR domains, which enable Cyp40 to interact with the MEEVD motif of Hsp90 (Davis et al. 2010; Pratt and Toft 1997). Interestingly, Cyp40 competes with the multidomain isoforms of the FKBP families, such as FKBP51 and FKBP52, for this exact binding motif of Hsp90 when forming Hsp90 multichaperone complexes in the cell (Pratt and Toft 1997). Furthermore, Cyp40 mediates the delivery of the

activated glucocorticoid receptor alongside the cytoskeleton to the nucleus by binding to the motor protein dynein (Galigniana et al. 2002; Owens-Grillo et al. 1995).

Comparable to the Cyps, all members of the FKBP family comprise one or more PPIase domains to which the specific FKBP inhibitor FK506 binds (Galat 2003; Harding et al. 1989; Schiene-Fischer 2014). The single-domain isoform FKBP12 shows the highest affinity to FK506 but no direct interaction with the enzyme components of C2, iota, or CDT toxins in vitro (Kaiser et al. 2011). Nevertheless, the multidomain isoform FKBP51 has been identified as an interaction partner of C2I, Ia, and CDTa (Kaiser et al. 2012). FKBP51 and also FKBP52 contain three TPR domains that enable them to interact with Hsp90 (Pratt and Toft 1997; Schiene-Fischer 2014). When part of the Hsp90 multichaperone complex as described for steroid hormone receptor activation, FKBP52 increases affinity of the hormone in glucocorticoid and androgen receptors, while FKBP51 is a negative regulator regarding receptor activity (Cheung-Flynn et al. 2005; Denny et al. 2000; Mamane et al. 2000; Riggs et al. 2003). Similar to Cyp40, FKBP52 binds to dynein supporting transport of the steroid hormone receptor complex along the cytoskeleton to the nucleus (Galigniana et al. 2001, 2010). Again, FKBP51 displays antagonistic behavior and delays this translocation to the nucleus (Wochnik et al. 2005). Interestingly, Hsp90 coprecipitates with C2I at the same time points in the same lysates from toxin-treated cells as Cyp40 and FKBP51 (Ernst et al. 2015; Kaiser et al. 2012). Moreover, a direct interaction was shown between Hsp90 and the enzyme components of C2, iota, and CDT toxins (Kaiser et al. 2011). However, the binding/translocation component C2IIa does not bind to Hsp90 or the PPIases, implying that the interaction with these host cell factors is limited to the enzyme components of clostridial ADP-ribosylating toxins (Ernst et al. 2015). Keeping in mind that chaperones and PPIases are involved in folding and refolding of proteins and that the enzyme components have to be at least partially unfolded during translocation into the cytosol to fit through the narrow pore formed by the B-components, it is plausible that the denatured conformations of C2I, Ia, and CDTa show an enhanced binding to the chaperones and PPIases (Haug et al. 2003a; Pratt and Toft 1997).

Up to now, all investigated ADP-ribosylating toxins require Hsp90/PPIases for their membrane translocation into the host cell cytosol, including DT and CT discussed later in this chapter. In contrast, toxins that display high structural homology and/or very similar uptake mechanisms but have a different enzyme activity like the *Bacillus (B.) anthracis* lethal toxin, a metalloprotease, or the large glycosylating toxins A and B of *C. difficile,* are independent of Hsp90/PPIases (for an overview, see Table 1) (Dmochewitz et al. 2011; Haug et al. 2003b; Kaiser et al. 2009, 2011, 2012; Zornetta et al. 2010). Thereby, the hypothesis was deduced that the requirement of Hsp90/PPIases might be a common and specific characteristic of ADP-ribosylating toxins. This hypothesis is supported by further investigations using recombinant fragments and fusion toxins based on the enzyme component C2I of the C2 toxin. It is known that the N-terminal adapter domain of C2I (C2IN), consisting of the first 225 amino acids, displays no enzyme activity but is as

Table 1 Overview of toxins that are dependent or independent of chaperones and PPIases

	Hsp90	Cyp	FKBP	Hsp70	
Toxins that require Hsps/PPIases					
C. botulinum C2 toxin	✓	✓	✓	n.d	ADP-RT
C. perfringens iota toxin	✓	✓	✓	✓	
C. difficile CDT toxin	✓	✓	✓	n.d	
P. luminescens PTC3 toxin	✓	✓	✓	n.d	
Corynebacterium diphtheriae toxin	✓	n.d	n.d	n.d	
Vibrio cholerae toxin	✓	n.d	n.d	n.d	
Toxins that do *not* require Hsp/PPIases					
C. difficile TcdA	–	–	–	n.d	Glycosyl transferase
B. anthracis lethal toxin	–	–	–	n.d	Metalloprotease
But cellular uptake of fusion toxins with ADP-RT activity requires Hsp90/PPIases					
C2IN-C3lim	✓	✓	✓	n.d	ADP-RT
LF_NDTA	✓	✓	n.d	n.d	
And the isolated ADP-RT domain of *P. luminescens* PTC3 toxin determines the interaction with Hsp90/PPIases					
HisTccC3hvr	✓	✓	✓	n.d	ADP-RT

Details are given in the text [summarized from data from Dmochewitz et al. (2011), Ernst et al. (2015, 2016), Haug et al. (2003b, 2004), Kaiser et al. (2009, 2011, 2012), Lang et al. (2014), Ratts et al. (2003), Taylor et al. (2010), Zornetta et al. (2010)]

✓ = respective host cell factor is required for the membrane translocation of the respective toxin

n.d. = not done

ADP-RT ADP-ribosyltransferase, *PPIase* peptidyl-prolyl *cis/trans* isomerase

efficiently taken up into the host cell cytosol as the full-length C2I (Barth et al. 1998a). However, the uptake of C2IN is independent of Hsp90 and PPIases (Kaiser et al. 2012). Only if fusion toxins, such as C2IN-C3lim, are constructed where an ADP-RT domain is fused to the adapter domain C2IN, Hsp90 and PPIases are required for the efficient membrane translocation of the fusion toxins into the host cell cytosol (Kaiser et al. 2012; Pust et al. 2007). The uptake of fusion toxins consisting of C2IN and proteins that are not ADP-RTs is also independent of Hsp90/PPIases, further supporting the hypothesis of a common and specific Hsp90/PPIase-dependent membrane translocation for ADP-ribosylating toxins (Kaiser et al. 2012). Unfortunately, it is not possible to express the C-terminal part of C2I, which harbors ADP-RT activity, as a functional enzyme on its own, most likely due to lack of proper protein folding. Therefore, the ADP-ribosylating PTC3 toxin of *Photorhabdus* (*P.*) *luminescens* was used as a model toxin (Lang et al. 2010). For this toxin, the expression and purification of its isolated His-tagged ADP-RT domain (HisTccC3hvr) are possible. Another important advantage of this isolated His-tagged ADP-RT domain is that it can be delivered via the PA_{63} pore of

the *B. anthracis* lethal toxin into the host cell cytosol (Beitzinger et al. 2012; Blanke et al. 1996; Lang et al. 2010), which enables the investigation of the membrane transport and role of host cell chaperones/PPIases in this process for an isolated ADP-RT.

Excursion: The *B. anthracis* toxins The anthrax toxins show various similarities to the clostridial binary ADP-ribosylating toxins concerning structural homology of the components, uptake route via endosomes, and the heptameric pore structure. Native anthrax toxin consists of three different protein components. The lethal factor (LF), a 90 kDa metalloprotease which cleaves MAP kinase kinases (Duesbery et al. 1998; Tonello and Montecucco 2009; Vitale et al. 1998) and edema factor (EF), an 89 kDa adenylyl cyclase (Leppla 1982, 1991), represent the two enzymatically active A-components. Both components are transported into the cytosol of target cells via the 83 kDa binding and translocation B-component protective antigen 83 (PA_{83}). Therefore, PA_{83} binds to its cell surface receptors, ANTXR1 and ANTXR2 (Young and Collier 2007), is then proteolytically activated by the endoproteinase furin, and oligomerizes into its oligomeric (heptamers and octamers) prepore form PA_{63}, which binds LF and EF (Young and Collier 2007). The receptor-bound toxin complexes are internalized into the cell via clathrin-coated pits (Abrami et al. 2003) by actin- and clathrin-dependent endocytosis and are delivered to acidic endosomes (Abrami et al. 2003, 2004, 2010; Friedlander 1986; Zornetta et al. 2010). The acidic condition triggers a dynamic change in the oligomeric PA_{63}, leading to its conversion into a membrane-spanning pore (Blaustein et al. 1989; Miller et al. 1999) through which LF and EF translocate into the cytosol, where they exhibit their respective enzymatic activity (Collier 2009; Young and Collier 2007). For translocation through the narrow PA_{63} pore, LF and EF need to be unfolded, which is mediated by the acidic pH of the endosomal lumen (Krantz et al. 2004).

It has been shown that the PA_{63} pore is able to transport His-tagged proteins via this uptake mechanism into the host cell cytosol. It was suggested that the positively charged His-tag interacts with specific structures in the PA_{63} pore lumen and thereby facilitates the binding and uptake of the His-tagged protein (Beitzinger et al. 2012; Blanke et al. 1996; Lang et al. 2010). Noteworthy, uptake of the natural enzyme component LF via PA_{63} is independent of Hsp90/PPIases (Dmochewitz et al. 2011; Zornetta et al. 2010). However, the specific pharmacological inhibitors of Hsp90/PPIases, Rad, CsA, and FK506 inhibit uptake of the isolated His-tagged ADP-RT domain HisTccC3hvr of the PTC3 toxin via PA_{63} (Lang et al. 2014). Moreover, Hsp90, CypA, Cyp40, and FKBP51 coprecipitate with HisTccC3hvr in lysates from toxin-treated cells, strongly suggesting that the ADP-RT domain determines the interaction with Hsp90 and PPIases which supports the hypothesis of a common and unique membrane translocation for ADP-ribosylating toxins. Noteworthy, the membrane translocation of the wild-type PTC3 toxin also depends on Hsp90 and PPIases, although the pore formed by the binding/translocation component of PTC3 is very different from the pore formed by the clostridial binary toxins (Lang et al. 2014). This demonstrates that the interaction between Hsp90 and

the PPIases with ADP-ribosylating toxins is not limited to one particular translocation pore type, but rather depends on the ADP-RT domain of the toxin.

Furthermore, the uptake of another fusion toxin, LF_NDTA, which artificially contains an ADP-RT domain, was investigated regarding the involvement of Hsp90 and PPIases. The fusion toxin LF_NDTA consists of the N-terminal 254 amino acids of LF genetically fused to the enzymatic active domain of DT, DTA (Arora and Leppla 1994). Thereby, LF_N mediates the uptake via the PA_{63} pore, and DTA exhibits the ADP-RT activity, enabling a sensitive detection of translocated LF_NDTA. Besides studying the involvement of host cell factors during anthrax lethal toxin uptake, LF_NDTA/PA_{63} represents also a variant ADP-ribosylating toxin for studying the requirement of host cell factors for this group of toxins.

In this context, by performing an in vitro translocation assay with LF_NDTA/PA_{63}-preloaded endosomal vesicles, it was demonstrated that translocation and release of this DTA fusion toxin from the lumen of acidified endosomal vesicles to the external milieu are indeed enhanced by the addition of ATP and crude cytosolic extracts to the translocation assay mixture (Tamayo et al. 2008). The successful translocation was hereby specifically demonstrated by measuring ADP-RT activity mediated by DTA (Tamayo et al. 2008). Furthermore, by using LF_NDTA, the requirement of both Hsp90 and Cyps for DTA translocation was observed (Dmochewitz et al. 2011). Thereby, Rad and CsA protect CHO-K1 cells from intoxication with LF_NDTA/PA_{63}. In contrast, intoxication of cells by native LF/PA_{63} is not impaired by these compounds, confirming the effect based on DTA (Dmochewitz et al. 2011). Moreover, it was shown that Rad and CsA prevent the PA_{63}-mediated release of active LF_NDTA, but not of LF, in vitro from purified endosomal vesicles into the cytosol. The usage of a specific antibody in this assay identified the isoform CypA as an essential Cyp involved in this process (Dmochewitz et al. 2011). Due to the fact that CsA is not isotype selective, Cyps other than CypA might be involved in this process. Besides, by using the dot blot method, a direct interaction of LF_NDTA, but not LF, with Hsp90 and CypA was detected in vitro (Dmochewitz et al. 2011). This interaction was also shown for native DTA and enzymatically inactive DTA (DTAE148S), revealing that the ADP-RT domain but not the activity is required for this interaction (Dmochewitz et al. 2011). Prior to the studies of Dmochewitz et al. with LF_NDTA, Ratts et al. established a functional role of Hsp90 for DTA translocation across the early endosomal membrane using the fusion protein $DAB_{389}IL\text{-}2$ (Ratts et al. 2003). Here, the native R-domain of DT is substituted with human interleukin-2 (IL-2), resulting in a specific targeting of the cytotoxic action only to cells that express the high-affinity IL-2 receptors (Bacha et al. 1988; Ratts and van der Spek 2002; Waters et al. 1990). In the first step of their experiments, protein complexes required for toxin translocation were isolated from both human and yeast cytosolic extracts and applied to mass spectrometry sequencing to identify individual proteins required for DTA translocation. Further on, specific inhibitors and neutralizing antibodies for immunoprecipitation were used in both the in vitro translocation assay and cytotoxicity assays to examine the role of certain proteins. It was demonstrated that the in vitro translocation of DTA from the lumen of

toxin-preloaded, partially purified endosomal vesicles through the DTB pore to the external milieu requires both the addition of ATP and a cytosolic translocation factor (CTF) complex, which includes the chaperone Hsp90 and the thioredoxin reductase (TrxR) (Lemichez et al. 1997; Ratts et al. 2003). Building on the results of Ratts et al. who performed their experiments with the fusion toxin DAB_{389}IL-2 and used the TrxR-specific inhibitor *cis*-13-retinoic acid, the group of Holger Barth tested another pharmacological TrxR inhibitor, auranofin, concerning its effect on the intoxication of cells by native DT (Schnell et al. 2015). Here, it was observed that HeLa cells are protected from DT intoxication in the presence of auranofin. Inhibitory effects of auranofin on the enzymatic activity of DTA, as well as on the receptor-binding of DTB, were excluded. Most importantly, the performance of an in vitro translocation assay during which the conditions in the early endosome are mimicked directly at the cytoplasmic membrane of intact cells allows an isolated look at this particular step and the effect of auranofin based on the inhibition of DTA translocation into the cytosol (Schnell et al. 2015). Thus, the functional activity of TrxR during DTA translocation of native DT was confirmed. However, whether or not TrxR is directly involved in reducing the interchain disulfide bond between DTA and DTB as hypothesized is still not known. So far, all studies addressing the role of host cell factors for DTA transport across cell membranes, except the experiments of Schnell et al. (2015) for TrxR, have been performed with DT-related fusion protein toxins. Therefore, it is still of question whether Hsp90 and Cyps also play a role during the uptake of native DT.

Concerning the role of host cell factors for anthrax toxin uptake, in contrast to LF_NDTA, the membrane translocation of the native A-component LF across the endosomal membrane through the PA_{63} pore is independent of Hsp90 and Cyps, LF also does not directly bind to CypA and Hsp90 in vitro (Dmochewitz et al. 2011). Nevertheless, LF delivery is also dependent on cytosolic factors such as ATP and COPI coatomer complex proteins. Abrami et al. demonstrated that a functional COPI coatomer complex was required for anthrax toxin action in intact cells in culture (Abrami et al. 2004). These results were confirmed by Tamayo et al. (2008), who performed an in vitro translocation assay of LF_NDTA from LF_NDTA/PA_{63}-loaded purified early endosomes and pull-down experiments using GST-LF_N (Tamayo et al. 2008). Here, immunodepletion of COPI coatomer complex proteins from crude cytosolic extracts reduced the in vitro translocation of LF_NDTA and the pull-down revealed an interaction of LF_N with ζ- and β-COP (Tamayo et al. 2008). Their investigations based on the results of Ratts et al. who identified a 10 amino acid motif in transmembrane helix 1 of the T-domain of DT, termed T1, which is conserved in the N-terminal segments of EF and LF of anthrax toxin and botulinum neurotoxin serotypes A, C, and D (Ratts et al. 2005). Interestingly, all these toxins have in common that they employ a similar route of entry into the cell, requiring the passage through an acidified early endosomal compartment to deliver their respective A-subunit into the cytosol (Bade et al. 2002; Pappenheimer 1977; Wesche et al. 1998). The relevance of this T1 motif for DT A-domain translocation was demonstrated by using a mutant of DAB_{389}IL-2 containing an amino acid exchange within this conserved motif and by applying a transfected cell line

expressing a T1 motif-containing peptide (Ratts et al. 2005). By performing pull-down experiments using the fusion protein GST-$DT_{140–271}$, they finally showed specific binding of β-COP to this region of the T-domain (Ratts et al. 2005). Therewith, Ratts et al. (2003) confirmed and extended the results from Lemichez et al. (1997). The latter observed that by using an N-terminal C-myc-tagged DT mutant (E148S) with low ADP-ribosylating activity (Barbieri and Collier 1987), there is a colocalization of DT with β-COP in tubular structures in electron microscopy (Lemichez et al. 1997). Furthermore, in their study, antibodies against β-COP prevent the in vitro translocation of DTA across the endosomal membrane of purified endosomes (Lemichez et al. 1997). Thus, both LF_N of anthrax toxin LF and DTB of DT interact with β-COP (Lemichez et al. 1997; Ratts et al. 2005; Tamayo et al. 2008). According to these results, COPI coatomer complex proteins play an essential role in the cellular uptake of anthrax lethal toxin and DT. In both cases, the interaction is mediated by the T1 motif, located either in transmembrane helix 1 of DT T-domain or in the N-terminal segment of LF.

