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Clostridial binary toxins: iota and C2 family portraits

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There are many pathogenic *Clostridium* species with diverse virulence factors that include protein toxins. Some of these bacteria, such as *C. botulinum*, *C. difficile*, *C. perfringens*, and *C. spiroforme*, cause enteric problems in animals as well as humans. These often fatal diseases can partly be attributed to binary protein toxins that follow a classic AB paradigm. Within a targeted cell, all clostridial binary toxins destroy filamentous actin via mono-ADP-ribosylation of globular actin by the A component. However, much less is known about B component binding to cell-surface receptors. These toxins share sequence homology amongst themselves and with those produced by another Gram-positive, spore-forming bacterium also commonly associated with soil and disease: *Bacillus anthracis*. This review focuses upon the iota and C2 families of clostridial binary toxins and includes: (1) basics of the bacterial source; (2) toxin biochemistry; (3) sophisticated cellular uptake machinery; and (4) host-cell responses following toxin-mediated disruption of the cytoskeleton. In summary, these protein toxins aid diverse enteric species within the genus *Clostridium*.

Keywords: actin, *Bacillus*, *Clostridium*, protein toxins

BASICS OF THE BACTERIAL PLAYERS

Species of *Clostridium* (derived from Greek “kloster” = spindle) are ubiquitous, anaerobic, spore-forming bacilli of the phylum *Firmicutes* (Latin “firmus” = strong and “cutis” = skin). These bacteria are commonly found throughout the world in soil, water, and gastrointestinal tracts of animals as well as humans. The G + C content of the genus ranges from 22 to 52%, with the majority around 28% (Jones and Keis, 2005). Many clostridia are harmless and quite versatile for solvent production (i.e., acetone, butanol, isopropanol from *C. acetobutylicum* and *C. beijerinckii*), nitrogen fixation (*C. pasteurianum*), biodegradation of natural polymers (cellulose, pectin, etc.) or hazardous materials (TNT, chlorinated solvents, etc.), debridement of necrotic tissue (application of collagenase from *C. histolyticum*), and novel anti-cancer treatments (*C. novyi*, etc.). However, there are notable exceptions and some of these clostridial pathogens for various mammals are presented in this review (Dürre, 2005; Songer, 2005).

Some *Clostridium* and related *Bacillus* species have developed common mechanisms for survival within, and outside of, numerous hosts. This is evidenced by the various diseases caused by these microorganisms that are often mediated by protein toxins, enzymes, and spores. *C. botulinum*, *C. difficile*, *C. perfringens*, as well as *C. spiroforme* are collectively associated with a multitude of animal and human diseases/intoxications such as gas gangrene, food poisoning, antibiotic-associated diarrhea, pseudomembranous colitis, and enterotoxemia. Anthrax attributed to *B. anthracis* also occurs in different mammals, and includes three forms: (1) cutaneous; (2) intestinal; and (3) inhalational. An ability to survive and thrive in diverse niches is a remarkable characteristic of these

spore-forming bacteria. This review particularly focuses upon different aspects of the iota and C2 families of binary toxins produced by four different clostridia.

CLOSTRIDIUM PERFRINGENS IOTA TOXIN

Clostridium perfringens, previously known as *Bacillus aerogenes capsulatus* and later *Clostridium welchii*, was first described by Welch and Nuttal in 1891 (Welch and Flexner, 1896; Lucey, 2004). In particular, the bacterium was isolated following a human autopsy (death due to an aortic aneurism) with profuse gas formation throughout the circulatory system and multiple organs. Microscopic examination of organ tissues revealed bacilli masses, especially where gas pockets formed within the tissue wall. The isolate was successfully cultured in anaerobic, not aerobic, media. This bacterium was non-motile and very similar in size/shape as *B. anthracis* previously described by Robert Koch; however, it was not *B. anthracis*. There was no overt pathogenesis of this unique isolate upon intravenous injection into rabbits, but bacterial introduction immediately followed by euthanasia reproduced post-mortem findings similar to the aforementioned human case. It was concluded that growth of *Bacillus aerogenes capsulatus* (*C. perfringens*) can occur in humans, and animals, as a post-mortem event. Welch and Flexner (1896) nicely describe many other human cases of *C. perfringens*-associated disease manifested as a pelvic abscess, pneumothorax, peritonitis, gas gangrene of extremities, etc. Under certain circumstances involving an anaerobic niche, many sites within the human body were recognized 120 years ago as hospitable for *C. perfringens* growth during life, and afterward in death.