The involvement of host cell factors for the uptake and mode of action of CT has been intensively studied in recent years by the group of Ken Teter. They identified Hsp90 as essential for CTA1 translocation from the ER to the cytosol (Taylor et al. 2010). By using surface plasmon resonance (SPR), Taylor et al. (2010) observed a direct binding of Hsp90 to the isolated CTA1 subunit in an ATP-dependent manner at the physiological temperature of 37 °C. This binding is blocked by the addition of the Hsp90 inhibitor GA. Concerning the functional role of Hsp90 for CT uptake, CT toxicity assays were performed in the presence and absence of GA, using the level of intracellular cAMP to assess toxicity. In the presence of GA, both cultured cells and ileal loops are protected from CT intoxication, therewith indicating a functional role of Hsp90 for CT intoxication. Besides, the knockdown of Hsp90 resulted in cells highly resistant to intoxication by CT. By applying GA in an established dislocation assay, Taylor et al. (2010) demonstrated that the efficient delivery of CTA1 into the cytosol requires the activity of Hsp90. In addition, GA was shown to inhibit the export of CTA1 from the ER into the cytosol, but it does not impair intracellular trafficking of CT to the ER (Taylor et al. 2010). Thereby, these results were the first demonstration of an Hsp90-dependent export of a soluble protein from the ER. In a further study, the same group investigated the co- and post-translocation roles of Hsp90 for CT intoxication in more detail (Burress et al. 2014). By performing a series of in vitro assays, it was demonstrated that Hsp90 is able to convert disordered CTA1 to a structured conformation and therewith restores its enzymatic activity (Burress et al. 2014). Thereby, refolding of CTA1 by Hsp90 and the maintenance of a high-affinity interaction between Hsp90 and the refolded toxin require ATP. Besides this, it was demonstrated that ATP hydrolysis by Hsp90 is also required for the actual export of CTA1 from the ER (Burress et al. 2014). Using SPR, Hsp90 was further shown to prefer binding to the unfolded conformation of CTA1 and the in vitro data also suggest that Hsp90 does not dissociate from CTA1 after toxin extraction from the ER. Thus, CTA1 refolding and extraction from the ER by Hsp90 seem to be coupled. Besides their investigations on Hsp90, Burress et al. (2014) also showed that cytosolic

ADP-ribosylation factor 6 (ARF6) in its GTP-bound form can bind to the CTA1-Hsp90 complex, and this association enhances the enzymatic activity of CTA1 (Burress et al. 2014). Therewith, they confirmed and extended their results obtained in Banerjee et al. (2014) and demonstrated that for optimal toxin activity, both host factors, Hsp90 and ARF6, are required (Banerjee et al. 2014). On the basis of their results, Burress et al. (2014) hypothesized an Hsp90-driven model for CT translocation from the ER in which Hsp90-mediated refolding of CTA1 leads to its inhibition of backsliding of CTA1 into the translocation pore, resulting in a unidirectional ER to cytosol export. Further on, Hsp90 remains associated with the refolded CTA1 and this complex then interacts with ARF6, which stimulates the activity of CTA1 at physiological temperature (Burress et al. 2014). According to these results, Hsp90-mediated translocation is not limited to the endosomal membrane, as demonstrated for the binary actin ADP-ribosylating clostridial toxins and DT, but also is required for toxin translocation from the ER via the ERAD pathway.

Taken together, Hsp90 activity is required for the membrane translocation of various bacterial ADP-RTs including clostridial binary toxins, DT and CT. Moreover, a functional involvement of PPIases of the Cyp and FKBP families was shown for the cellular uptake of clostridial actin ADP-ribosylating binary toxins and recombinant ADP-RT containing fusion toxins. Interestingly, the interaction with Hsp90 and PPIases seems to be specific for and restricted to ADP-RT toxins since the membrane translocation of toxins that display a different enzyme activity is independent of Hsp90 and PPIases suggesting a novel and unique membrane translocation mechanism for ADP-ribosylating bacterial toxins.

4 Hsps, Cyps, and FKBPs as Novel Drug Targets: Inhibition of Membrane Translocation of ADP-RT Domains Prevents Intoxication of Cells with Particular Bacterial Protein Toxins

The novel findings summarized in this article that chaperones and PPIases facilitate the membrane translocation of bacterial ADP-ribosylating toxins should also be of medical relevance: If the membrane translocation of the ADP-RT subunit of the toxins into the host cell cytosol can be inhibited by specific pharmacological inhibitors of the identified host cell chaperones/PPIases, the cytosolic substrate would not be modified by the toxin. This means that the toxin-induced cytotoxic effects would not occur, and therefore, the toxin-associated clinical symptoms should be prevented and/or diminished. For Hsp90 inhibition, novel derivatives of Rad and GA were already developed for the treatment of cancer and characterized in vitro and in vivo. Such compounds show improved tolerance and less adverse effects compared to Rad or GA in clinical trials (Li and Buchner 2013). The specific pharmacological inhibitors of Cyps and FKBPs, respectively, CsA and FK506, are already applied as drugs (Borel et al. 1976; Clipstone and Crabtree 1992; Liu et al.

1991; Schreiber et al. 1991). Both CsA and FK506 have an immunosuppressive effect and therefore are mostly used for organ transplantations to avoid organ rejection (Borel et al. 1976). The immunosuppression by FK506 and CsA proceeds analogously. CsA binds to the PPIase site of Cyps, and this CsA/Cyp complex is now able to bind to the protein phosphatase calcineurin, resulting in its inhibition. As a consequence, the transcription factor nuclear factor of activated T cells (NF-AT) is not dephosphorylated by calcineurin and therefore cannot activate the transcription of interleukins in the nucleus, resulting in a decreased activation of T lymphocytes (Clipstone and Crabtree 1992; Liu et al. 1991; Schreiber et al. 1991). Since CsA inhibits the membrane translocation of the ADP-RTs of clostridial binary toxins and likely other ADP-ribosylating toxins, this compound should be considered as a novel therapeutic approach to diminish the toxin-induced symptoms in some food-borne intoxications associated with bacterial ADP-RT toxins. However, the immunosuppressive effect of CsA might be counterproductive in patients suffering from infection with toxin-producing bacteria. Therefore, the effect of the tailored CsA derivative VK112 that lacks immunosuppressive effects, but still inhibits PPIase activity (Prell et al. 2013) on intoxication of cells with clostridial ADP-ribosylating toxins, was tested (Ernst et al. 2015). As expected and comparable to CsA, VK112 efficiently protects cells from intoxication with binary actin ADP-ribosylating toxins from clostridia and inhibits the membrane transport of their ADP-RT components into the cytosol (Ernst et al. 2015).

Therapeutic strategies that aim at chaperones or PPIases as novel drug targets tackle the toxin-induced diseases not on the bacterial level like the antibiotics, but specifically on the molecular level (i.e., the toxins). This implicates that such inhibitors might still be able to protect cells from intoxication even if the toxin has already been taken up into cells because the compounds inhibit the intracellular membrane translocation and therefore the uptake of the "toxic" subunit into the cytosol. For example, diphtheria is treated by the application of diphtheria antitoxin that neutralizes DT in the patient. However, cell-bound or already internalized DT is not affected by the antitoxin. Moreover, novel strategies that aim at the toxin level are especially important if the disease is caused by the toxins in the absence of their producing bacteria, as in many food-borne intoxications. If there is no causative treatment available except for antibiotic treatment, or if antibiotic treatment becomes difficult due to increasing numbers of hypervirulent bacteria strains that resist traditional antibiotics, novel therapeutics are clearly needed now (Barth 2011; WHO position paper 2010).

5 Conclusion and Future Directions

Taken together, there is substantial evidence that membrane translocation of the clostridial ADP-ribosylating C2, iota, and CDT toxins is facilitated by the host cell chaperone Hsp90 and PPIases of the Cyp and FKBP families. Moreover, DT and CT depend on Hsp90 for the membrane translocation of their enzyme domains into

the host cell cytosol. The interaction between CTA1 and Hsp90 has been investigated in more detail showing that Hsp90 not only facilitates membrane translocation but also restores enzyme activity of CTA1 in an ATP-dependent manner. Whether Hsp90 acts in a comparable way with ADP-RTs of other bacterial toxins such as the clostridial toxins or DT has to be elucidated.

So far, it is not known whether DT and CT also require Cyps and FKBPs for membrane translocation like the clostridial ADP-RTs. Regarding the fact that an Hsp90 multichaperone complex, containing Cyps and FKBPs as cochaperones, was described for the activation of steroid hormone receptors in the cell, it might be conceivable that Hsp90 and PPIases also act in a concerted manner during the membrane translocation of bacterial ADP-RTs. Diseases caused by bacterial ADP-ribosylating toxins such as diphtheria, cholera, and other enteric diseases still pose a severe threat to human and animal health. Finally, a better understanding of the membrane translocation mechanism and therefore identification of novel interaction partners of bacterial ADP-ribosylating toxins potentially harbor medical impact. Hsp90 and PPIases might serve as novel drug targets for specific pharmacological inhibitors and/or improved derivatives such as the CsA derivative VK112 that successfully inhibit membrane translocation of the clostridial ADP-ribosylating toxins. Moreover, all results so far suggest that an Hsp90/PPIase-dependent membrane translocation is a special and common characteristic of only ADP-ribosylating toxins. Toxins with different enzyme activities such as the glycosylating toxin TcdA of *C. difficile* do not require Hsp90 or PPIases for their membrane translocation into the host cell cytosol. This might implicate that novel therapeutic strategies based on Hsp90/PPIases as drug targets not only might be applied against clinical symptoms of one unique disease, but also might be effective against several diseases caused by ADP-ribosylating toxins that affect different parts of the body.

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Cross References

Stiles BG, Clostridial binary toxins: basic understandings that include cell-surface binding and an internal “coup de grace”

Multivalent Inhibitors of Channel-Forming Bacterial Toxins

Goli Yamini and Ekaterina M. Nestorovich

Abstract Rational design of multivalent molecules represents a remarkable modern tool to transform weak non-covalent interactions into strong binding by creating multiple finely-tuned points of contact between multivalent ligands and their supposed multivalent targets. Here, we describe several prominent examples where the multivalent blockers were investigated for their ability to directly obstruct oligomeric channel-forming bacterial exotoxins, such as the pore-forming bacterial toxins and B component of the binary bacterial toxins. We address problems related to the blocker/target symmetry match and nature of the functional groups, as well as chemistry and length of the linkers connecting the functional groups to their multivalent scaffolds. Using the anthrax toxin and AB5 toxin case studies, we briefly review how the oligomeric toxin components can be successfully disabled by the multivalent non-channel-blocking inhibitors, which are based on a variety of multivalent scaffolds.

Contents

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1 Introduction

1.1 Multivalency in Nature

A Burdock—clawed my Gown—
Not Burdock's—blame—
But mine—
Who went too near
The Burdock's Den
—Emily Dickinson

Many nature-lovers are familiar with the pesky burr, the plant seed-sacks that attach themselves to clothing or to animal fur in order to travel to wider fertile planting areas. The famous "Queen Recluse," American legendary poet Emily Dickinson gave a dark poetic description of the burdock sticking to her gown. In a more practical way, Swiss electrical engineer George De Mestral examined the cockleburs, which clung to his clothes and his dog's fur after his summer walks in the Alps, through a microscope. De Mestral discovered that the burrs were covered by multiple small hooks which were readily able to attach to the diminutive loops on the clothing and fur of those passing near the "*Burdock's Den*." In 1955 after more than eight years of experimenting with different types of material, from natural to synthetic, De Mestral invented "the zipperless zipper," the product known today as Velcro®, the word that originates from French "*vel ours*" (velvet) and "*cro chet*" (hook). The product was made up of two strips of fabric, one covered by thousands of small hooks and the other with thousands of small loops designed to give an ideal and firm match. Increasing the material surface led to an increase in the mutual effect. Originally receiving skepticism from the fashion designers, today Velcro® production has become a multimillion dollar industry. Another fascinating manufacturing example is related to the U.S. military, developing special climbing gloves that would enable soldiers to climb vertical surfaces. The invention is inspired by the unique feet of the gecko species that are covered by an array of tiny long tubular filaments, the so-called *setae* with branched tips, each containing hundreds of miniature fibers. This exceptional surface architecture allows the lizards to cling equally effectively on various hydrophobic and hydrophilic surfaces due to dry adhesion of gecko setae by van der Waals forces (Autumn et al. 2002). With a single van der Waals interaction being lower than 2 kJ/mol, the numerous points of contact provide sizable amount of energy to secure tight but reversible binding. These types of multivalent interactions are ubiquitous in nature, where strong non-covalent binding is achieved by operation of multiple recognition events between multivalent ligands and receptors. Additional examples include adhesion of viruses to their target cell receptors, antibody–antigen interaction, intercellular

recognition, and many others. Through the billions of years of evolution, nature has optimized these interactions ensuring that an array of weak molecular recognition events is acting in a powerful balanced concert (Mahon and Fulton 2014). Therefore, design and investigation of the synthetic multivalent compounds mimicking nature's ability for multiple recognition events have tremendous prospects for the rational drug design goals (Fasting et al. 2012).

1.2 Multivalent Ligands to Combat Multivalent Targets

Multivalency is one of the best modern tools to transform relatively weak, non-covalent monovalent interactions into a strong binding by creating multiple points of contact between multivalent ligands and their receptor interfaces (Fasting et al. 2012). Traditionally, multivalent interaction is defined as specific simultaneous binding of multiple *functional groups* attached onto an inert platform, named *scaffold,* to multiple target receptor sites (Choi 2004). Numerous natural or designed multivalent molecules are built either on linear scaffolds or on preorganized, rigid, and conformationally defined scaffolds that allow for the suitable positioning and density of the functional group ligands. The proper display of the functional groups is frequently intended to match arrangement and distances between receptors on the complementary surface. The design and synthesis of the multivalent drug molecules involves covalent attachment of multiple copies of the previously identified functional groups onto a scaffold using molecular fragments, called *linkers*, or *spacers*. It is expected that the linker would serve as at least an "innocent observer" (Fasting et al. 2012) securing appropriate spacing between the functional groups. The linkers could also be designed to favorably contribute to the ligand/receptor interaction (Choi 2004). In addition, parameters such as linker length, conformational rigidity or flexibility, and chemical nature are frequently considered as important factors in the multivalent compound design (Mammen et al. 1998b; Shewmake et al. 2008; Krishnamurthy et al. 2007; Kane 2010). This chapter is written to discuss some of the recent advances in designing multivalent inhibitors of the channel-forming bacterial toxins. We also highlight several prominent examples of using the multivalent molecules in other aspects of ion channel research and provide examples of the multivalent inhibitors that counteract the channel-forming bacterial toxins without direct pore blockage. The multivalent inhibitors targeting non-pore-forming bacterial toxins will be described briefly in Sect. 3; for additional examples, we address the reader to the recent reviews published on the subject (Branson and Turnbull 2013; Weisman et al. 2015).

2 Multivalent Inhibitors of Channel-Forming Bacterial Exotoxins

Bacterial exotoxins are polypeptides or proteins secreted by pathogenic bacteria during invasion to destroy or damage host cells. Many of them oligomerize on the cell surface constituting the ideal symmetrical multivalent receptor targets for rational design of multivalent antitoxin ligands (Joshi et al. 2008; Ivarsson et al. 2012). Nowadays when biological origins of many diseases are investigated on a molecular level, with methods such as *X*-ray crystallography, cryo-EM, and all-atom molecular dynamic simulations, providing deep insight into structure/function relationships of the biomolecules, rational drug discovery strategies concurrently evolve. Below we discuss the recent efforts to rationally design multivalent antitoxins specifically focusing on ones tuned to interact with the multivalent targets made of oligomeric pore-forming bacterial toxins. In a way, this approach represents the development of an original idea related to attachment of an influenza virus to a target cell that suggests that multivalent attachment can be blocked effectively by an inhibitor that is itself multivalent (Mammen et al. 1998a).

2.1 Pore-Forming Bacterial Toxins as Targets for Multivalent Blocker Development

In 2001, Joseph E. Alouf had estimated that 35 % (115 out of 325) of bacterial exotoxins, identified by that time, attack mammalian cells damaging the cytoplasmic phospholipid bilayer membranes (Alouf 2001). Most of these exotoxins do so by forming transmembrane pores which allow the uncontrollable flow of ions down their electrochemical gradients leading to reduction in the membrane potential and eventually to the collapse of the plasma membrane barrier function (Bernheimer 1996). To reflect the mechanism of action of these toxins, they are often referred to as *membrane-perforating* or *pore-forming bacterial toxins* (*PFTs*). When added into model bilayer membrane bathing solutions, many PFTs spontaneously form large (nS conductance), stable (last for hours) oligomeric pores ideally suitable for molecular-sensing approaches that can be expanded to rational design of the effective multivalent pore blockers. One of the most striking examples is heptameric β-barrel α-hemolysin (αHL) of *Staphylococcus aureus*. The biosensing properties of this toxin, its stability, and structural robustness have determined its use for the stochastic resistive-pulse sensing of a variety of substrates, ranging from small molecules to polymers and biomacromolecules. Interestingly, even though these applications are not directly related to the toxic properties of αHL, they provide a deep insight into single molecule biophysics of the particle/channel binding reactions proved to be helpful for rational channel blocker design approaches.

Being secreted as a water-soluble 293-amino acid monomeric polypeptide, αHL forms heptameric pores in target cell membranes (reviewed in Berube and Bubeck Wardenburg 2013). In 1981, Oleg Krasilnikov and coauthors reconstituted αHL proteins into bilayer lipid membranes for the first time and recorded formation of large, stable, and slightly anion-selective channels of ~1 nS conductance in 1 M KCl solution (Krasilnikov et al. 1981). The channels remained in an open low-noise state for hours. The 1.9-Å resolution crystal structure of the heptamer revealed a hallow mushroom-like 100 Å × 100 Å (length by diameter) channel made of stem, cap, and rim domains with two narrow 0.9-nm and 0.6- to 0.7-nm constriction zones (Song et al. 1996). In the last twenty years after gaining momentum from the pioneering articles by Kasianowicz and Bezrukov (Bezrukov and Kasianowicz 1993; Kasianowicz and Bezrukov 1995), who suggested to employ αHL as a nanoscopic cuvette for reaction dynamic studies, this PFT was explored as a biological nanosensor to detect a wide variety of analytes. Among the multiple virulence factors secreted by *S. aureus*, in the context of this review, it is worth mentioning bicomponent octameric γ-hemolysin (Hlg), leukocidin (Luk), and Panton–Valentine leukocidin (PVL). These toxins are formed as a result of the interaction between two distinct polypeptides named F and S components (Kaneko and Kamio 2004; Alonzo and Torres 2014).

2.2 *Binary Bacterial Toxins as Targets for Multivalent Blocker Development*

In contrast to the PFTs damaging host cells by acting on their cell membranes, AB-type exotoxins employ a different mode of intoxication enzymatically modifying specific intracellular targets (Geny and Popoff 2006). These toxins are frequently secreted as single-chain polypeptides containing at least two functionally distinct domains, the binding B domain that docks to specific surface receptors and the active A domain that modifies certain substrates in the cytosol. Alternatively, the AB-type toxins, the so-called binary toxins, are made of two (or three) individual non-linked proteins with one acting as a binding/translocation B factor and another as an enzymatic A factor acting intracellularly (Barth et al. 2004). The B components of the binary toxins, after binding to their specific cell receptors, self-assemble to form ring-shaped oligomeric prepores able to bind several A components. These complexes are subsequently endocytosed and the oligomers are converted into the membrane-spanning ion-conductive pores reported to mediate the A component transport from the acidic endosomal environment into the cytosol. When incorporated into model lipid bilayers, B components of the binary toxins form robust and structurally stable β-barrel channels, apt for single-channel single molecule investigation (Blaustein et al. 1989; Schmid et al. 1994; Knapp et al. 2002). Therefore, for the purposes of this review, we will focus on the multivalent pore blockers designed to inhibit the 2nd group of the AB-type toxins.

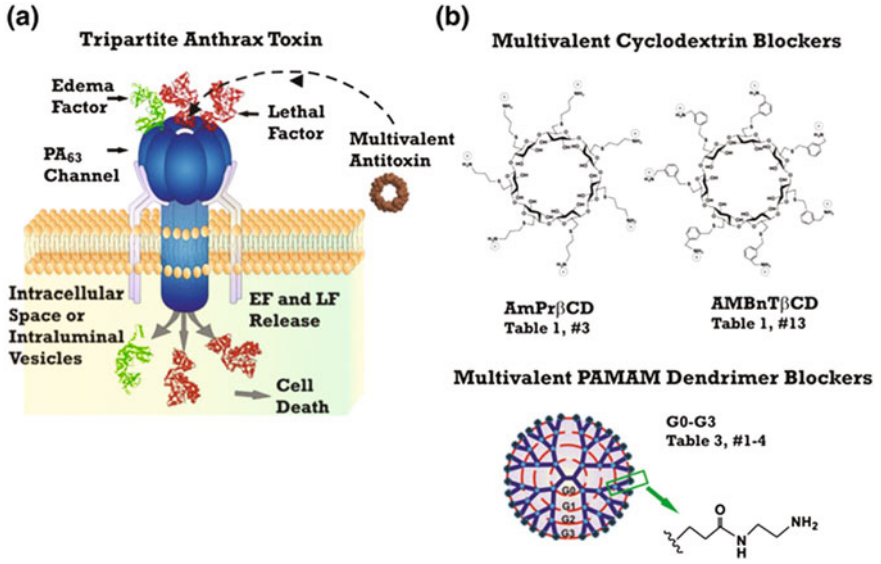


Fig. 1 Multivalent channel-blocking inhibitors of PA_{63} of anthrax toxin. **a** Illustration of the idea: a multivalent blocker as effective inhibitor of PA_{63} of tripartite anthrax toxin. **b** The most prominent examples of the multivalent blockers are based on cyclodextrin (Karginov et al. 2005) (*top*) and dendrimer (Forstner et al. 2014) (*bottom*) scaffolds

The existing efforts to develop pore-forming bacterial toxin inhibitors mainly concentrate on targeting the anthrax toxin (Fig. 1a) and a family of related clostridial binary toxins, C2, iota, and CDT (Nestorovich et al. 2011; Roeder et al. 2014). Following intentional dissemination of *Bacillus anthracis* spores via the "*anthrax letters*" in Sept. 2001, quite remarkable developments were made in understanding molecular details of anthrax toxin uptake (Moayeri et al. 2015; Liu et al. 2015). Briefly, the tripartite anthrax toxin is released as three separate proteins, lethal factor (LF), edema factor (EF), and protective antigen (PA) that self-assemble at the host cell surface to form complexes contributing to the symptoms of anthrax. LF is a Zn-metalloprotease that cleaves MAP kinase kinases (Duesbery et al. 1998; Vitale et al. 2000) and Nlrp1 (Levinsohn et al. 2012). EF is a Ca^{2+} and calmodulin-activated adenylyl cyclase (Leppla 1982, 1984). PA, named this way for its ability to produce the protective antibodies in the anthrax vaccines, is a receptor-binding and translocation component that is important for the intracellular delivery of LF and EF. The anthrax toxin uptake occurs in several stages. After binding to the cellular CMG2 and TEM8 receptors and being proteolytically cleaved, PA oligomerizes to make heptameric (Petosa et al. 1997) and/or octameric (Kintzer et al. 2009, 2010) ring-shaped prepores. The oligomeric $(PA_{63})_7$ prepore formation creates three (Mogridge et al. 2002) or four (Kintzer et al. 2009) LF- and EF-binding sites. The toxic AB complexes then undergo the receptor-mediated endocytosis (Pilpa et al. 2011). Subsequently, the acidic environment of the

endosome causes substantial conformational changes of the PA oligomers leading to their insertion into endosomal membranes and formation of cation-selective channels (Blaustein et al. 1989). 2.9-Å cryo-EM imaging of the channel showed an elongated "flower-on-a-stem" heptamer with an external diameter ranging from ~27 to ~160 Å with 75-Å long bud and 105-Å long stem (Jiang et al. 2015) with channel radius varying from ~16 Å to as low as ~3.5 Å. The broadly accepted model of anthrax toxin translocation asserts that the PA oligomer acts as an effective translocase, capable of unfolding and translocating LF and EF into the cytosol using the proton gradient across endosomal membranes ($pH_{endosome} < pH_{cytosol}$) (Zhang et al. 2004a, 2004b; Krantz et al. 2006). The translocation was shown to be facilitated by a ring of phenylalanyl residues at position 427 of $(PA_{63})_7$ (ϕ-clamp) (Krantz et al. 2005) and a substrate binding α-clamp (Brown et al. 2015; Feld et al. 2010). Kasianowicz and colleagues developed an alternative model showing that the anthrax toxin is able to catalyze the rupture of endosomal membranes allowing for the toxic complexes to be released into the cytosol (Nablo et al. 2013). Interestingly, PA_{63} was shown to deliver LF not only into the cytosol but also inside endosomal intraluminal vesicles that later fuse and release LF into the cytosol (Abrami et al. 2013).

A group of clostridial binary channel-forming toxins (Knapp et al. 2015a): C2 (Ohishi and Odagiri 1984; Aktories et al. 1986; Simpson 1984), iota (Simpson et al. 1987; Stiles and Wilkins 1986), and CDT (Popoff et al. 1988) are related both structurally and functionally. They are made of two components where the A subunit acts through mono-ADP-ribosylation of G-actins (Aktories and Wegner 1989; Barth et al. 2015) and the B subunit binds and mediates delivery of the A subunit into the cytosol (Barth and Stiles 2008). PA and clostridial binary toxin B subunits have high degrees (from 27 to 38 %) of amino acid homology and are made of four distinct domains, each involved in the host receptor interaction, oligomerization, channel formation, and binding of the A subunits (Petosa et al. 1997; Schleberger et al. 2006). Similar to PA_{63}, the proteolytically activated B subunits form ring-shaped heptamers, called *prepores*, on the host cell surface or in solution (Barth et al. 2000). The cell-bound AB complexes, C2I/C2IIa, Ia/Ib, and CDTa/CDTb are then internalized by receptor-mediated endocytosis (Blocker et al. 2001; Stiles et al. 2002; Nagahama et al. 2009; Pust et al. 2010) and enzymatic components translocate across the endosomal membranes into the cytosol. This process may involve use of the pores formed by binding/translocation components of the toxins as translocation corridors (Barth et al. 2000; Stiles et al. 2002; Bachmeyer et al. 2001; Blocker et al. 2003; Gibert et al. 2007). In mildly acidic conditions (pH < 6.6) in vitro, the B subunits of C2 and iota toxins form cation-selective ion channels (Schmid et al. 1994; Knapp et al. 2002). The phenylalanine clamp preserved in position 428 (C2IIa) and 454 (Ib) was reported to catalyze unfolding of C2I and Ia in the course of their transport across endosomal membrane (Knapp et al. 2015b; Lang et al. 2008; Neumeyer et al. 2008). Remarkably, PA_{63} is able to bind and translocate His-tagged A component of the

C2 toxin (C2I). These similarities suggest that the universal approaches, for instance, those targeting the channel-forming components of the toxins, should be considered in rational design of broad-spectrum binary toxin inhibitors.

2.3 Cyclodextrin-Based Channel Blockers

When designing multivalent inhibitors of the oligomeric bacterial toxins which are frequently arranged into the centrosymmetric structures, one may consider settling ligands into a rigid cyclic scaffold (Fig. 1b) with a controlled number of pre-assembled attachment sites to achieve their proper positioning (Choi 2004). Cyclodextrins (CDs), cyclic oligomers of glucose which are typically composed of six (αCD), seven (βCD), and eight (γCD) subunits represent a well-studied example of the rigid scaffolds. Non-modified CDs are amphipathic molecules shaped like truncated cones. The CD's exterior is hydrophilic due to presence of the outward-facing 2- and 3-OH groups on the wider rim and 6-OH group on the narrow rim. The hydroxyl groups can be easily derivatized by covalent attachment of different ligands (Choi 2004; Wenz 1994), which provides unlimited opportunities for their selective rational modifications (Khan et al. 1998). The hydroxyl groups at positions 2 and 3 form hydrogen bonds that are required to keep the molecule rigid, as a result, the hydroxyl groups at position 6 are frequently considered as a favorable site to introduce substitutions (Khan et al. 1998). Szejtli estimated that by 2004 over 15,000 αCD, βCD, and γCD derivatives had been reported and their properties were investigated in a number of diverse applications (Szejtli 2004). The hydrophobic interior of CD's cavity defines its remarkable ability to form water-soluble "host–guest" inclusion complexes with otherwise poorly soluble small molecules and macromolecule fragments. This unique property has determined a long history of CD use in pharmaceutical, agrochemical, environmental, cosmetic, and food industries. In addition, CDs typically do not elicit immune responses and have relatively low toxicity in animals and humans (Davis and Brewster 2004). We specifically want to refer our readers to an excellent review on history of CDs that covers many different aspects of the 120 years of development of these "novel" compounds (Crini 2014).

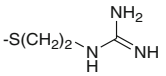
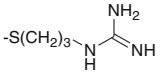
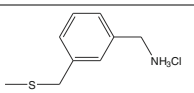
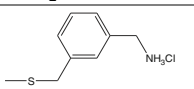
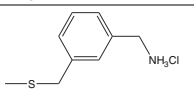
The original idea (Karginov et al. 2005) to rationally modify CDs into effective antitoxins was based on the wealth of earlier research on stochastic sensing of organic analytes by an αHL channel containing a non-covalently bound βCD as a molecular adapter (Gu et al. 1999). The approach was designed to transform a water-filled lumen of αHL into a sensor element capable of detecting a number of small organic molecules that normally would not bind to this channel. To do so, Bayley and colleagues equipped an αHL incorporated into bilayer lipid membranes with CDs that were able to enter inside the pore lumen causing partial ($\sim$60 %) reversible blockages of the ion current. Because of the partial mode of the current blockage, the authors used the system to detect and quantify a variety of guest molecules, such as adamantanamine hydrochloride, adamantine carboxylic acid,

promethazine, and imipramine, which presumably interact with the hydrophobic cavity of the βCD. The symmetry match between heptameric αHL pore and sevenfold symmetrical βCD secured the comfortable fit between the channel and adapter molecules. At the same time, sixfold symmetrical αCDs and eightfold symmetrical γCDs were also shown to reversibly interact with this heptameric channel (Gu et al. 1999).

The rationally modified cyclodextrin molecules were also investigated as potent channel-blocking antitoxins against αHL (Karginov et al. 2007; Ragle et al. 2010) and binary bacterial anthrax (Karginov et al. 2005), C2, iota (Nestorovich et al. 2011), and CDT toxins (Roeder et al. 2014). As channel-forming components of the binary bacterial toxins are known to be preferentially selective to cations (Blaustein et al. 1989), nearly any tested positively charged ligands were shown to reversibly block K^+ current through PA_{63} (Krantz et al. 2005), C2IIa (Bachmeyer et al. 2003; Beitzinger et al. 2013; Bronnhuber et al. 2014), and Ib (Knapp et al. 2002) channels in a wide nM–mM range of effective concentrations. The significant increase in efficiency of the cationic compounds was achieved when multiple copies of positively charged ligands were covalently linked to a cyclodextrin scaffold (Karginov et al. 2005, 2006b). Thus, synthetic tailor-made sevenfold symmetrical βCDs (d ≈ 15 Å) carrying seven amino groups, each attached to the 6th position of the ring via a variety of hydrophobic linkers (Fig. 1b, top), effectively blocked PA_{63} current (Nestorovich et al. 2010) (Table 1). To optimize the 7+βCD compounds, the authors focused on a number of structural characteristics of these molecules, such as length and chemical nature of the linkers connecting functional groups to the CD scaffold, nature of functional groups, and ligand/blocker symmetry complementarity.

Length and chemical nature of linkers Multivalent compounds are frequently designed by connecting multiple copies of a functional group to a scaffold with a linker (Choi 2004). These linkers are not only used as the covalent connectors for the ligands but also secure proper spacing to allow for the optimal fit between the multivalent target and the multivalent molecule. To determine an optimal length of the spacers, which tether the amino groups to the βCD, a group of hepta-6-thioaminoalkyl derivatives was investigated. By combining planar lipid bilayer measurements and cell assay studies, the authors reported that the alkyl linkers (3–8 CH_2-linkers) were almost equally effective, with IC_{50} values from 0.6 to 4.6 nM in planar bilayers and from 0.3 to 7.5 μM in cell assays (Karginov et al. 2006a). Shorter spacers were less effective (Table 1), which is apparently related to the reduced size of these blockers and/or restricted mobility of the linkers. 7+βCDs with longer linkers (9–10 CH_2-linkers) did show some evident channel blockage at low-nM concentrations but induced instability in the bilayer membranes and toxicity to the RAW cells. Positive linear correlation ($R^2 = 0.84$) between the planar lipid bilayer and cell assay measurements supports the concept that 7+βCDs inhibit anthrax toxins by blocking the PA_{63} channel. The most significant increase in 7+βCD pore blocking activity was achieved when a single phenyl group was introduced into each thio-hydrocarbon linker (Karginov et al. 2006a). Three

Table 1 Multivalent positively charged cyclodextrin-based blockers of PA_{63} channel ion current and LT cytotoxicity

No.	Cyclodextrin	Substituent	Inhibition of PA_{63} channels in model lipid membranes, IC_{50} (nM)	Inhibition of LT cytotoxicity, IC_{50} (μM)
Hepta-6-aminoalkyl *β*-cyclodextrin derivatives (Karginov et al. 2006a)				
1	*β*	$-NH_2$	140 ± 90	20 ± 9
2	*β*	$-S(CH_2)_2NH_2$	3.5 ± 0.9	7.8 ± 2.4
3	*β*	$-S(CH_2)_3NH_2$	0.57 ± 0.39	2.9 ± 1.0
4	*β*	$-S(CH_2)_4NH_2$	1.1 ± 0.5	5.1 ± 2.4
5	*β*	$-S(CH_2)_5NH_2$	3.8 ± 1.0	7.5 ± 2.4
7	*β*	$-S(CH_2)_6NH_2$	0.97 ± 0.38	0.6 ± 0.3
8	*β*	$-S(CH_2)_7NH_2$	4.6 ± 3.2	1.9 ± 1.1
9	*β*	$-S(CH_2)_8NH_2$	2.4 ± 0.95	0.3 ± 0.1
10	*β*	$-S(CH_2)_{10}NH_2$	27.0 ± 17.0	2.6 ± 0.7
Hepta-6-guanidinealkyl *β*-cyclodextrin derivatives (Karginov et al. 2006a)				
11	*β*		5.3 ± 3.2	8.9 ± 6.0
12	*β*		12.6 ± 9.0	12.2 ± 2.9
Hepta-6-arylamine *β*-cyclodextrin derivative (Karginov et al. 2006a; Yannakopoulou et al. 2011)				
13	*β*		0.13 ± 0.10	0.8 ± 0.5
Cationic α- and γ-cyclodextrin derivatives (Yannakopoulou et al. 2011)				
14	α	$-NH_2$	1200 ± 300	>100
15	γ	$-NH_2$	170 ± 50	12 ± 3
16	α		29 ± 5	45 ± 13
17	γ		2.8 ± 1.3	5.4 ± 0.8

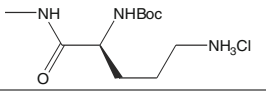
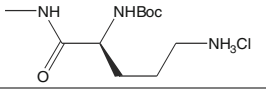
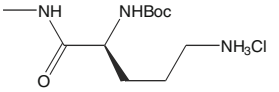
hepta-6-arylamine *β*CD derivatives (AMBnT*β*CDs) with the methylamino groups located in 2nd, 3rd, and 4th position of the aromatic ring were reported to protect RAW cells with IC_{50} values being as low as 0.5 ± 0.2 μM. When tested using the planar lipid bilayer technique, AMBnT*β*CD blocked PA_{63} channel conductance with K_D = 0.13 ± 0.1 nM. AMBnT*β*CD was found to completely protect Fisher F344 rats from LT. Moreover, in combination with ciprofloxacin, the antibiotic used to treat *Bacillus anthracis* infection, AMBnT*β*CD significantly increased mice

survival in an infection mode of anthrax (Moayeri et al. 2008). AMBnTβCD represents an interesting example of a multivalent compound with linkers favorably contributing to a contact formed between target and ligand. The exact mechanism of phenyl group contribution to the blocking activity of AMBnTβCD is not clear at the moment. On one hand, the small-molecule cationic compounds containing aromatic fragments were reported to block PA_{63} more effectively than their aliphatic molecular counterparts (Krantz et al. 2005). This effect was explained by the stabilizing aromatic–aromatic, π–π or cation–π interactions between aromatic groups on the blockers and phenylalanyl residues of the ϕ-clamp. On the other hand, inclusion of the aromatic fragments can change conformation and flexibility of the linkers optimizing the binding. To understand the physical forces responsible for the channel/blocker interaction, the binding reaction was studied in solution with different electrolyte concentrations and under a wide range of applied transmembrane voltages (−100 to +200 mV) (Bezrukov et al. 2012). It was demonstrated that in the 7+βCD/PA_{63} binding reaction, salt concentration-independent short-range forces prevail. At relatively low salt concentrations, βCD residence time inside the channel was influenced by long-range Coulomb interactions. The existence of an additional electrostatic component was indicated by an increase in the blocker residence time as a function of transmembrane voltage.

Nature of functional groups To determine if the nature of tethered positively charged groups is critical for effective binding, the aminoalkyl substituents were compared with a group of hepta-6-guanidine β-CD derivatives, in which positive charges were distributed between two nitrogens on the guanidine group (Table 1, compounds 11 and 12) (Karginov et al. 2006a). No increase in the blocker activity was detected; instead these compounds showed slightly lower PA_{63} channel-blocking and cell-protective activity.

Symmetry of the cyclodextrin scaffolds The original idea behind design of the effective PA_{63} channel blockers was to build the sevenfold symmetrical blocker molecules to complement the heptameric structure of the channel. However, later it was demonstrated that this requirement is not strict because both 6+αCD and 8 +γCD were able to inhibit channel conductance (Yannakopoulou et al. 2011). 6 +αCD/PA_{63} binding was noticeably weaker (IC_{50} = 1.2 μM in planar bilayers and >100 μM in cell assays), whereas 8+γCD/PA_{63} binding was comparable with that of 7+βCD/PA_{63} both in planar bilayers and in cell assays (Table 1, compounds 14-17). At the same time, the sevenfold symmetry of the blockers turned out to be fundamentally important when the symmetry match idea was extended to the cyclodextrin antitoxins designed to block ion current through αHL (Karginov et al. 2007). Thus, a seven positively charged hepta-6-substituted βCD derivative, IB201, effectively inhibited ion current through αHL in planar bilayers and protected rabbit red blood cells from αHL-triggered hemolysis. In a murine model of *S. aureus* infection, IB201 was reported to prevent the αHL-mediated alveolar epithelial cell lysis and mortality (Ragle et al. 2010). The sixfold and eightfold symmetrical IB201 analogues were not effective (Table 2). The molecular details of the αHL inhibition by IB201 were revealed using single-channel planar lipid bilayer experiments.