There are five toxinotypes (A–E) of *C. perfringens* classically based upon four lethal, dermonecrotic toxins (alpha, beta, epsilon, and iota). These “major” protein toxins are neutralized by type-specific antisera in mouse lethal and guinea-pig dermonecrotic assays. Today, multiplex polymerase chain reactions (PCR) are usually employed for rapid typing of isolates (Sawires and Songer, 2005). The iota toxin is exclusively produced by type E strains and implicated in sporadic diarrheic outbreaks among calves and lambs (Bosworth, 1940; Billington et al., 1998). Although *C. perfringens* iota toxin was initially described in 1940 by Bosworth, its binary nature was elucidated 45 years later by exploiting cross-reacting antiserum against *C. spiroforme* (Stiles and Wilkins, 1986). The two proteins that comprise iota toxin were then designated as iota a or Ia (slow moving) and iota b or Ib (fast moving), based upon electrophoretic mobility in crossed-immunoelectrophoresis. Ia or Ib are separately non-toxic, as is the case for individual components from any toxin described in this review. However, an Ia–Ib mixture forms a potent cytotoxin that rapidly kills mice, causes dermonecrosis in guinea pigs, induces rounding of various cell types *in vitro*, and elicits fluid accumulation in rabbit ileal loops. Later studies revealed that Ia is a mono-ADP-ribosyltransferase specific for actin (Schering et al., 1988). Although Ib lacks discernible enzymatic activity, it binds to a cell-surface protein(s) and subsequently translocates Ia into the cytosol of a targeted cell via lipid rafts and clathrin-independent endocytosis (Stiles et al., 2000; Hale et al., 2004; Nagahama et al., 2004; Gibert et al., 2011).

Recent studies by Nagahama et al. (2011) suggest a slight paradigm shift for the clostridial binary toxins, pending cell type. For instance, they investigated the effects of Ib (no Ia) upon eight different cell lines. Although there were no effects of only Ib (high ng/ml) upon six lines, viability and ATP levels rapidly decreased in A431 (human epithelial carcinoma) and A549 (human lung adenocarcinoma) cells. Future experiments will surely reveal more interesting attributes of Ib, without Ia, upon cells.

CLOSTRIDIUM SPIROFORME TOXIN

Similar to the classic rod-shaped *C. perfringens* and enteric-acting iota toxin, the distinctly coiled *C. spiroforme* also causes diarrheic deaths that are spontaneous or antibiotic-induced in rabbits (Borriello and Carman, 1983; Carman and Evans, 1984) and perhaps humans (Babudieri et al., 1986). Although further linkage with human disease has not been confirmed, *C. spiroforme* was originally isolated from human feces (Kaneuchi et al., 1979), as is the closely related *Coprobacillus cateniformis* (Kageyama and Benno, 2000). Clearly, rabbits are very susceptible to *C. spiroforme*-induced diarrhea during stress involving lactation, old age, weaning, and an altered diet (Carman and Evans, 1984). This bacterium is not commonly associated with the intestinal flora of healthy animals (Borriello and Carman, 1983; Carman and Evans, 1984). Furthermore, *C. spiroforme* isolated from outbreaks throughout Italy have become rather resistant to antimicrobials commonly used for treating infected rabbit colonies (Agnoletti et al., 2009). This latter point raises a daunting issue of disease management in the future.

The major virulence factor produced by *C. spiroforme* is an iota-like toxin called CST. The Sa and Sb components of CST are respectively analogous to Ia and Ib of *C. perfringens* iota toxin,