Table 2 α, β, and γCD blockers of αHL channel ion current and toxicity (Yannakopoulou et al. 2011)

No.	Cyclodextrin	Substituent	Inhibition of a single αHL channel in model lipid membranes, IC_{50} (nM)	Inhibition of αHL toxicity, IC_{50} (μM)
1	α		>5000	>100
2	β		~50	3.3 ± 2.3
3	γ		>5000	>100

Thus, application of IB201 blocker to the side of the membrane that corresponds to the cap side of the heptamer resulted in αHL switching to a weakly conductive state with a residual conductance ranging from 1 to 15 % of the open-channel conductance. The αHL/IB201 binding was reported to be significantly influenced by the transmembrane voltage, negative from the side opposite to the blocker addition. Moreover, within the time period of the measurements (several hours), IB201 binding to αHL was irreversible resembling the voltage-gating type of the channel closure. Interestingly, the symmetry complementarity was not required for the CD passive diffusion across the monomeric 14-stranded β-barrel bacterial porin, CymA, of *Klebsiella oxytoca* (Pajatsch et al. 1998, 1999). Recently, Winterhalter and colleagues suggested an interesting model to describe the passive diffusion of CDs through CymA (van den Berg et al. 2015).

2.4 Dendrimer-Based Channel Blockers

Dendrimers are rigid highly divaricate starburst-shaped polymers, composed of regularly repeated branches originating from a central core. This architecture offers multiple unique opportunities for development of multivalent ligands (recently reviewed in Wu et al. 2015). Dendrimer synthesis processes are well established. The controlled production of monodispersed dendrimers, where each consecutive growing step results in a new "generation" with an increased diameter and doubled number of surface groups, determines dendrimer surface valence (Tomalia and Frechet 2002; Wijagkanalan et al. 2011). Dendrimer properties, such as their

nanoscale dimensions, rigidity, stability, monodispersity, highly regulated multivalency, and ability to form host–guest complexes, have resulted in numerous emergent medical and industrial applications, including their use as antibacterial, antiviral, and antiparasitic agents (Helms and Meijer 2006). The void interior of dendrimer molecules has been used to incase drugs for targeted delivery or biomarkers for imagining purposes. The outer shell is densely packed with a well-controlled number of terminals, which can be modified with a wide variety of functional groups to vary their biological activity and bioavailability. The starburst configuration makes dendrimers particularly useful for analyzing modes of multivalent compound interaction within confined objects, such as nanopores (Martin et al. 2007; Ficici et al. 2015; Forstner et al. 2014; Bustamante et al. 2000). Dendrimer-related studies on ion channels are limited. In 2007, Howorka's group used sulfhydryl-reactive polyamido amine (PAMAM) dendrimers of generations 2, 3, and 5 with a mixed surface of terminal hydroxyl/amine groups to couple with αHL channels containing engineered cysteine residues (Martin et al. 2007). This type of modification was expected to change the stochastic-sensing properties of αHL under placement of charged and dense dendrimer polymers into their lumen. The authors reported that PAMAM dendrimers acted as both an ion-selectivity filter and a molecular sieve for the passage of small molecules and biopolymers. The extent of this modification was dependent on the PAMAM generation; the bigger G5 dendrimers did not enter the *cis* entrance, while G3 was able to couple inside the pore. Fluorescently labeled starburst amino-terminated dendrimers were used for rapid nuclear pore patch-clamp sizing, as an inert class of particles that can enter pores without irreversibly affecting their gating (Bustamante et al. 2000). Polypropylenimine dotriacontaamine G3 and G4 dendrimers were tested for their ability to block *E. coli* E69 pore-forming Wza K30 capsular polysaccharide transporter (Kong et al. 2013). However, no detectable interaction of the dendrimers with the Wza pore was detected. At the same time, rationally designed tetrameric G1 poly(propylene imine) dendrimer decorated with four arylpiperazine moieties (Cappelli et al. 2011) was shown to be effective in activating the pentameric serotonin 5-HT3 receptor (Paolino et al. 2014). 5-HT3 receptors are ligand-gated ion channels that open upon binding of serotonin, allowing for the influx of Na^+ and K^+ and creating an excitatory response in neurons. The group designed two homotetravalent compounds with shorter, TETRA-S and longer, TETRA-L linkers. Interestingly, the correct geometry of the bioactive moiety was only achieved with the longer TETRA-L. TETRA-L was shown to be more potent than the corresponding monovalent ligand (threefold difference) and the bivalent ligand (twofold difference). In another study, transport of dye-labeled amino-terminated PAMAM dendrimers through silica colloidal nanopores, as a function of the nanopore and dendrimer size, was investigated as a potential application in size-selective separations (Ignacio-de Leon and Zharov 2011).

Recently, commercially available amino-terminated PAMAM dendrimers of different generations and imperfect dendrimers, known as dendrons, were tested for their ability to block channel-forming components of binary anthrax and C2 toxins (Table 3, Fig. 1b, bottom) (Forstner et al. 2014). According to the manufacturer

Table 3 Multivalent PAMAM dendrimer- and dendron-based blockers of PA_{63} channel ion current (Forstner et al. 2014)

No.	Generation	Measured diameter (Å)	Number of surface NH_2 groups	Inhibition of PA_{63} channels in model lipid membranes, IC_{50} (nM)
PAMAM-NH_2 dendrimers				
1	0	15	4	128 ± 44
2	1	22	8	5.3 ± 2.6
3	2	29	16	7.15 ± 4.7
4	3	36	32	5.0 ± 1.4
5	4	45	64	2.4 ± 1.3
6	8	97	1024	0.22 ± 0.08
7	10	135	4096	0.16 ± 0.07
PAMAM-OH dendrimers				
8	2	n/a	0	142 ± 36
9	3	n/a	0	45 ± 14
PAMAM-SA dendrimers				
10	2	n/a	0	> 150000
PAMAM-COONa dendrimers				
11	0.5	n/a	0	> 400000
75%OH/25%NH_2 PAMAM dendrimers				
12	2	n/a	4 (ave)	122 ± 35
PAMAM-NH_2 dendrons				
13	0 dendron	n/a	2	26 ± 7
14	1 dendron	n/a	4	4.9 ± 0.7
15	2 dendron	n/a	8	4.2 ± 0.9

(Dendritech®), PAMAM dendrimers are synthesized based on an ethylene diamine core and amidoamine repeat branching structure and come in generations (G0–G10) with increasing molecular diameter (d = 15–135 Å) and doubled number of reactive surface sites with every subsequent generation (ranging from z = +4 to +4096). All but the smallest G0 dendrimers were reported to inhibit PA_{63} channels at low-nM and C2IIa channels at high-nM concentrations in the model bilayer membranes. Interestingly, the IC_{50} values (0.16 − 230 nM) for these commercially available dendrimers compare well with the ones for the very first rationally designed β-CD blocker AmPrβCD (0.55 nM), which was selected out of dozens of related compounds. At that, G1, G2, and G3 dendrimers were found to have higher *per-functional group* activity in inhibiting PA_{63} and C2IIa in comparison with G0 and G4, G8, and G10 dendrimers. Moreover, dendrimers of generation 2 and higher affected the morphology of the tested cells by their own. G0 and G1 did not interfere with cell morphology and viability but protected HeLa cells from intoxication with C2 toxin by inhibiting His-C2I delivery into the host cell cytosol.

Based on the molecular modeling of PA_{63} oligomeric channel (Lee et al. 2012) and its negative-stain electron microscopy image (Katayama et al. 2008), the authors expected 15–45 Å G0–G4 dendrimers to enter and block PA_{63} channels, similarly to that observed with the 7+βCD inhibitors. However, not only smaller G0–G4 but also G8 (d = 97 Å) and G10 (d = 135 Å) dendrimers were shown to effectively inhibit both PA_{63} and C2IIa channels. This finding is in agreement with a recently published article addressing PAMAM dendrimer behavior inside the confined space of αHL channel (Ficici et al. 2015). Using single-channel measurements and molecular dynamics simulations, the authors show that PAMAM permeation inside the αHL lumen is not determined by the apparent hydrodynamic size of the dendrimers but rather by their generation-dependent conformational flexibility. Easily compressible G1 and G2 dendrimers were able to completely enter inside the stem opening of αHL, G3 did not fully enter the barrel. Small fragments of G4 and G5 dendrimers, for example, several branches, were still able to enter inside the pore.

To address the problem of toxicity, several modified dendrimers were investigated (Table 3). In particular, PAMAM G2 and G3 dendrimers functionalized with neutral hydroxyl (compounds 8 and 9), but not with anionic succinamate and carboxyl surface groups (compounds 10 and 11), inhibited PA_{63} conductance in planar lipid bilayers. Thus, G2-OH and G3-OH were 20 and 9 times less effective than the amino-terminated dendrimers of the same generation. This finding was explained by the positively charged tertiary amino groups at the branching points of the PAMAM core structure interacting with the negatively charged PA_{63} lumen. The favorable therapeutic window can sometimes be achieved by partial surface modification to lower the density of the attached ligands, or by degradation of the dendrimers to the "fractured" or "imperfect" dendrons (compounds 13–15) (Tang et al. 1996). The mixed surface 75%OH/25%NH_2 G2 PAMAM dendrimer (compound 12) was ~17 times less active compared to its 16-positively charged G2-NH_2 analogue (compound 3). However, activity of the G1-NH_2 dendron, functionalized with 4 positively charged amines (compound 14), was ~26 times higher (IC_{50} = 4.9 ± 0.7) compared with that of the G0-NH_2 dendrimer (compound 1), which also carries four positive charges (IC_{50} = 128 ± 44). The authors explain this effect either by increase in mobility of the surface primary amino groups of the dendrons or by improved access of the tertiary amino groups to the binding sites in the PA_{63} lumen.

2.5 *Blocking Channel-Forming Bacterial Toxins Without Blocking the Channel*

While the antitoxins discussed above were mostly design to block the toxin channels directly, the channel-forming bacterial toxins could also be inhibited by multivalent molecules that do not permeate into the channels. Kane's group has successfully designed a number of ligands attached to several multivalent scaffolds

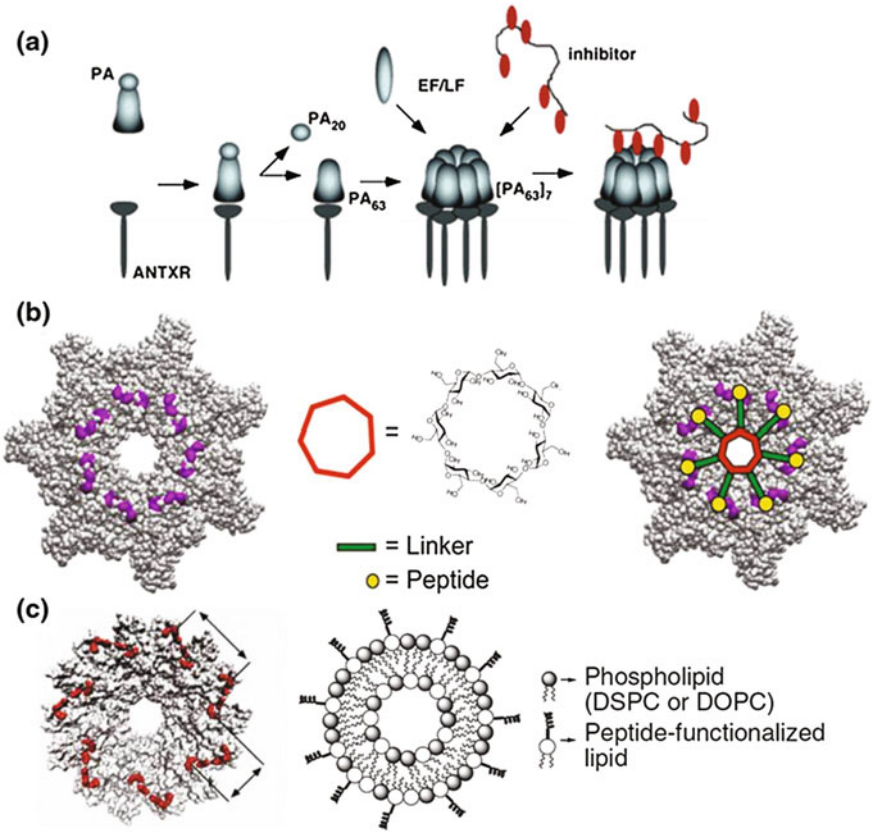


Fig. 2 Multivalent non-channel-blocking inhibitors of PA_{63} of anthrax toxin. **a** Peptide-based inhibitors. **b** Cyclodextrin-based inhibitor of anthrax toxin complexes assembly. **c** Liposome-based inhibitor of anthrax toxin complexes assembly. Reprinted with permission from Joshi et al. (2011), Rai et al. (2006), and Gujraty et al. (2005). © 2005, 2011. American Chemical Society. © 2006. Nature Publishing Group

(Vance et al. 2009) for disabling the channel-forming component of anthrax toxin either by blocking its cellular receptors (Basha et al. 2006) or by blocking PA_{63} interaction with LF and EF (Mourez et al. 2001). We focus here on the 2nd type of inhibitors (Fig. 2), because they are designed to directly bind to the PA_{63} oligomers. Using the peptide phage-display library, Mourez et al. identified an inhibitory peptide (HTSTYWWLDGAP) that was able to weakly (IC_{50} = 150 μM) bind the $(PA_{63})_7$ prepores and inhibit their interaction with the enzymatic components of anthrax toxin (Mourez et al. 2001). When multiple copies of this peptide were covalently attached to an *N*-acryloyloxysuccinimide (pNAS) polymer backbone, the resulting polymer inhibited LF and EF binding to PA_{63} with a 7500-fold improvement in efficacy, and protected rats from anthrax toxin (Fig. 2a). The group has also used a reversible addition fragmentation chain transfer (RAFT) technique

to optimize the synthesis process to get more control over the spacing of functional groups and the ability to copolymerize pNAS with non-active acrylamide (Gujraty et al. 2008). It was also reported that although multivalency can greatly enhance ligand potency, overcrowding of the binding sites can have the opposite effect (Gujraty et al. 2006). Instead of a pNAS backbone, Joshi et al. used activated poly-L-glutamic acid to conjugate HTSTYWWLDGAP peptide copies, and reported IC_{50} values of 20 nM per-peptide basis, which were comparable to the polyacrylamide-based inhibitors (Joshi et al. 2006). More recently instead of using the peptide decorated polymeric molecules, the group synthesized polypeptide repeats to interpose multiple instances of modified inhibitory HTSTYWWLDGAP (LIG) peptides with flexible peptide linkers in the sequence of SE[LIG–(SE)m]n to assure optimal linker length, flexibility, and ligand density (Patke et al. 2014). Guided by molecular dynamics simulation data, the authors created and tested a range of monodisperse candidate inhibitors with $(H)_{10}$-SE[LIG$(SE)_5]_7$ compound made of decahistidine tag that aids in the purification of the polypeptides, five sequential repeats of serine and glutamic acid, and seven HTSTYWWLDGAP repeats. The IC_{50} value of this blocker was 4 ± 0.5 nM per inhibitory peptide, exceeding the corresponding monovalent ligands by fourfold. When seven copies of HTSTYWWLDGAP peptide were attached to a β-cyclodextrin core with polyethylene glycol (PEG) linkers (Fig. 2b), the resulting compound effectively neutralized anthrax lethal toxin, being 100,000-fold more effective than the monovalent peptide (Joshi et al. 2011). The authors varied the linker length to determine that PEG_{11} allowed for an optimal fit, matching the 30-Å distance from the center of the PA_{63} oligomer to the peptide-binding faces.

Significant increase in the ligand activity was achieved when ~50-Å liposomes (Fig. 2C) were decorated with multiple copies of HTSTYWWLDGAP peptides (IC_{50} = 20 nM on a per-peptide basis) (Rai et al. 2006). This strategy was refined with design of liposome containing raft-like membrane microdomains to create regions of optimal ligand density (Rai et al. 2007). A major advancement of this approach was recently suggested in a study where artificial liposomes, containing higher than in vivo concentrations of cholesterol and sphingomyelin, were tailored to effectively compete with host cell membranes for toxin binding (Henry et al. 2015). These "membrane mimicking" nanoparticles efficiently sequestered a significant number of toxins secreted by a variety of staphylococcal and streptococcal pathogens and treated fatal invasive diseases in mouse models.

Calix[*n*]arenes (C[*n*]s), cone-shaped phenol–formaldehyde macrocycles, provide another easily adaptable scaffold for the design of high-affinity multivalent ligands (Varejao et al. 2013; Baldini et al. 2007). In a pioneering study, para-sulfonated calixarenes were found to effectively block outward rectifying chloride channels, showing subnanomolar inhibition constants and exceptionally long blockage times (Singh et al. 1995). Modified calix[*4*]arenes were also reported to effectively interact with tetrameric voltage-dependent potassium channels by binding to their surface in a reversible manner (Martos et al. 2009). Several p-sulfonato-C[*n*]s were effective in inhibiting staphylococcal pore-forming cytolysins, Hlg, Luk, and PVL (Laventie et al. 2013). These heterooctameric toxins are made via interaction of the

two distinct class F and class S polypeptides. Even though the original compound selection was based on the potential ability of these compounds to block the upper ring of the Luk pores, the actual mechanism of toxin inhibition is probably different. The authors suggested that p-sulfonato- C[*n*]s act by preventing the binding of class S proteins to membranes and eventual formation of Luk pores.

3 Representative Examples of Multivalent Inhibitors of Other Bacterial Exotoxins

Multisubunit organization is inherent to a number of clinically relevant non-pore-forming bacterial exotoxins. One of the most prominent examples is represented by AB5 toxins of the cholera toxin family, which include cholera toxin, *E. coli* heat-labile enterotoxin, Shiga-like toxins (SLTI and SLTII), and pertussis toxin (Beddoe et al. 2010). AB5 toxins are made of a single catalytic A subunit, which disrupts essential host functions intracellularly, and a homopentameric B subunit, responsible for binding to glycan receptors on the target cell. Blocking these multivalent B subunit/receptor interactions would inhibit the toxin's intracellular uptake. Extensive structural information on these proteins and their complexes with different small ligands made the AB5 family toxins an attractive model system for design of multivalent ligands (Fig. 3). These inhibitors act by blocking the initial cell host receptor-binding step during the AB5 toxins uptake (Fan and Merritt. 2002). A representative of this group is Shiga-like toxin of *E. coli* that binds to a specific glycolipid, globotriaosylceramide, Gb3 to enter host cells. Ling et al., resolving a 2.8 Å crystal structure of Shiga-like toxin I complexed with an analogue of the Gb3 trisaccharide, has shown that three trisaccharide molecules bind to each monomer of the B-pentamer complex resulting in 15 trisaccharides interacting with a single pentamer of the toxin (Ling et al. 1998). While the individual affinity of the carbohydrate–protein interaction was low, it was outdone by the multivalent toxin binding; the feature that required employment of the multivalent inhibitors "tailored" to the structure of the B-subunit pentamer (Kitov et al. 2000). The authors designed a decavalent water-soluble carbohydrate ligand, named STARFISH, with 1- to 10 million-fold higher in vitro inhibitory activity against Shiga toxin B-pentamer compared with that of monovalent ligands. Interestingly, crystallographic study of the complex formed between Shiga toxin I B-pentamer and STARFISH showed a binding pattern which was different from the one projected by the rational design of the compound. It turned out that STARFISH molecules bind to two B-subunit monomers from separate toxin molecules, forming a molecular sandwich between two pentamers, instead of bridging two sites on a single molecule. The 2:1 toxin/ligand complexes were formed even though STARFISH concentration was sufficient to form a 1:1 complex. However, solution-phase measurements predicted that 1:1 complexes could also form (Mulvey et al. 2003). Nevertheless, the study represents an example of unusually

high efficacy (nM) for glycoconjugates of such size and valency protecting susceptible cells against prolonged exposure to SLTI and more clinically significant SLTII. The same group has extended the study designing a more active 2nd-generation multivalent inhibitor that was capable of simultaneously interacting with several binding sites on the same B component subunit (Mulvey et al. 2003). In vivo studies have shown only modest activity of the lead STARFISH inhibitor in protecting mice against Shiga toxin. A modified version of STARFISH, called Daisy, designed to simultaneously occupy binding sites 1 and 2 in the STLI and STLII subunits, was capable of protecting mice from the lethal effects of both SLTI and SLTII. Bundle's group created a class of optimized inhibitors where polymeric scaffolds were used to display covalently preorganized heterobifunctional ligand pairs, designed to mediate formation of specific complexes of an endogenous multivalent protein with a multivalent target (Fig. 3a) (Kitov et al. 2008). The

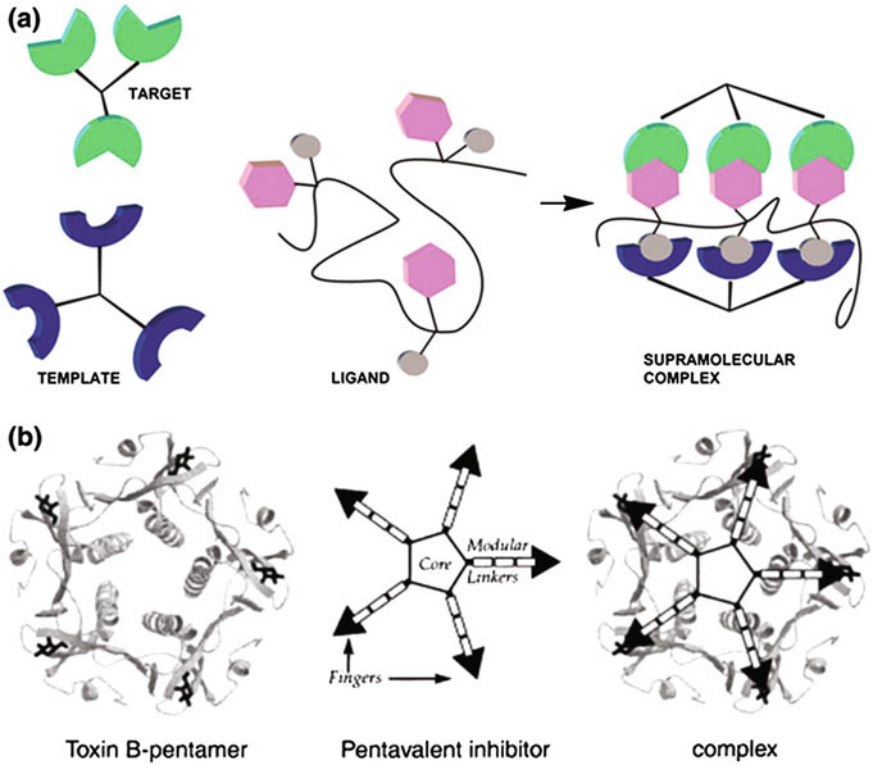


Fig. 3 Multivalent inhibitors of AB5 bacterial toxins. **a** A schematic representation of polymeric preordered heterobifunctional ligands. **b** Conceptual design of pentavalent inhibitors based on symmetrical core, variable number of linker units, and monovalent fingers blocking the toxin receptor binding site. Reprinted with permission from Kitov et al. (2008) and Merritt et al. (2002). © 2008 National Academy of Sciences, USA. © 2002 American Chemical Society

polymeric inhibitors/adaptors containing preordered heterobifunctional ligands that recognize Shiga toxin Type 1 and human serum amyloid P components have shown a significant increase in in vivo activity to protect mice against Shigotoxemia.

Unlike the Shiga toxins, cholera toxin and *E. coli* heat-labile enterotoxin's B-pentamers contain only one set of five host cell receptor binding sites. This property simplifies rational design of the effective multivalent inhibitors against these toxins. However, large, ~45 Å separation between the non-adjacent binding sites creates some challenges (Fan and Merritt 2002). First, a series of pentavalent 1-*β*-amidated D-galactose ligands attached to an acylated pantacyclen 5 core with a number of flexible linkers of different lengths was suggested (Fan et al. 2000). The compounds with an ideal match to the size of the B-pentamer were >100,000-fold more effective (0.56 μM vs. 58 mM) compared to the monovalent ligands. This approach was further refined when pentavalent inhibitors based on more effective monovalent ligands were constructed (Fig. 3b) (Merritt et al. 2002). Thus, the inhibitor carrying five copies of m-nitrophenyl-α-galactoside (MNPG) instead of *β*-D-galactose was >100-fold more effective (0.9 μM vs. 15 μM). Using dynamic light scattering and *X*-ray crystallography, the authors provided proof for a 1:1 toxin/ligand association that occurs through single-site binding interaction of the pentavalent inhibitor with the surface of the toxin B-pentamer. The MNPG moiety was later investigated as a substructure to anchor hydrophobic ring systems able to bind in a variety of modes within the receptor binding site of both cholera toxin and *E. coli* enterotoxin B-pentamers (Pickens et al. 2002). The group has later extended the study replacing the morpholine ring core of the blockers by cyclic peptide cores. This approach, together with manipulation of the linker length, allows for the controlled tuning of the multivalent inhibitor dimensions to obtain an optimal match with the target (Zhang et al. 2004c).

Recent advancements in search of multivalent AB5 toxin inhibitors include ganglioside GM1 oligosaccharide ligands attached on corannulene (IC_{50} = 5 nM) (Mattarella et al. 2013), calixarene (IC_{50} = 450 pM) (Garcia-Hartjes et al. 2013), and the inactive cholera toxin B subunits (104 pM) (Branson et al. 2014). It was shown that the multivalent GM1-inhibitors with mismatched valencies can show significant increase in potency compared to the monovalent ligand, apparently due to formation of higher-order structures (Sisu et al. 2009; Fu et al. 2015). About 3-orders of magnitude affinity increase was observed by structure-based design of heterobifunctional ligands that were able to dimerize two different multivalent proteins. In a particular example of cholera toxin, the bifunctional ligands allowed for dimerization of the B-pentamer with human serum amyloid P component (SAP) (Liu et al. 2005). Similarly, markedly enhanced activity of the multivalent ligands was achieved with creation of a multivalent heterofunctional inhibitor-adapter, called "BAIT" capable of capturing a Shiga toxin subunit by one ligand of a heterobivalent headgroup and an endogenous SAP trap proteins by the other ligand (Solomon et al. 2005).

4 Concluding Remarks

Despite the massive vaccination campaigns and the 1928 Fleming's discovery of penicillin that revolutionized modern medicine, bacterial infections remain a significant cause of morbidity and mortality worldwide. The well-known microbial resistance problem is entangled by the ability of many pathogens to produce a wide variety of virulent factors, including the endotoxins that act outside the bacterial cell being not susceptible to the antibiotic action. Therefore, search for the novel antidotes counteracting toxin poisoning is an important complementary approach to development of new antibacterial therapies. If implemented successfully, the antitoxins would allow for more time and energy for the infected organisms to respond to the bacterial infection and build protective immunity. In the last two decades, the concept of multivalent constructs decorated with ligands that have proven affinity to the target bacterial toxin have advanced and developed (Weisman et al. 2015). Many bacterial toxins form radially symmetrical oligomeric structures. This structural property could be used for rational design of multivalent ligands exploring the inhibitor–toxin symmetry match idea, where the ligands would simultaneously occupy complementary symmetrical binding sites of the target toxin. In this chapter, we discussed several examples when this principle was successfully used in rational development of the multivalent blockers acting in the limited confinements of pore-forming bacterial toxins including the channel-forming component of the binary bacterial toxins. It was estimated that the most effective multivalent channel-blocking antitoxins compare well with the blockers of classical ion-selective channels of neurophysiology (Table 3 in Nestorovich and Bezrukov 2012). However, in contrast to the blocker of the ion-selective channels of excitable cells, which are designed to be highly channel-specific, the toxin pore blockers offer advantages of being universally active in inhibiting a number of structurally related proteins. Thus, the sevenfold symmetrical cationic AMBnTβCD blocker was protective against anthrax (Karginov et al. 2005), C2, iota (Nestorovich et al. 2011), and CDT toxins (Roeder et al. 2014). Another extensively studied family of bacterial toxins is represented by the AB5-type bacterial toxins, for which a great number of multivalent inhibitors design tactics was suggested. As we briefly discuss above, several smart tuning strategies focused on the available structural data has allowed for the design of multivalent AB5 toxin inhibitors that are over 10 million-fold more effective in the in vitro interaction with the toxin complexes compared to the monovalent ligands of the same type.

There is little doubt that the strategies for the multivalent molecule applications, already manifold, will continue to emerge stronger (Fasting et al. 2012; Badjic et al. 2005). The main caveat here is related to challenges that the developers of these compounds will face transferring them from laboratories to clinics. Traditions given by the widely used classical Lipinski's "rule of five" (Lipinski et al. 1997), designed to predict the drug-likeness for orally available small-molecule compounds, automatically excludes many multivalent ligands from the pool of potential therapeutic

agents. We hope the dogmatic drug discovery practices will become more flexible with many successful exceptions reported from this otherwise very informative rule (Zhang and Wilkinson 2007; Walters 2012; Doak et al. 2014). In our days of "*the dawning era*" of polymer therapeutics (Duncan 2003), advances in synthetic polymer chemistry have already led to considerable progress in changing the pharmaceutical community perceptions of polymer-based drug candidates (Duncan 2011, 2014; Duncan and Vicent 2013). Thus, two polymer-based drugs Neulasta®, used to prevent neutropenia, and Copaxone®, used to treat multiple sclerosis, were listed in the US top 10 selling therapeutic agents of 2013 (U.S. Pharmaceutical Sales.). Many other macromolecule-based compounds are being reviewed for a number of emergent applications in the field of nanomedicine (Duncan and Gaspar 2011). To summarize, we believe the rational strategies currently being developed will pave the road toward discovery and approval of effective multivalent drugs tuned to act against different targets, including those formed by bacterial exotoxins.

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Toxin Transport by A-B Type of Toxins in Eukaryotic Target Cells and Its Inhibition by Positively Charged Heterocyclic Molecules

Roland Benz and Holger Barth

Abstract A-B types of toxins are among the most potent bacterial protein toxins produced by gram-positive bacteria. Prominent examples are the tripartite anthrax toxin of *Bacillus anthracis* and the different A-B type clostridial toxins that are the causative agents of severe human and animal diseases and could serve as biological weapons. The components of all these toxins comprise one binding/transport (B) subunit and one or two separate, non-linked enzymatically active (A) subunits. The A and B subunits are separately produced and secreted by the pathogenic gram-positive bacteria and must assemble on the surface of eukaryotic target cells to form biologically active toxin complexes. The B components are cleaved by proteases to generate the biologically active species that binds to receptors on the surface of the target cells and form there oligomers which bind the A subunits. The AB complexes are internalized by receptor-mediated endocytosis and reach early or late endosomes that become acidified. Subsequently, the B components form channels in endosomal membranes that are indispensable for the transport of the enzymatic subunits across these membranes into the cytosol of target cells via the trans-membrane channels. In addition to the channels formed by the B components, host cell factors including chaperones and further folding helper enzymes are involved in the import of the enzymatic subunits into the cytosol of eukaryotic cells. Positively charged heterocyclic molecules, such as chloroquine and related aminoquinolinium and azolopyridinium salts have been shown in recent years to bind with high affinity to the channels formed by the B components of binary toxins. Since binding to the B components is also a prerequisite for transport of the A components across the endosomal membranes the channel blockers also prevent transport of the A subunits into the host cell cytosol. The inhibition of toxin uptake

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into cells by such pharmacological compounds should also be of clinically interest because the toxins are the major virulence factors causing anthrax on the one hand and severe enteric disease on the other hand. Therefore, the novel toxin inhibitors should be attractive compounds for an application in combination with antibiotics to prevent or treat the diseases associated with binary toxins. Here the different processes involved in channel block in vitro and inhibition of intoxication of living target cells are reviewed in some detail.

Contents

1 Interaction of Binary A-B Type Toxins with Mammalian Target Cells and the Concept of Pore Blockers as Anti-toxins

Binary protein toxins are a special family of A-B type protein toxins which consist of two non-linked proteins, an enzyme (A) component and a separate binding/transport (B) component. The A and B components alone are not toxic and must assemble in solution or on the surface of mammalian target cells to form biologically active toxin complexes. Binary toxins are produced by various pathogenic bacteria and represent important virulence factors, which cause severe human and animal diseases because the toxin-mediated cell damage is the underlying reason for the clinical symptoms. To exhibit their cytotoxic effects the A subunits act as enzymes in the cytosol of their human or animal target cells and the

B components serve for efficient transport of the A subunits into the cytosol. Thus, if the transport of the A subunits into the cytosol can be blocked, cells are protected from intoxication and the clinical symptoms should not occur. Therefore, pharmacological inhibitors which specifically interfere with the transport of the A subunits into the cytosol should be of particular interest to prevent and/or treat diseases associated with such toxins and in past years, we and others developed and characterized various inhibitors of binary bacterial toxins including positively charged heterocyclic molecules. These anti-toxins specifically bind to the B components and inhibit their ability to deliver the A components across cell membranes into the cytosol thereby protecting cells from intoxication. The anti-toxins described in this article are efficient pore blockers for the B components of the binary toxins in vitro and in membranes of living cells. Thereby, they prevent the transport of the A components from endosomal vesicles into the cytosol and protect cells from intoxication, as schematically depicted in Fig. 1 for anthrax toxin, which is

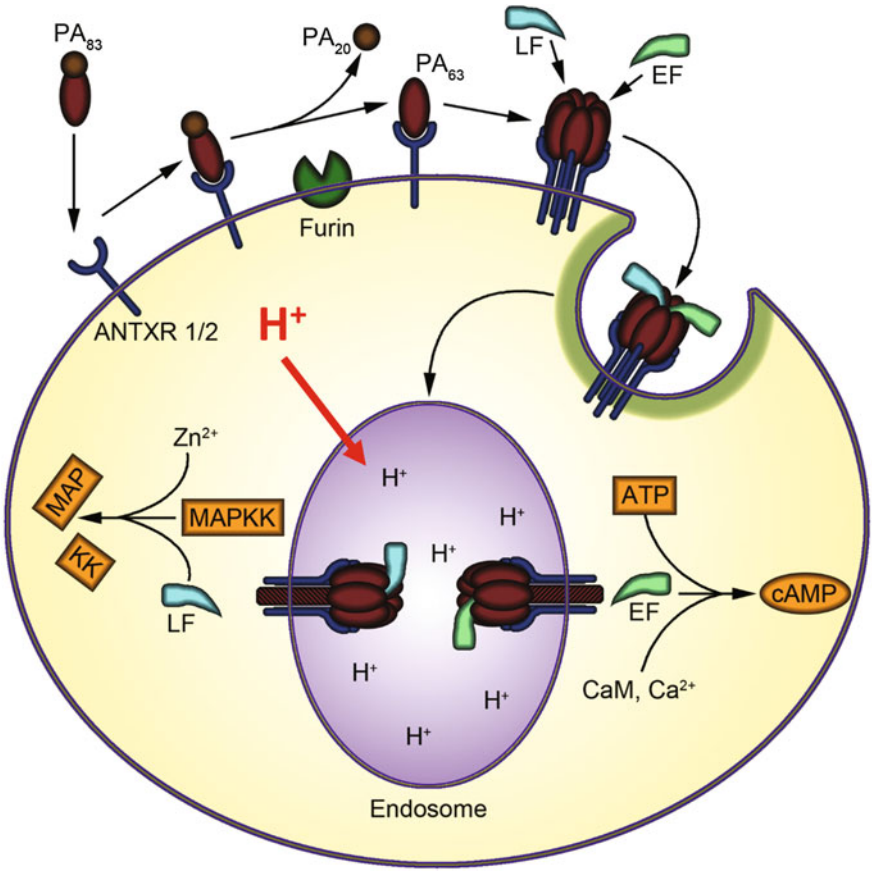

Fig. 1 Intoxication mechanism of anthrax toxin. Adapted from Young and Collier (2007). Courtesy of Christoph Beitzinger

composed of one binding protein (PA) and two separate enzymatic subunits edema factor (EF) and lethal factor (LF). Ekaterina Nestorovich also describes this concept in her article in this book; however, the compounds identified as pore blockers are different.

The role of the B components for the delivery of the A components into the cytosol of target cells is of particular importance to understand the mode of action of such pharmacological toxin inhibitors. In brief, the B components have two different functions during toxin uptake into cells: first, they bind to their cell surface receptors where they generate a docking platform for the A components and trigger receptor-mediate endocytosis of the AB complexes. Second, in acidified endosomal vesicles, the B components change their conformation and insert into endosomal membranes to form trans-membrane pores which serve as translocation channels for the A components. Although the enzymatic components of anthrax- and C2-toxin differ considerably in their enzymatic activity and in their primary structures, their B components PA_{63} and C2IIa, respectively, share a significant overall sequence homology of about 35%, which means that they are closely related in structure and in function (Mock and Fouet 2001; Barth et al. 2004; Young and Collier 2007; Aktories and Barth 2011; Knapp et al. 2016).

The A components of the binary toxins investigated so far unfold to translocate through the narrow channels formed by the B pores across the endosomal membranes into the host cell cytosol. There, they exploit specific host cell factors including chaperones and protein folding helper enzymes to become refolded into their enzymatically active conformation, which then modifies its specific cellular substrate molecules, depending on the individual toxin, and triggers the characteristic cytotoxic effects.

In this article, we focus on chloroquine and related 4-aminoquinolones as pore blockers for binary bacterial toxins. These compounds inhibit the transport pores of the binary anthrax toxins on the one hand and the binary toxins from pathogenic clostridia on the other hand. However, there are striking similarities regarding cellular uptake, intracellular membrane transport and the structures and functions of the B components between anthrax and clostridial binary toxins although the activities of their A components, the cytotoxic effects and the clinical symptoms caused by the toxins are different. The binary anthrax and clostridial toxins are extensively reviewed in some excellent articles of this book to which we refer for a detailed understanding of these toxins.