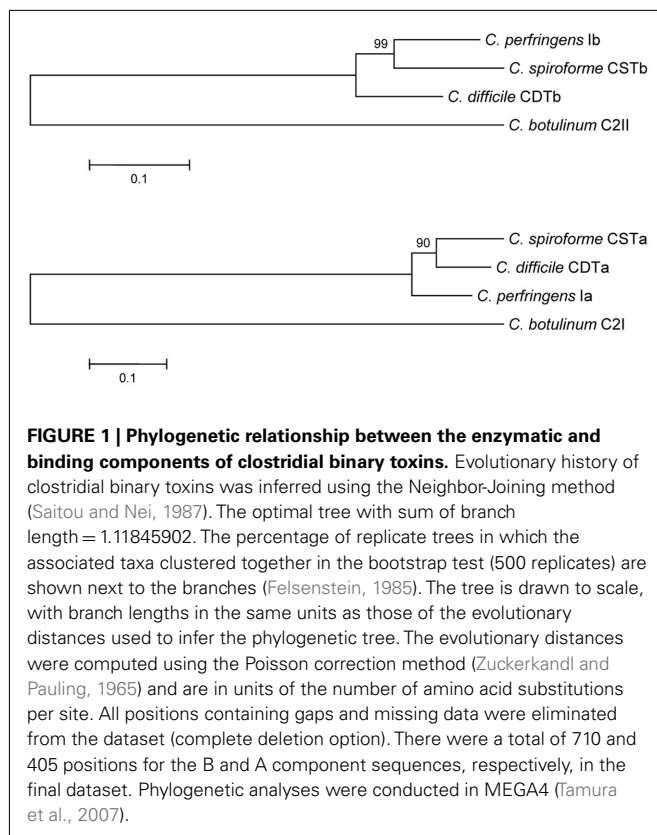
as first determined by crossed-immunoelectrophoresis and neutralization studies with *C. perfringens* type E antiserum (Stiles and Wilkins, 1986; Popoff et al., 1989; Simpson et al., 1989). It was erroneously thought that *C. perfringens* type E caused various diarrheic outbreaks within rabbit colonies, as type E antiserum neutralizes the cytotoxic cecal contents from enterotoxemic rabbits *in vitro* (Katz et al., 1978; Borriello and Carman, 1983). However, *C. perfringens* type E was never isolated and the real breakthrough came in 1983 correlating disease with enteric presence of *C. spiroforme* (Borriello and Carman, 1983). Spores were selected from cecal contents via heat (80°C/10 min) or ethanol (50%/1 h at room temperature) resistance and subsequently plated onto blood or egg yolk agar incubated anaerobically at 37°C. Simply based upon cell morphology and arrangement, there are distinct differences between *C. perfringens* and *C. spiroforme*. There are now less laborious, PCR-based techniques for detecting *C. spiroforme* via ribosome- and toxin-specific genes (Drigo et al., 2008).

CLOSTRIDIUM DIFFICILE TOXIN

The final member to enter the iota family is CDT (Popoff et al., 1988; Perelle et al., 1997a). *C. difficile* was first recognized as a major pathogen in the 1970s regarding its role in pseudomembranous colitis and antibiotic-induced diarrhea in humans (Carroll and Bartlett, 2011). This bacterium increasingly causes many life-threatening problems, especially in hospitals throughout the world via emerging “epidemic” strains (O’Conner et al., 2009; Kim et al., 2011).

Initial discovery and isolation of *C. difficile* (originally named *Bacillus difficilis*) are credited to Hall and O’Toole (1935) following studies of intestinal flora in newborn (up to 10 day old) infants. Their pioneering studies involving guinea pigs and rabbits injected with culture filtrates of *B. difficilis* (*C. difficile*) suggested a soluble exotoxin(s). The species name is derived from the French word for “difficult,” as these anaerobes did not readily ferment sugars with available techniques. Unlike adults, the intestinal tracts of infants colonized by *C. difficile* and containing large molecular-weight, Rho-glucosylating toxins A and B are interestingly not indicative of disease. In addition to humans, CDT-producing *C. difficile* colonize the digestive tracts of cattle (Houser et al., 2010), horses (Thean et al., 2011), and pigs (Thakur et al., 2010). Other mammals may also act as sources of *C. difficile* for human infection (Keel and Songer, 2006; Avbersek et al., 2011). *C. difficile* is found in commercially available meats (Gould and Limbago, 2010) and vegetables (Metcalf et al., 2010). Detection of the bacterium in clinical samples is typically done via toxins A and B (protein or DNA) assays (Barbut et al., 2011).

Like the other binary toxins, CDT consists of two components (CDTa and CDTb) that respectively share high amino acid sequence identity with *C. perfringens* Ia and Ib (Figure 1). This relatedness is further demonstrated by interchanging protein components between CDT, CST, and iota toxin (not C2 though) to form biologically active chimeras (Popoff et al., 1989; Perelle et al., 1997b; Gülke et al., 2001). Obvious structural and functional commonalities exist between these toxic proteins of *C. difficile*, *C. perfringens*, and *C. spiroforme*. It does not appear a random coincidence that these intestinal, spore-forming pathogens possess iota-family toxins.



For *C. difficile*, there have been many studies among hospital patients suggesting that CDT is linked to particularly virulent, epidemic strains (Geric et al., 2003, 2006; Barbut et al., 2007; Blossom and McDonald, 2007; Miller et al., 2010; Bacci et al., 2011); however, definitive proof correlating CDT levels in feces and disease severity is lacking. In contrast, other studies do not correlate CDT with disease severity (Goldenberg and French, 2011). To help resolve this, and other, CDT-based issues a recently developed ELISA can possibly correlate binary toxin levels in feces, or from cultured isolates, to disease severity (Carman et al., 2011). When one compares the enteric/systemic effects of other clostridial binary toxins upon various mammals, quite plausibly CDT adds a synergistic or additive twist to *C. difficile*-associated disease in humans and animals. In fact, a recent Danish study by Bacci et al. (2011) suggests higher fatality rates in patients with *C. difficile* containing the CDT genes versus those strains without. Further correlation of CDT concentrations in the gut, and severity of *C. difficile* disease, becomes rather complicated with a sporulating bacterial pathogen that produces two other potent toxins, A and B. Add to this relative antibiotic resistance of an isolate and it becomes very difficult to unequivocally ascertain the role CDT plays in *C. difficile* disease. Perhaps use of CDT-targeted gene knockouts of *C. difficile*, with an animal infection model, could be useful for future studies (Kuehne et al., 2010)?

CLOSTRIDIUM BOTULINUM C2 TOXIN

Clostridium botulinum, initially identified as *Bacillus botulinus*, was first described in 1895 by Emile van Ermengem following a social

gathering in Belgium where contaminated ham was served to the guests (Devriese, 1999). Some of these people died due to botulism, caused by a protein neurotoxin (BoNT). Similar to the *C. perfringens* typing toxins, BoNT types A–G of *C. botulinum* are classically determined by mouse lethal assays with BoNT-specific antisera. As with the other clostridia though, PCR-based detection is becoming more common for identifying the different toxinotypes (Fach et al., 2011).

Unlike BoNTs, the binary C2 enterotoxin produced by *C. botulinum* types C and D lacks neurotoxicity but is implicated in fatal enteric outbreaks among waterfowl. The toxin consists of C2I (enzyme) and C2II (cell-binding and translocation) proteins (Ohishi, 1983a,b), which do not complement iota-family toxin components. C2 toxin is cytopathic for many different cell types and induces vascular permeability, necrotic–hemorrhagic lesions, as well as lethal fluid accumulation into the lungs and intestinal tracts of various animals (Ohishi et al., 1980; Simpson, 1982; Ohishi and Miyake, 1985; Kurazono et al., 1987). In 1986, Aktories and co-workers discovered that C2I mono-ADP-ribosylates globular (G) actin (Aktories et al., 1986). This was the first report of any bacterial toxin that modifies actin and subsequently destroys the cytoskeleton.

BIOLOGY OF CLOSTRIDIAL BINARY TOXINS

Clostridial binary toxins are composed of enzymatic (A) and cell-binding/translocation (B) proteins released separately from the bacterium, subsequently assembling upon targeted eukaryotic cells. The iota-family members are *C. difficile* CDT, *C. perfringens* iota, and *C. spiroforme* CST (Table 1) based upon high sequence homology, immunological cross-reactivity, and interchangeable components that generate biologically active chimeras. The lone representative of the C2 family is from *C. botulinum* and distinct from the iota family in many ways (Figure 1). Interchangeable protein components of the iota-toxin family share 80–85% sequence identity, but the signal peptides are less conserved (40–61% identity). There is only 31–40% identity between C2 and iota-family toxins which is slightly higher than the 26–30% identity between *B. anthracis* protective antigen (PA) and clostridial B components. The A and B components of iota-family toxins are respectively synthesized with a leader peptide consisting of 29–49 and 39–47 residues (Popoff, 2000). In contrast, C2 toxin components are sporulation-linked and thus lack a signal peptide. These findings correlate with iota-family proteins secreted during logarithmic growth, while the C2 toxin is produced during sporulation (late logarithmic) and released after sporangium lysis (Nakamura et al., 1978). Various commonalities between clostridial and bacillus binary toxins, along with production of spores, suggest overlapping evolutionary paths between these genera.

The AB components of all *Clostridium* binary toxins are encoded by distinct genes possessing 27–31% G + C content (Popoff, 2000). The A and B genes are transcribed in the same orientation from a common operon. The A gene is located 40–50 nucleotides upstream of the B, with an exception being the C2 genes separated by 247 nucleotides (Perelle et al., 1993; Fujii et al., 1996; Gibert et al., 1997; Kimura et al., 1998). There are also other genetic differences as *C. difficile* CDT and *C. spiroforme* CST are chromosome-encoded versus the plasmid-localized *C. botulinum*

Table 1 | Clostridial binary toxins.