1.1 Anthrax Toxin

The different A-B types of toxins are often major virulence factors of gram-positive bacteria. A well-known example for an A-B type of toxin is the tripartite anthrax toxin of *Bacillus anthracis* which may serve as a potent biological weapon. The plasmid-encoded toxin comprises a receptor-binding moiety termed protective antigen (PA) and two different enzymatically active components, lethal factor

(LF) and edema factor (EF) (Friedlander 1986; Mock and Fouet 2001; Collier and Young 2003; Young and Collier 2007). Both EF and LF require the binding component PA for delivery into the cytosol of target cells via the endosomal pathway following acidification of the endosomes. EF and LF exhibit enzymatic activity in the target cells that can lead to cell death. Lethal factor (90 kDa) is a highly specific zinc-dependent metalloprotease targeting mitogen-activated protein kinase kinases (MAPKKs), e.g. MEK2, thereby initiating a still poorly understood mechanism leading to subsequent death by apoptosis of some types of macrophages and to the inhibition of the release of pro-inflammatory mediators like nitric oxide, tumor necrosis factor-alpha (TNF-α) and interleukin-1 β (IL-1 β) from macrophages (Hanna et al. 1993; Menard et al. 1996; Pellizzari et al. 1999). Edema factor (89 kDa) is a calmodulin- and Ca^{2+}-dependent adenylate cyclase, interfering with cell signalling by uncontrolled increase of the cytosolic cAMP level, thereby altering water homeostasis and destroying the balance of intracellular signalling pathways (Mock and Fouet 2001; Lacy and Collier 2002; Dixon et al. 2000).

Monomeric protective antigen (83 kDa) is secreted by *B. anthracis* as a water-soluble precursor form (PA_{83}). Proteolytic cleavage of a 20 kDa N-terminal fragment (PA_{20}) by furin-like proteases leads to the activated PA_{63} monomer which is able to oligomerize into heptamers following binding to cell surface receptors (Young et al. 2007; Petosa et al. 1997). The receptors are tumor endothelial marker 8 (TEM8) (Bradley et al. 2003) also termed ANTXR1 and capillary morphogenesis gene-2 as the second anthrax toxin receptor (ANTXR2) (Scobie et al. 2003). This heptameric prepore can bind up to three molecules of EF and/or LF with high affinity (Cunningham et al. 2002; Escuyer and Collier 1991; Elliott et al. 2000; Kintzer et al. 2010). The cell surface exposed receptor-PA_{63}-EF-LF complex is subjected to clathrin-dependent endocytosis (Abrami et al. 2003). Acidification of the endosome results in translocation of the enzymatic components into the target cells' cytosol (Miller et al. 1999; Nassi et al. 2002). This translocation mechanism with some modifications is common to several other A-B type toxins, such as C2 toxin from *Clostridium botulinum* or iota toxin from *Clostridium perfringens* (see below) (Barth et al. 2002).

The crystal structures of the water-soluble heptamers or octamers of PA_{63} are known since many years (Petosa et al. 1997; Kintzer et al. 2009). Similarly, hypothetical models of the membrane-spanning functional pore PA_{63} are also known since some time (Nguyen 2004; Katayama et al. 2010). Attempts to crystallize the 3D structure of the membrane form failed for some time so far. However, the membrane form of the PA_{63} heptamers was recently studied using cryoelectron microscopy, which allowed very high resolution of the heptamers at 2.9-Å resolution (Jiang et al. 2015). All determinants of the 3D structure of the heptamer, which have been found to play an important role in EF and LF binding and transport could be visualized in the complex. The Φ-clamp (F427) and the negatively charged amino acids E398 and D425 of PA_{63} are localized in the constriction of the heptamer (Jiang et al. 2015). These amino acids are involved in binding, unfolding and translocation of the LF and EF components through these trans-membrane pores in the endosomal membrane (Krantz et al. 2005; Zhang et al.

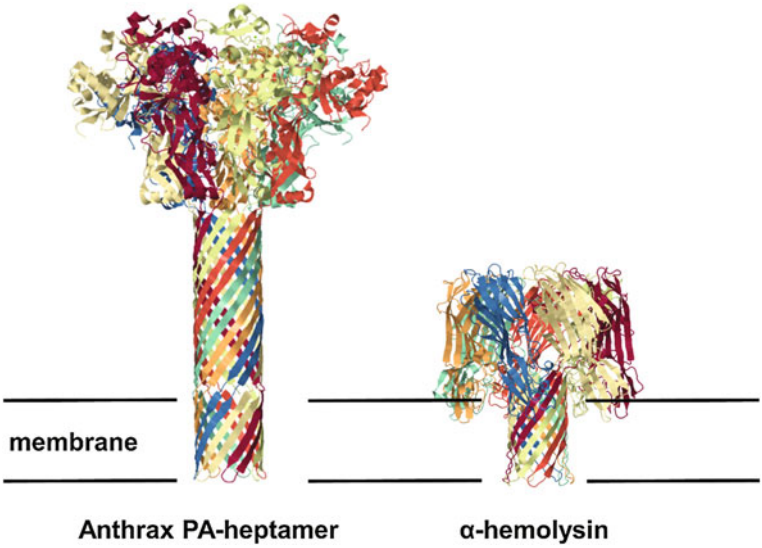

Fig. 2 Comparison of the 3D-structures of the anthrax PA pore (PDB ID: 3J9C) and the α-hemolysin channel (PDB ID: 7AHL) of *Staphylococcus aureus*. The two structures are shown in their membrane-inserted forms. The *black lines* indicate the position of the membrane surfaces

2004a, b; Jiang et al. 2015). The 14 *ß*-strands of the PA_{63} heptamer are slightly twisted against the plain perpendicular to the membrane surface, which is typical for *ß*-barrel cylinders, such as the gram-negative bacterial porins (Benz et al. 1986) or alpha-hemolysin from *Staphylococcus aureus* (Song et al. 1996) (see Fig. 2). The *ß*-barrel cylinder of the PA_{63} heptamer is quite long [around 10 nm (Jiang et al. 2015)], which means that only a minor fraction of the cylinder is localized in the membrane. The *ß*-barrel cylinder of the PA63 heptamers is considerably longer than that of α-hemolysin (Song et al. 1996). The *ß*-barrel cylinder is in the case of α-hemolysin of *Staphylococcus aureus* only little longer than the membrane thickness (see Fig. 2).

Experiments with lipid bilayer membranes demonstrated that either a pH-gradient or a voltage across the membrane is sufficient to initiate transport of the enzymatic components of anthrax toxin across membranes (Zhang et al. 2004b; Krantz et al. 2006). Other factors seem not to be involved in effector translocation. This is in some contrast to effector transport mediated by clostridial A-B type of toxins where several other factors are involved in protein translocation (see below). This means also that transport by PA_{63} heptamers appears to be less specific as compared to clostridial A-B toxins. In fact, it has been demonstrated that PA_{63} is able to bind the enzymatic component C2I of C2-toxin (Kronhardt et al. 2011). Similarly, the binding component C2II of C2-toxin binds LF and EF in vitro. However, whereas Pa_{63} is able at high concentration to transport C2I into human umbilical vein endothelial cells (HUVECs) a similar effect of C2II on EF or LF could not be

observed (Kronhardt et al. 2011). This means definitely that PA_{63} is more promiscuous than the binding proteins of the clostridial A-B toxins. Binding and transport of foreign proteins by PA_{63} channels can be enhanced by addition of several positively charged amino acids to the N-terminal end of the proteins (Blanke et al. 1996; Beitzinger et al. 2012). Therefore, that PA_{63} is able to transport foreign proteins into target cells either as fusion proteins with the N-terminal end of EF or LF or with a cluster of positively charged amino acids, such as an His-tag, at the N-terminus.

Here we review the binding properties of chloroquine-based 4-aminoquinolines to the protective antigen channel in artificial membranes leading to a dose-dependent decrease of membrane conductance and the effect on cytotoxicity in Vero cells and J774A.1 cells. On- and off-rate constants of in vitro-binding to the PA channels were determined by the current noise analysis indicating a strong relationship between compound structure and binding kinetic to the PA channels.

1.2 Binary Clostridial Toxins

The family of binary clostridial toxins comprises the actin ADP-ribosylating toxins with the prototypic C2 toxin from *C. botulinum* on the one hand and the iota-like toxins including *C. perfringens* iota toxin, *C. difficile* CDT and *C. spiroforme* toxin on the other hand. Because the structure, mode of action and cellular uptake of these toxins is extensively reviewed in other chapters of this book, the binary actin ADP-ribosylating toxins are only shortly introduced in this chapter.

Clostridium (C.) botulinum C2 toxin consists of two distinct proteins, which are released separately into the medium (Ohishi et al. 1980). The A component C2I ($\sim$50 kDa) mono-ADP-ribosylates G-actin at arginine 177 (Aktories et al. 1986; Vandekerckhove et al. 1988) which inhibits actin polymerization (Wegner and Aktories 1988). In cells, this results in the breakdown of the actin cytoskeleton and rounding up of adherent cells (Wiegers et al. 1991) and finally triggers apoptosis (Heine et al. 2008). The B component C2II (80 or 100 kDa, depending on the strain) becomes proteolytically activated and the active species C2IIa forms ring-shaped heptamers (Barth et al. 2000; Schleberger et al. 2006) which bind to the cell receptor, an asparagine-linked carbohydrate structure (Eckhardt et al. 2000), form complexes with C2I (Barth et al. 2000; Blöcker et al. 2003a; Ohishi and Yanagimoto 1992) and mediate the internalization of C2IIa/C2I complexes into cells via receptor-mediated endocytosis (Barth et al. 2000). In addition, C2IIa facilitates the subsequent transport of C2I from the lumen of acidic endosomal vesicles into the host cell cytosol. To this end, the C2IIa heptamers form trans-membrane pores in endosomal membranes which serve as translocation channels for C2I (Barth et al. 2000; Blöcker et al. 2003a, b; Haug et al. 2003b). It became evident that host cell chaperones such as Hsp90 and Hsp70 as well as peptidyl-prolyl cis/trans isomerases of the cyclophilin and FK506 binding protein families are necessary for the trans-membrane transport of C2I (Barth et al. 2000; Haug et al. 2003a; Kaiser et al. 2009, 2012) (Fig. 3).

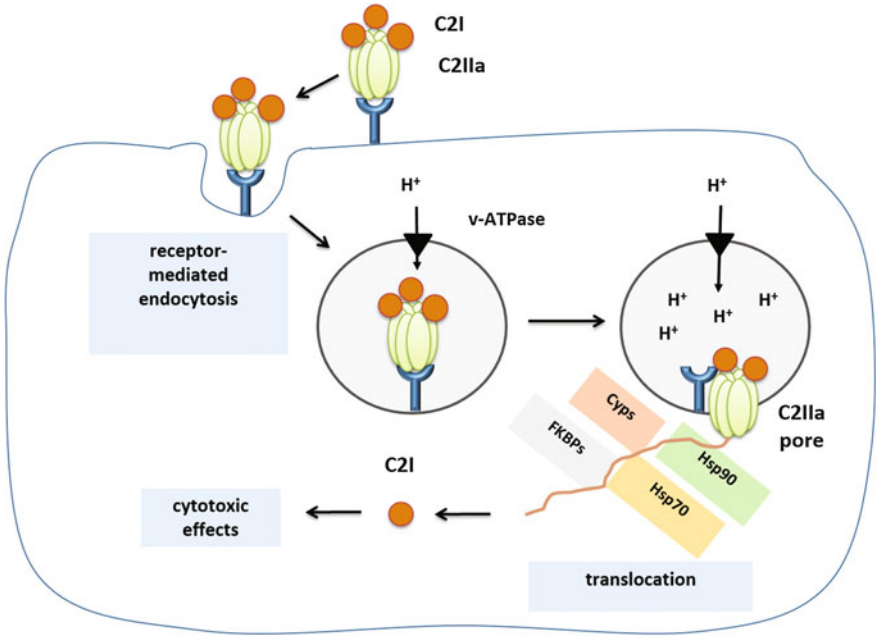

Fig. 3 Current model of the cellular uptake of *C. botulinum* C2 toxin. Explanations are given in the text

In artificial lipid bilayer membranes, C2IIa heptamers also form ion-permeable channels (Bachmeyer et al. 2001; Barth et al. 2000; Schmid et al. 1994). This in vitro model was used for the identification of C2IIa as a drug target and the characterization of many pharmacological compounds that block the translocation channel in membranes and thereby inhibit the translocation of C2I into the cytosol of living cells. Besides C2 toxin, iota toxin of *C. perfringens* (Blöcker et al. 2001; Perelle et al. 1996; Schering et al. 1988; Stiles and Wilkins 1986a, b), CDT of *C. difficile* (Gülke et al. 2001; Perelle et al. 1997; Popoff et al. 1988), *C. spiroforme* toxin (Popoff and Boquet 1988), are further binary clostridial actin ADP-ribosylating toxins.

2 Binding of the A Components to the Trans-Membrane Channels of the B Components

2.1 Binding Affinity of the Enzymatic Components to the Binding Protein Channels

The binding proteins of anthrax-toxin of *B. anthracis*, C2-toxin of *C. botulinum* and iota toxin of *C. perfringens* are able to form ion-permeable channels in artificial and

biological membranes as described above (Blaustein et al. 1989, 1990; Schmid et al. 1994; Finkelstein 1994; Knapp et al. 2002). However, the formation of ion-permeable channels is not the primary reason for channel formation by the binding protein heptamers of the different A-B toxins. In fact, these channels serve basically as protein conducting nanopores for the transport of the enzymatic subunit A into the target cells. The transport occurs in different distinct steps. The first step is always the binding of the subunit A to some sort of binding site within the vestibule of the heptamers formed by the component B of the same toxin. This binding process was studied in detail for a variety of toxins using the lipid bilayer assay. For these studies, the binding proteins channels were reconstituted into artificial lipid bilayer membranes from one side, the cis-side. Addition of the enzymatic components to the trans-side did not result in any effect on the membrane conductance (Bachmeyer et al. 2001). However, when the enzymes were also added to the cis-side, the membrane conductance decreased in a dose-dependent fashion as has been demonstrated in different investigations (Bachmeyer et al. 2003; Neumeyer et al. 2006a, b). Analysis of the titration data was performed in the same way as it is shown below see (Fig. 6) for block of many C2IIa-channels by the heterocyclic compound chloroquine and yielded via a Langmuir adsorption isotherm (Fig. 7) the half saturation constants or the stability constants for binding of the enzymatic components to the binding component channels. All of these investigations are consistent with the assumption that the interaction between channel and enzymatic component occurs via a single hit process although up to three of them can bind to one single channel (Halverson et al. 2005; Neumeyer et al. 2006a; Cunningham et al. 2002; Mogridge et al. 2002). This indicated that the binding of the first enzyme to the channels is monitored and that the binding of the one to two further A components cannot be monitored in electrophysiological experiments. Probably, further binding of additional enzymes to the channels may occur with a much lower affinity than for the first one also because of limited space in the vestibules (Ren et al. 2004; Jiang et al. 2015).

The data shown in Table 1 demonstrate an extremely high affinity of the binding between enzymatic components and the PA_{63}-channel with half saturation constants in the nanomolar range. The results of binding studies suggested that ion–ion interactions are involved in binding of the enzymatic components to the PA_{63}-channel. This is also reflected by the strong effect of His_6-tags at the N-terminal end of LF and EF on binding to the PA_{63}-channel and supports the view that the interaction between enzymatic components and the PA_{63}-channel occurs via the positive charge at the N-terminus of the components (Pannifer et al. 2001; Chauhan and Bhatnagar 2002; Cunningham et al. 2002). Besides the two rings of seven negatively charged ions formed by the residues E398 and D425 the putative binding site in the vestibule of the PA63-channel contains also seven phenylalanines (F427). This Φ-clamp plays also an important role in transport of EF and LF into target cells and is presumably also involved in unfolding of the enzymatic components during transport (Sellman et al. 2001; Krantz et al. 2005).

The binding between PA_{63}-channels and EF and LF has been studied in detail in many investigations. Of interest was also in the past the interaction between binding

Table 1 Stability constants K for the inhibition of the anthrax PA_{63}-channel by EF, LF, His_6-EF and His_6-LF in lipid bilayer membranes added to the cis-side of the membranes. The data represent means of at least three individual titration experiments. K_S is the half saturation constant. Constants for His_6-EF and His_6-LF binding are given for comparison. Taken from (Neumeyer et al. 2006a, b)

Ionic strength (mM)	K (10^8 M^{-1})	K_S (nM)	K (10^8 M^{-1})	K_S (nM)
EF			LF	
50	16.1	0.62	22.5	0.44
150	1.45	6.9	3.62	2.76
300	0.29	34.5	0.45	22.2
1000	0.037	280	0.046	220
His_6-EF			His_6-LF	
50	7.9	1.27	31.6	0.316
150	62.5	0.16	55.2	0.181
300	13.7	0.73	41.6	0.240
1000	3.4	2.93	5.4	1.86

The PA channels were reconstituted into diphytanoyl phosphatidylcholine/*n*-decane membranes. The aqueous phase contained the indicated KCl concentration and about 10 ng/ml PA_{63}; added to the cis-side of the membranes T = 20 °C

protein channels and the enzymatic proteins of other AB-type of toxins. The most prominent of them are the clostridial C2- and iota-toxins that are both ADP-ribosylating toxins (Ohishi et al. 1980; Perelle et al. 1997). Table 2 shows the half saturation constants for the binding of the enzymatic units to the corresponding channels at an ionic strength of 150 mM KCl. The binding affinity of the enzymatic subunits to the C2IIa- and the iota b channels was in the same range as shown above for anthrax toxin. It is however, interesting to note that a His_6-tag at the C-terminus of C2I had no effect on its binding to C2IIa-channels (Beitzinger et al. 2012) in contrast to the results with anthrax PA_{63} (see Table 1). This has presumably to do with the structure of the translocation channel itself. Whereas the

Table 2 Stability constants K and half saturation constants K_S for the binding of the enzymatic subunits to binding protein channels of C2- and iota toxin

Toxins	Binding component	Enzymatic subunit	K (10^8 M^{-1})	K_s (nM)
C2	C2IIa	C2I	0.37	27
	C2IIa	His_6-C2I	0.34	29
iota	iota b	iota a	33	0.3

Stability constants K for the binding of C2I and iota to the C2II and iota b channels, respectively, reconstituted in lipid bilayer membranes from diphytanoyl phosphatidylcholine/n–decane. The aqueous phase contained 150 mM KCl, buffered to pH 5.5–6 using 10 mM MES-KOH; T = 20 °C. Measurements were performed at a membrane potential of 20 mV. The data represent the means of at least three individual titration experiments. K_S is the half saturation constant, i.e. 1/K. The data were taken from Kronhardt et al. (2011); Beitzinger et al. (2012)

important amino acids involved in binding of the enzymatic components to the vestibule F427, E398 and D425 (PA_{63}), F428, E399 and D426 (C2IIa) and F415, D386 and D423 (iota b) are well preserved for all three binding components, the number of negatively charged groups in the channel itself varies. The Pa_{63}-channel heptamer has the highest density of negative charges within the β-barrel cylinder (E302, E308 and D315 per monomer), followed by C2IIa (E307 per monomer) and iota b (which has no negatively charged amino acid inside the pore) (Neumeyer et al. 2008). These negatively charged groups inside the channel are presumably involved in the interaction with the His_6-taged enzymatic components and provide the additional driving force for their binding to the PA_{63}-channel.