Toxin and components (kDa)	Gene location	Associated disease
C. PERFRINGENS IOTA		
Ia (45)	Plasmid	Calf/lamb enterotoxemia
Ib (94 precursor/81 activated)		
C. SPIROFORME CST		
Sa (44)	Chromosome	Rabbit enteritis/potential rare cases in humans
Sb (92 precursor/76 activated)		
C. DIFFICILE CDT		
CDTa (48)	Chromosome	Additional virulence factor in pseudomembranous colitis/post-antibiotic enteritis
CDTb (99 precursor/75 activated)		
C. BOTULINUM C2		
C2I (49)	Large plasmid	Avian hemorrhagic enteritis
C2II (80 or 100 precursor/60 or 80 activated)		

C2 and *C. perfringens* iota toxins (Popoff, 2000; Li et al., 2007; Sakaguchi et al., 2009). Originally, the *C. botulinum* C2 toxin genes were thought to be chromosomal but later studies revealed a quite large (107 kb) plasmid with 123 potential open reading frames (Sakaguchi et al., 2009). Plasmid from *C. perfringens* type E that contains the iota-toxin genes is also unique in that it can encode another toxin, *C. perfringens* enterotoxin, which is: (1) associated with human food poisoning; (2) sporulation-linked; and (3) possesses a different mode of action versus the clostridial binary toxins (Miyamoto et al., 2011). Additionally, these type E strains can be mistyped as *C. perfringens* type A due to sequence variability within the Ia gene that is typically targeted by PCR.

STRUCTURE AND FUNCTION OF B COMPONENTS

As Table 1 shows for each toxin, the cell-binding B components are produced as precursors activated outside of the bacterium by various serine-type proteases from bacteria, the mammalian host, or that added *in vitro*. The resultant loss of an N-terminal peptide (~20 kDa) evidently induces conformational changes that facilitate homoheptamerization, either in solution or on the cell surface. The B oligomers bind to cell-surface receptors, form complexes with respective A component(s), facilitate internalization, and ultimately release A into the cytosol. There is no enzymatic activity attributed to B components from any clostridial binary toxin.

It was initially reported in 1949 that iota toxin requires proteolytic activation for mouse lethal and guinea-pig dermonecrotic effects (Ross et al., 1949). Additional clues were provided years later, revealing that an Ib precursor (designated as Ibp) was the target of exogenously added, or culture-derived, serine-type proteases (Barth et al., 2004). Trypsin proteolysis of fractionated, early cultures of *C. perfringens* type E increased ELISA readings for Ibp but not Ia. These findings suggest a conformational change in “activated” Ibp exposing cryptic epitopes recognized by Ib-specific antibodies. There was also increased guinea-pig dermonecrosis

and mouse lethality of Ibp, following proteolysis, in conjunction with untreated Ia. Subsequent cloning and sequencing revealed proteolysis of Ibp near A₂₁₁ (Perelle et al., 1993), which promotes oligomerization of Ib into SDS-stable heptamers on cell membranes and lipid rafts (Hale et al., 2004; Nagahama et al., 2004); however, Ib heptamers formed in solution are rather unstable (Blöcker et al., 2001; Nagahama et al., 2002; Stiles et al., 2002). Vero cell-bound Ibp does not form oligomers and is not activated over time *in vitro* with, or without, exogenous trypsin or chymotrypsin (Stiles et al., 2002). The cell-targeting domain of Ib/Ibp is in the C-terminus and quite distal from the N-terminal activation site. To date, activation and cell-binding studies similar to those for Ib have not been conducted with *C. difficile* CDTb or *C. spiroforme* Sb; however, there are likely many similarities in the biology of these B components based upon sequence homology with Ib.

Following proteolysis of Ibp, Ia readily docks with the Ib oligomer (Stiles et al., 2000). There are also voltage-dependent, ion-permeable channels formed in artificial lipid membranes by Ib oligomers, but not Ibp monomers (Knapp et al., 2002). These channels are blocked by Ia. Ib oligomers formed in solution are structurally fragile and upon binding to Vero cells do not cause potassium release, are readily digested by pronase, and do not promote Ia-induced cytotoxicity (Blöcker et al., 2001; Nagahama et al., 2002). The pronase studies suggest that solution-generated Ib oligomers, once bound to cells, remain exposed and do not insert into lipid membranes.