2.2 *Influence of Aqueous Ionic Strength and Size of the Enzymatic Components on Binding Affinity*

Table 1 shows a considerable dependence of the binding affinity of the enzymatic components on the bulk aqueous KCl concentration. The stability constant, K, for EF- and LF binding to the PA_{63}-channel decreased by more than two orders of magnitude for an increase of the ionic strength from 50 mM to 1 M KCl. This is caused by a substantial ionic-strength dependent negative potential, which attracts cations and repels anions. The strong dependence of the stability constant for binding on ion strength may be explained in a quantitative way by the Debye-Hückel theory (Neumeyer et al. 2006b). Figure 4 shows a fit of the stability constants for LF binding to the PA_{63}-channel using the Debye-Hückel theory. The results suggest that the vestibule of the PA_{63}-channel has a diameter of about 1.4 nm and is highly negatively charged (six negatively charged groups; $q = -9.6 \times 10^{-19}$ As). The stability constant for binding of LF (one positive charge at the N-terminus) to the channel is in the absence of the charges about 1.6×10^7 l/mole, which is already very high, but low ionic strength increases this constant by more than thousand-fold at 50 mM KCl because of the large number of negatively charged groups involved in the binding site.

The amino-terminal parts of LF (268 amino acids; LF_N) and EF (254 Amino acids; EF_N) are sufficient to confer the ability to associate with the PA_{63}-heptameric channels. They can be used in particular to drive the translocation of unrelated polypeptides fused to LF_{1-254} into target cells in a PA_{63}-dependent manner (Leppla et al. 1999; Zhang et al. 2004a; Beitzinger et al. 2013). It was suggested that the binding of full-length and truncated versions of EF and LF to the PA_{63}-channel shows roughly the same affinity (Elliott et al. 2000; Cunningham et al. 2002; Lacy et al. 2002). Careful analysis of the binding of EF_N and LF_N to the PA_{63}-channel has shown, however, that the full-size enzymatic components EF and LF have a much higher affinity to the PA_{63}-channel than EF_N and LF_N as a comparison of the

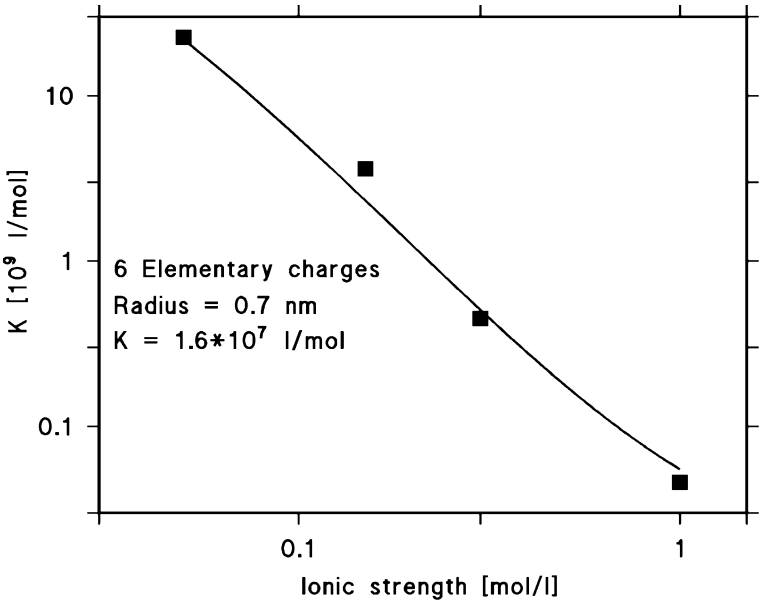

Fig. 4 Stability constants of LF binding to the PA_{63}-channel as a function the ionic strength (equal to the KCl concentration) in the aqueous phase. The *solid line* shows the fit of the stability constants as a function of the ionic strength of the aqueous phase using the Debye-Hückel theory and assuming that six negatively charged groups ($q = -9.6 \times 10^{-19}$ As) are located within the binding site of the PA_{63}-channel and interact with the positively charged N-terminus of LF. The radius of the binding site (channel opening) was assumed being approximately 0.7 nm. The size of the binding site as derived from these considerations shows relatively good agreement with the 3D-structure of PA_{63} (Jiang et al. 2015). The number of charges has to be considered as more tentative because not all seven E398 and seven D425 of the PA_{63}-channel may be negatively charged at pH 5–6 because of their close vicinity in the binding site

results of Tables 1 and 3 clearly shows (by factors of 24 and 7, respectively). This results indicates that additional interactions must exist between the C-terminal part of the enzymatic components EF and LF (amino acids 255 and 269 onwards, respectively) and the vestibule domain of the PA_{63}-channel (Leuber et al. 2008). Nevertheless, the N-terminal parts of EF and LF are able to mediate transport of foreign proteins, for example vaccines, into target cells via the PA63-channels (Leppla et al. 1999; Zhang et al. 2004a; Beitzinger et al. 2012; Chandra et al. 2007; Kong et al. 2009; Arévalo et al. 2017). In addition, an N-terminal extension of positively charged amino acids (for example, a His_6-tag) at a given foreign protein is also very often sufficient for its transport into eukaryotic cells by PA_{63} (Blanke et al. 1996; Kronhardt et al. 2011; Beitzinger et al. 2013).

Table 3 Stability constants K for the inhibition of the anthrax PA_{63}-channel by EF_N, LF_N (left column), His_6-EF_N and His_6-LF_N (right column) in lipid bilayer membranes

Ionic strength [mM]	K (M^{-1})	K_S (nM)	K (M^{-1})	K_S (nM)
	EF_N		**His_6-EF_N**	
150	6.0×10^6	167	2.4×10^8	4.2
	LF_N		**His_6-LF_N**	
150	5.0×10^7	20	1.0×10^9	0.97

The PA_{63}-channels were reconstituted into diphytanoyl phosphatidylcholine/n-decane membranes. The aqueous phase contained 150 mM KCl, 10 mM MES, pH 6.0, and about 10 ng/ml PA_{63}; T = 20 °C. Taken from Leuber et al. (2008)

3 Interaction of Positively Charged Heterocyclic Molecules with the B Component Channels

3.1 Chloroquine and Related 4-Aminoquinolones

Simpson noticed as early as 1989 that clostridial toxins act via receptor-mediated endocytosis on target cells (Simpson 1989). The heavy chain (the B component) bound to a cell surface receptor. The subsequent addition of the light chain (component A) produced cell poisoning. Positively charged quaternary ammonium ions such as ammonium chloride and methylamine hydrochloride could inhibit internalization of heavy chain–light chain complex of the toxins, but they had no influence on ADP-ribosylation (Simpson 1989). Similarly, quaternary ammonium ions could also block the anthrax PA_{63}-channel (Finkelstein 1994). Chloroquine, which represents a positively charged heterocyclic molecule (see Fig. 5) was able to block the channels formed by C2IIa (Schmid et al. 1994). Similarly, chloroquine was also able to block intoxication of Vero cells in a cell-based assay (Bachmeyer et al. 2001). This effect could also be the result of pH homeostasis across the endosomal membrane by the compound, i.e. neutralizing the acidification and subsequently blocking the translocation of C2I into the cytosol. Therefore, an alternative method was used to study the block of intoxication. C2-toxin was added to the surface and the transport of C2I across the endosomal membrane was mimicked by acidification of the external medium in the presence of bafilomycin A1 that permits the transfer of the enzymatic component directly from the plasma membrane into the cytosol. The results of these experiments demonstrated that chloroquine significantly inhibited the cytopathic effects induced by C2-toxin by binding to the channel formed by C2IIa (see also below; Bachmeyer et al. 2001; Blöcker et al. 2003b).

The block of binding protein channels of anthrax-, C2- and iota toxin by positively charged heterocyclic molecules was studied in detail using titration experiments with artificial lipid bilayer membranes (Bachmeyer et al. 2001, 2003). These experiments were performed in the following way: the channels were reconstituted from the cis-side into the membranes until equilibrium was obtained. Then the different channel blockers were added to both sides of the membrane under stirring

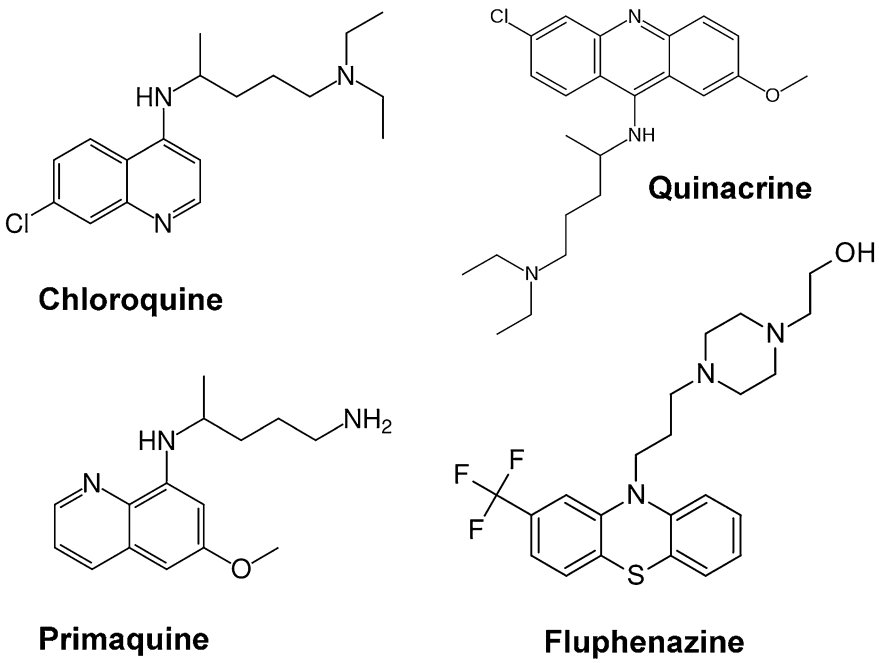

Fig. 5 Structure of chloroquine and related inhibitors of channel function of the binding proteins PA_{63}, C2IIa and iota b

to allow equilibration. The membrane current decreased in a dose-dependent manner as it is shown in Fig. 6 for an experiment, where C2IIa binding protein channels were titrated with chloroquine. The data of the experiment of Fig. 6 and similar experiments were analyzed using either Langmuir adsorption isotherms (see Fig. 7) or Lineweaver–Burk plots and yield the stability constant for binding. The half saturation constant K_S is equal to the inverse stability K.

In several studies we demonstrated that chloroquine and homologues bind to the binding component channels of anthrax- (PA_{63}) and C2-toxin (C2IIa) from *C. botulinum* and inhibit channel formation in vitro and intoxication in vivo at micromolar concentration (Bachmeyer et al. 2001, 2003). Protective antigen (PA) of anthrax toxin shares significant sequence identity (33%) with C2II, suggesting that the two proteins have similar modes of action. The structure of PA_{63} is known in its heptameric prepore and membrane-spanning pore form (Petosa et al. 1997; Jiang et al. 2015). Table 4 shows the results of titration experiments with the different 4-aminoquinolones chloroquine, quinacrine and fluphenazine. Interestingly, a higher affinity of these compounds was observed to the PA_{63}-channels (Orlik et al. 2005) as compared to C2IIa-channels (Bachmeyer et al. 2003; see Table 4). The affinity between the PA_{63}- and the C2IIa-channels and the 4-aminoquinolones increased for most KCl concentrations in the series chloroquine, quinacrine and fluphenazine. Again, the decrease of the ionic strength from

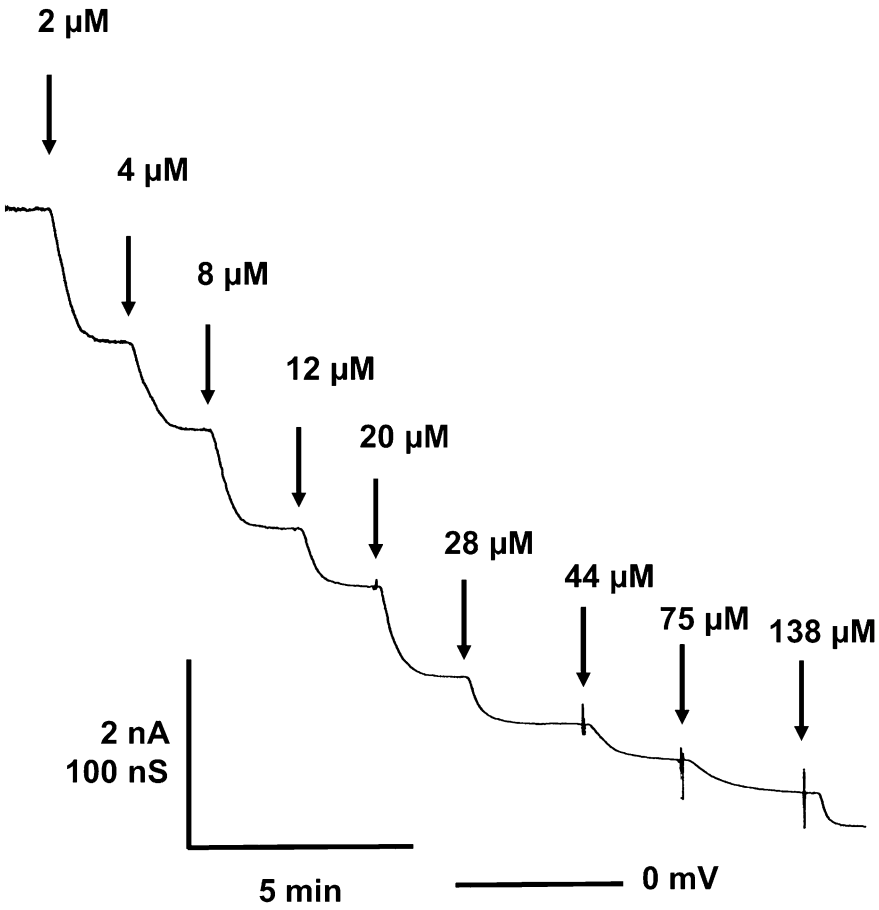

Fig. 6 Titration experiment of C2IIa-induced membrane conductance with chloroquine. The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 10 ng/ml (=170 pM) C2IIa protein (added to the cis-side of the membrane), 150 mM KCl, 10 mM MES, pH 6.0 and chloroquine at the concentrations shown in the Figure. The temperature was constant at 20 °C and the applied voltage was 20 mV. The membrane contained about 3250 C2IIa-channels. The *line* labelled with 0 mV represents zero level of conductance (i.e. $V_m = 0$)

3 M KCl to more physiological conditions (150 mM KCl), led to a substantial increase of the stability constants similar to the situation described above for the interaction between binding protein channels and enzymatic components (see Table 1). This result is consistent with the assumption that ion–ion interactions between positively charged inhibitors and negatively charged binding site within the vestibule of the binding protein channels are responsible for inhibitor binding. The inhibitors used in these studies are well-known drugs (e.g. quinacrine for treatment of giardiasis, chloroquine against malaria, fluphenazine is used in

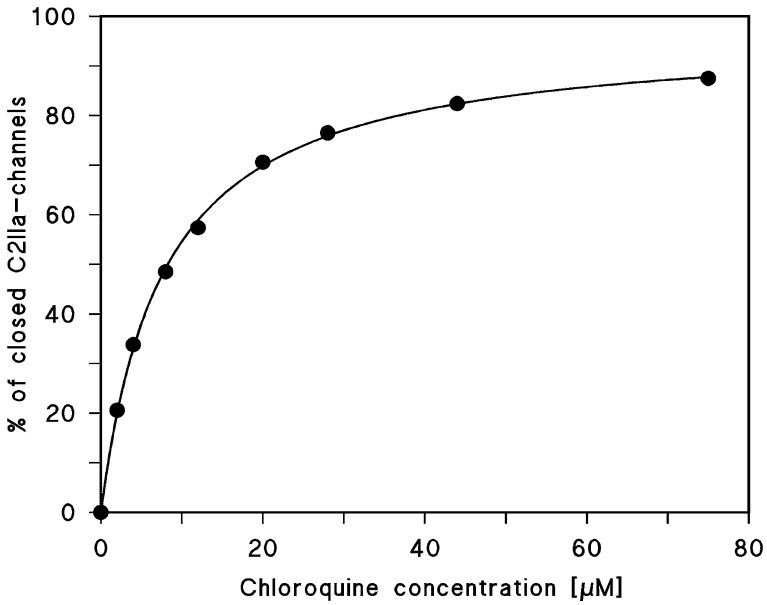

Fig. 7 Langmuir isotherm of the inhibition of C2IIa-induced membrane conductance (about 3250 C2IIa-channels) by chloroquine. The *line* corresponds to the data points taken from the titration experiments in Fig. 6. The fit of the data was performed using the Langmuir eqn. $\frac{(G_{max}-G(c))}{G_{max}} = A \cdot \frac{K \cdot c}{(K \cdot c+1)}$. The stability constant, K, for binding of chloroquine to the C2IIa-channels was (129,400 ± 4000) 1/M and the maximum degree of blockage was A = 97 ± 0.80%. K_S = 7.7 μM

anti-psychotic medication) that are applied here in concentrations that should allow also in vivo applications. The test of the inhibition of anthrax toxin-mediated intoxication of macrophage-like cells by the compounds of Table 4 is possible and is described below (Kreidler et al. 2017).

The stability constants for binding of the chloroquine analogs to the PA_{63}- and C2IIa-channels were also investigated using other electrolytes in the aqueous phase. The use of MEM solution had no substantial influence on the stability constants for the binding of chloroquine and its analogs to the PA_{63}- and the C2IIa-channels (see Table 4). Again, the affinity to PA_{63} was generally higher by factors between about 5 and 12 than that observed for the C2IIa-channels. This is not easy to understand because we discussed already above that the structural elements within the binding sites in the two channels (C2IIa and PA_{63}) are virtually the same. The same is also given for the iota b channels with an even much lower affinity for chloroquine binding (Knapp et al. 2015; Kronhardt et al. 2016). However, the beta barrel channel formed by Pa_{63} contains a higher excess of negatively charged amino acids than that formed by C2IIa or iota b, which could be one reason for the higher affinity of the 4-aminoquinolones to the PA_{63}-channel as it is already discussed above.

Table 4 Stability constants K for the inhibition of the anthrax PA_{63}-channel by chloroquine and related compounds in lipid bilayer membranes. The data represent means of at least three individual titration experiments taken from Orlik et al. (2005). The results of similar titration experiments performed with C2IIa of C2-toxin are given for comparison (K (C2II)) and were taken from Bachmeyer et al. (2003)

Compound	K (10^3 M^{-1})	K_S (μM)	K (C2IIa) (10^3 M^{-1})
0.15 M KCl			
Chloroquine	680	1.5	110
Quinacrine	12,300	0.081	870
Fluphenazine	5400	0.186	2100
0.3 M KCl			
Chloroquine	240	4.17	n.m.
Quinacrine	3700	0.27	n.m.
Fluphenazine	3600	0.28	n.m.
1 M KCl			
Chloroquine	55	18.2	20
Quinacrine	740	1.35	170
Fluphenazine	2100	4.76	620
3 M KCl			
Chloroquine	30	33.3	n.m.
Quinacrine	890	1.12	320
Fluphenazine	2000	5.00	n.m.

The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained the indicated KCl concentration and about 10 ng/ml activated PA_{63}; T = 20 °C. n.m. means not measured

3.2 *Rate Constants of Binding Kinetics Between Positively Charged Heterocyclic Molecules and Anthrax PA_{63}-Channels*

The titration experiments suggested that channel block by the different heterocyclic molecules of Tables 4 and 5 occurred in an association–dissociation reaction. This means that the kinetics of their binding to the channels could be measured using the current noise analysis of the binding reaction (Bachmeyer et al. 2003; Orlik et al. 2005). Open channel-forming toxins such as C2IIa from *C. botulinum*, or the open PA_{63}-channels exhibit $1/f$ noise that is probably related to the structure of the heptameric channel and may be caused by flickers of the channel structure (Nekolla et al. 1994; Wohnsland and Benz 1997; Bezrukov and Winterhalter 2000). The addition of the 4-aminoquinolones led to a complete change of the spectral density $S(f)$ of current noise. The dependence of the current noise is now given by a so-called Lorentzian function of the frequency f, indicating a chemical reaction between channels and inhibitors. Analysis of the Lorentzian function allows the evaluation of the rate constants and the stability constants for ligand binding to the

Table 5 Stability constants K for the inhibition of C2IIa-, PA_{63}-channels by chloroquine, primaquine, quinacrine and fluphenazine and iota b channels by chloroquine in lipid bilayer membranes.

Compound	K/M^{-1}	$K_S/\mu M$
Block of C2IIa channels; MEM solution		
Chloroquine	95,000	10.5
Primaquine	25,000	40
Quinacrine	790,000	1.25
Fluphenazine	1800,000	0.55
Block of PA_{63} channels; MEM solution		
Chloroquine	590,000	1.7
Primaquine	150,000	6.7
Quinacrine	10,500,000	0.095
Fluphenazine	8950,000	0.11
Block of iota b channels; 150 mM KCl		
Chloroquine	5000	0.00020

The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained MEM solution (containing basically about 145 mM Na^+ and 5.33 mM K^+, pH 7.4) and activated C2IIa or recombinant PA_{63} in concentrations of about 50 ng/ml and about 10 ng/ml, respectively; T = 20 °C. Taken from Kreidler et al. (2017). The data for chloroquine binding to iota b-channels was taken from Knapp et al. (2015)

PA_{63}- and the C2IIa-channels. The results of this analysis is given in Table 6, which shows the rate constants of the on- and the off-ate constants for binding of the 4-aminoquinolones to the binding site in a single-hit mechanism.

The on-rates for the binding of the different ligands to the PA_{63}-channel were between about 1.4×10^7 1/(M s) and 4.2×10^7 1/(M s) in 1 M KCl, and between 128×10^7 1/(M s) and 363×10^7 1/(M s) for 150 mM KCl, which means that it did not vary much with the structures of the different ligands. Similar results were obtained for ligand binding to the C2IIa-channel (see Table 6). However, the influence of ionic strength on the on-rate constants was more substantial. This means that the ionic strength has a much higher effect on the on-rate of ligand binding than their individual structures. These results suggested that the high on-rate of binding for all ligands is already close to that of diffusion-controlled reaction processes (Eigen et al. 1964). The off-rate constants of binding of the different ligands to the channels showed much higher variations and increased from fluphenazine (14 s^{-1}) over quinacrine (17 s^{-1}) to chloroquine (971 s^{-1}) at 1 M KCl. Similar results were obtained at 150 mM KCl with the difference that the constants were larger by a factor of about ten. Binding kinetics of the heterocyclic molecules to the C2IIa-channels was basically similar as their binding to the PA_{63}-channels with the difference that the off-rates were higher suggesting a smaller affinity of the molecules to the C2IIa-channel (Bachmeyer et al. 2003). These data illustrated that the binding affinity of the ligands to the channels is basically controlled by the off-rate constants, which are by far less sensitive to ionic strength conditions than the on-rate constants.

Table 6 Parameters of ligand-induced current noise of anthrax PA_{63}-channels and the C2IIa-channels (given in parenthesis)

Compound	$k_1/10^6$ $M^{-1}S^{-1}$	k_{-1}/s^{-1}	$K/10^3$ M^{-1}
1 M KCl			
Chloroquine	42 (74)	970 (2600)	43.3 (28)
Quinacrine	13.7 (34)	16.9 (210)	850 (161)
Fluphenazine	33.7 (34)	14.1 (55)	2770 (620)
150 mM KCl			
Chloroquine	363 (140)	350 (1600)	1030 (88)
Quinacrine	128 (120)	9.6 (210)	14,120 (570)
Fluphenazine	140 (62)	10.1 (44)	14,100 (1410)

The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained either 1 M or 150 mM KCl and about 10 ng/ml PA or C2IIa at the cis-side. k_1 and k_{-1} were derived from a fit of the corner frequencies as a function of the ligand concentration. K is the stability constant for ligand binding derived from the ratio k_1/k_{-1}. The data represent the mean of at least three individual experiments with the same inhibitor taken from Orlik et al. (2005). Binding parameters of ligand binding to C2IIa-channels taken from Bachmeyer et al. (2003) are given in parenthesis for comparison

3.3 Azolopyridinium Salts

The binding protein channel blockers, such as chloroquine or fluphenazine show some common structural motifs: the positively charged groups and the aromatic heterocyclic rings (see Fig. 8). The size of the heterocycles has an important impact on the off-rate of binding (see Table 6). Chloroquine with two conjugated aromatic rings shows a much higher off-rate than quinacrine and fluphenazine with three conjugated aromatic rings (see Table 6; Orlik et al. 2005). In a recent study to identify novel pharmacological inhibitors of anthrax toxins, we observed that heterocyclic azolopyridinium salts (HA-substances) protect macrophages, the target cells for anthrax lethal toxin, from intoxication (Beitzinger et al. 2013). The substances were able to block the translocation of the enzyme subunit of lethal toxin (i.e. lethal factor (LF) plus PA_{63}) from acidified endosomes into the host cell cytosol. In additional experiments, we could also demonstrate that the HA-substances (see Fig. 9) are also able to block intoxication of HeLa cells by C2-toxin (Bronnhuber et al. 2014). The binding of these HA-substances to PA_{63}- and C2IIa-channels reconstituted into lipid bilayer membranes was studied in detail (see Table 7). From the many compounds, which were employed HA 1383, HA 1495 and HA 1568 had the highest affinity to both PA_{63}- and C2IIa-channels indicating again that the size of the heterocyclic aromatic compounds has the highest impact on inhibitor function. In addition, the presence of the NO_2-group on the side chain of HA 1383 seems to boost its affinity to the binding protein channels. Interestingly, the pore blockers did not cause adverse effects in cultured cells even after prolonged incubation periods and therefore, they might be attractive lead compounds for novel therapeutics against anthrax (Beitzinger et al. 2013).

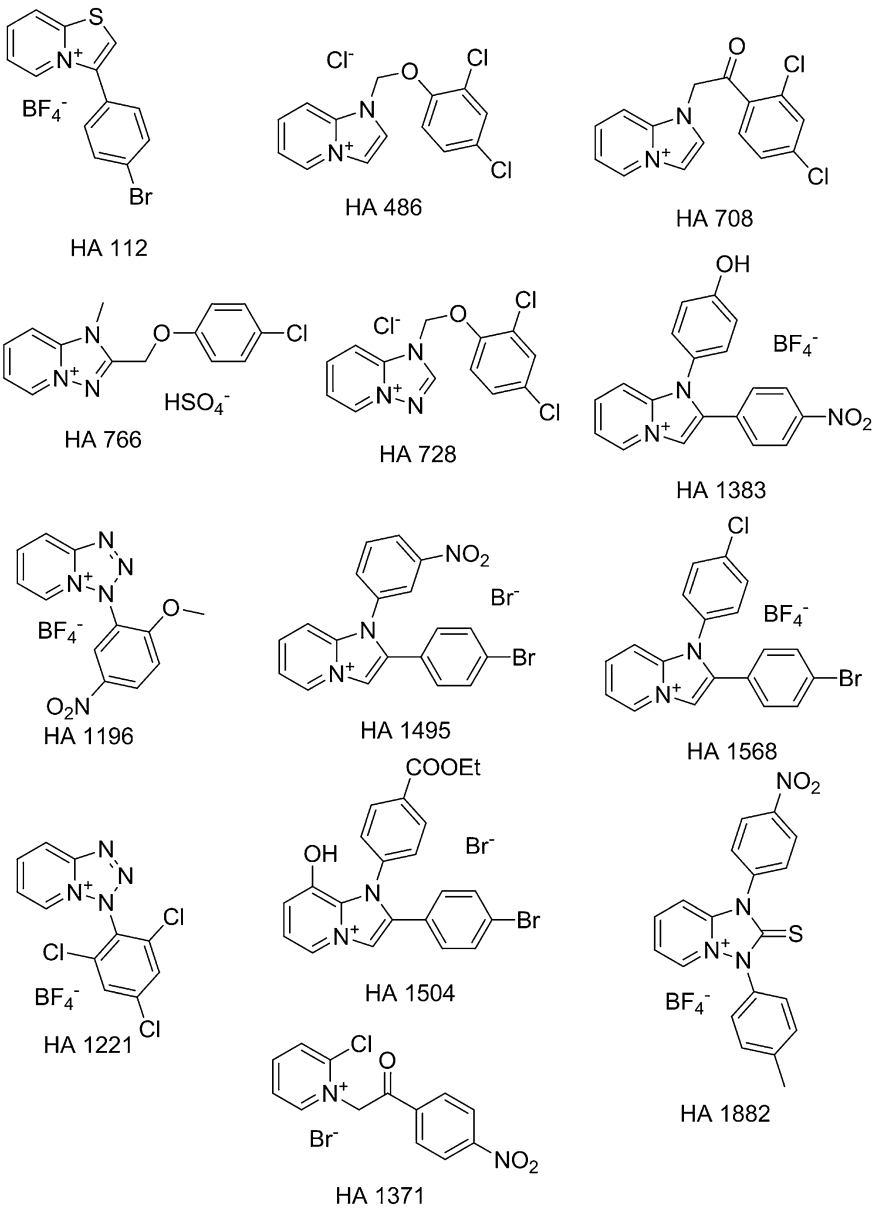

Fig. 8 Structures of heterocyclic compounds (azolopyridinium salts) used for the block of C2IIa- and PA_{63}-channels. For more detailed information about these substances and their synthesis see (Palkó et al. 2006; Hajós and Messmer 1984; Messmer et al. 1986; Timári et al. 1990; Beitzinger et al. 2013)

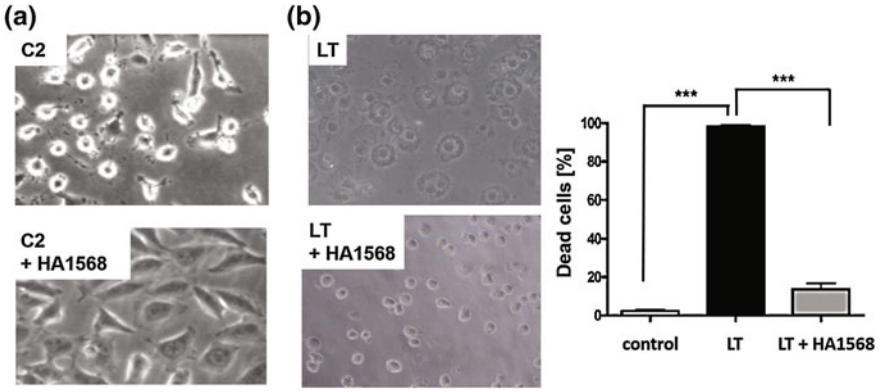

Fig. 9 Protective effect of a heterocyclic pore blocker (exemplarily shown for the compound HA1568) on the intoxication of cultured cells with *C. botulinum* C2 toxin (**a**) and *B. anthracis* lethal toxin (**b**). **a**. Effect of C2 toxin (C2I, 100 ng/mL + C2IIa, 200 ng/mL) on HeLa cells with or without 15 min pretreatment with HA1568 (modified from Bronnhuber et al. 2014). **b**. J774A.1 macrophages were pretreated for 30 min at 37 °C with HA1568 (100 mM) and LT (PA_{63}, 1 µg/mL + LF, 1 µg/mL) was added. After 2 h of incubation pictures were taken and the percentage of dead (i.e. lysed) cells determined (mean ± S.D., n = 3) (modified from Beitzinger et al. 2013)

Table 7 Stability constants K and half saturation constants K_s for the binding of different heterocyclic azolopyridinium salts to C2IIa- and PA_{63}-channels, when added to the cis-side of lipid bilayer membranes

Heterocyclic compound	K (L/mol)	K_S (µmol/L)	K (L/mol)	K_S (µmol/L)
	C2IIa		PA_{63}	
HA 112	5900	168	18,500	57
HA 486	12,000	83	38,000	27
HA 708	5600	180	11,300	89
HA 728	4230	240	11,800	85
HA 766	11,800	84	29,400	36
HA 1196	330	3000	3800	260
HA 1221	1100	950	6000	170
HA 1371	1200	830	3200	310
HA 1383	97,200	10.3	749,000	1.3
HA 1495	22,300	45	274,000	3.6
HA 1504	4500	220	82,900	12
HA 1568	95,000	10.5	478,000	2.7
HA 1882	1500	650	7900	130

The data represent means of at least three individual titration experiments. Membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 150 mM KCl, 10 mM MES-KOH, pH 6.0, and at the cis-sides of the membranes about 10 ng/ml C2IIa or PA_{63}; T = 20 °C. The data for block of C2IIa-channels were taken from Bronnhuber et al. (2014) and those for block of PA_{63}-channels from Beitzinger et al. (2013)

3.4 *Inhibition of Intoxication of A-B Type of Toxins by Heterocyclic Molecules*

All compounds that were identified in the in vitro lipid bilayer model as blockers for the trans-membrane channels formed by the B components of the binary toxins were subsequently tested for their inhibitory effects against intoxication with the respective binary toxins in cell-based cytotoxicity tests. First, the individual compounds were applied together with the toxin into the culture medium to test whether there is a delay of the intoxication process. In brief, cells were incubated with the respective toxin in the presence and absence of the test compound and the intoxication of cells was monitored by measuring the characteristic toxin-induced endpoints. For the binary clostridial actin ADP-ribosylating toxins the typical changes of cell morphology, i.e. cell rounding, was monitored by a microscopic analysis (Fig. 9, exemplary shown for HA1568; modified from Bronnhuber et al. 2014) and for the anthrax lethal toxin, the amount of viable macrophages was measured by MTS assay and monitoring the characteristic lysis of cells (Fig. 9, exemplary shown for HA1568; modified from Beitzinger et al. 2013) because lethal toxin triggers apoptosis of these cells.

For all compounds, which delayed intoxication of the cultured cells, the underlying molecular mechanism was investigated in more detail. Therefore, the effects of the compounds on the enzyme activity of the A components of the toxins and on the individual steps of toxin uptake into the cytosol of target cells including toxin binding to the cell surface, endocytosis and the pH-dependent membrane transport were tested. Taken together, no compound showed inhibitory effects on

Table 8 Summary of the individual inhibitors and their protective effects on the intoxication of cultured cells with the binary toxins

Compound	Target cells	Toxin (EC_{50})	Reference
Chloroquine	Vero, HeLa	C2 (33.7 μM)/LT	Bachmeyer et al. (2001); Kreidler et al. (2017)
4-Aminoquinaldine	Vero	C2	Bachmeyer et al. (2001)
Quinine	Vero	C2	Bachmeyer et al. (2001)
4-Amodiaquine	Vero	C2	Bachmeyer et al. (2001)
Quinacrine	HeLa	C2 (4.7 μM)/LT	Kreidler et al. (2017)
Primaquine	Vero, HeLa	C2 (41.2 μM)/LT	Bachmeyer et al. (2001); Kreidler et al. (2017)
Fluphenazine	HeLa	C2 (2.5 μM)/LT	Kreidler et al. (2017)
HA1383	HeLa/J774A.1	C2/LT	Beitzinger et al. (2013); Bronnhuber et al. (2014)
HA1495	HeLa	C2/LT	Beitzinger et al. (2013); Bronnhuber et al. (2014)
HA1568	HeLa	C2/LT	Beitzinger et al. (2013); Bronnhuber et al. (2014)
C280	Vero	C2/Iota	Kronhardt et al. (2016)

the enzyme activity but all tested substances inhibited the transport of the A components across cell membranes which is in line with the in vitro results that the compounds block the translocation channels of the toxins in lipid membranes. Table 8 summarizes the individual inhibitors and their protective effects on the intoxication of cultured cells with the binary toxins.

4 Conclusions

In the past years a series of heterocyclic compounds have been identified and characterized that efficiently block the trans-membrane pores of bacterial binary AB_7 protein toxins formed by the heptameric B components of these toxins in vitro and in living cells. Thereby, the compounds inhibit the trans-membrane transport of the enzymatically active A subunits through the B_7 pores from acidified endosomal vesicles into the cytosol of mammalian cells. If the A subunits cannot reach their cellular substrate molecules in the host cell cytosol, cells are protected from intoxication and thus the disease which is caused by the respective toxin should not occur or should have a milder course. In particular, the pores of binary clostridial enterotoxins which cause severe diseases in humans and animals and the related binary anthrax lethal toxin can be efficiently inhibited. By applying such compounds, cultured cells were indeed protected from the cytotoxic effects.

In contrast to established antibiotics which target only the toxin-producing bacteria during an infection but have no direct effect on the toxins or anti-toxins (i.e. antibodies) that target only the extracellular toxins in the body before they are internalized into their target cells, such pore blocking compounds act on the toxins which are already internalized as depicted schematically in Fig. 10. Moreover, most of the heterocyclic compounds did not exhibit obvious adverse effects on cultured cells by their own right and some of them such as chloroquine are licensed drugs

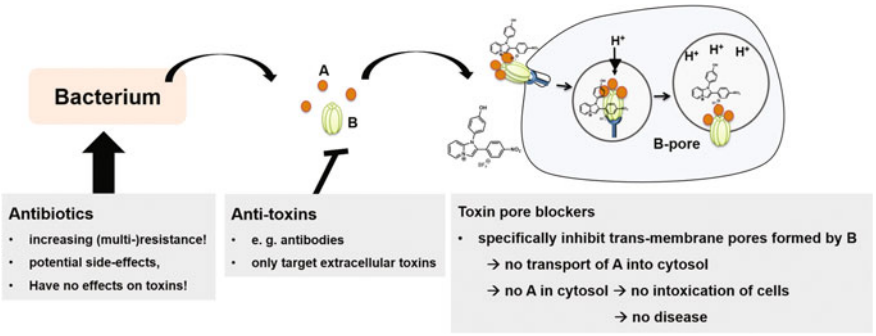

Fig. 10 Possible treatment of infections with A-B type of toxins. The block of the import of the enzymatic component *A* into the target cell by block of the binding protein *B* pore is depicted with the heterocyclic azolopyridinium salt HA 1568 on the *right side* of the figure

against other diseases and therefore characterized regarding their toxicology and pharmacokinetics. Therefore, the heterocyclic compounds reviewed in this article might serve as lead compounds for development of novel therapeutic strategies against diseases that are associated with binary bacterial toxins, e.g. in combination with antibiotics.

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