There are other proteases like pepsin, proteinase K, subtilisin, alpha-chymotrypsin, thermolysin, as well as the zinc-dependent *C. perfringens* lambda protease that activate Ibp more efficiently than trypsin. Besides Ibp, Ia also undergoes proteolysis by some of these same enzymes with an additional loss of 9–13 amino acids from the N-terminus after cleavage of leader peptide (Gibert et al., 2000). Proteolysis of Ia leads to increased cytotoxicity of Vero cells, when combined with Ib. It is still uncertain whether proteolysis of Ia affects docking efficiency to cell-bound Ib, translocation into cells, and/or enzymatic activity. Proteolysis effects upon A components from other clostridial binary toxins has not been reported to date. As the iota-family members are enteric, it is perhaps an evolutionary advantage to become activated by many different proteases from not only the host microbe, but also neighboring bacteria and eukaryotic host. Proteolytic activation, and subsequent resistance to proteolysis-based inactivation, is a common theme with clostridial toxins from various species.

Structure–function studies have been done with iota toxin, targeting Ib via deletion mutagenesis and antibody studies (Marvaud et al., 2001). Similar studies are lacking in the literature for B components from CST and CDT. Deletion of just 10 residues from the C-terminus (domain 4) effectively prevents Ib binding to Vero cells. C-terminal peptides of Ib containing more than 200 amino acids represent competitive inhibitors of iota cytotoxicity *in vitro*. On the other end, deletion of 27 N-terminal residues prevents Ia docking and intoxication, yet has little effect upon Ib binding to the cell surface.

Studies with monoclonal antibodies (Mabs) against an N-terminal epitope within residues 28–66 reveal no effect upon Ib binding or cytotoxicity (Marvaud et al., 2001). It is possible that these immunoreagents do not occupy the Ib site necessary for Ia

docking and/or perhaps are displaced upon Ib oligomerization or docking of Ia. An obvious void in the literature involves affinity constants for A–B docking amongst clostridial binary toxins, which evidently does not occur at an appreciable rate in solution versus on a cell surface.

Two other Mabs recognize unique Ib epitopes within the C-terminus (residues 632–655), protecting against iota cytotoxicity via distinct mechanisms. One Mab prevents Ib binding to cells while the other does not; however, this latter antibody efficiently prevents Ib oligomerization on the cell surface. These latter results further demonstrate the importance of Ib oligomerization on iota-toxin activity, which is a common theme amongst clostridial binary toxins. Unfortunately, from an antibody probe perspective, none of the N- or C-terminal binders recognize Ib bound to the cell surface.

Each Mab against Ib recognizes Ibp or *C. spiroforme* Sb in an ELISA and Western blot, but not *B. anthracis* PA (Marvaud et al., 2001). A similar effort with CDTb is obviously lacking. Surprisingly, C2II (activated form designated as C2IIa) is recognized by one of the C-terminal binding Mabs in an ELISA; however, in contrast to iota toxin this immunoreagent does not neutralize C2 cytotoxicity. C2IIa and Ib bind unique receptors via their C-terminus and share little sequence homology within the C-terminal domain (Fritz et al., 1995; Blöcker et al., 2000). Although previous efforts have targeted the N- and C-termini of Ib through different techniques, a more thorough understanding of the clostridial binary toxins could perhaps now be gleaned by focusing upon internal domains 2 and 3.

Like the iota-family toxins, the 80 kDa (or 100 kDa) C2II precursor of C2 toxin is activated by trypsin into 60 kDa (or 80 kDa) C2IIa (Blöcker et al., 2000). Size differences in precursor and activated C2II can vary, depending upon strain and C-terminal extension that increases toxin potency (Sterthoff et al., 2010). C2IIa, but not the C2II precursor, forms stable homoheptamers in solution (Barth et al., 2000; Kaiser et al., 2006). Electron microscopy of C2IIa oligomers on lipid bilayers reveals donut-, as well as horseshoe-, shaped heptamers with inner (20–40 Å) and outer (110–130 Å) diameters (Barth et al., 2000) further confirmed by Schleberger et al. (2006) via modeling. In similar fashion as the iota toxin, the C2II precursor binds to cells but is not activated by surface proteases and does not dock C2I (Ohishi, 1987; Ohishi and Yanagimoto, 1992).

C2IIa forms ion-permeable, cation-selective channels in artificial black lipid bilayer membranes that are blocked by complementary C2I (Schmid et al., 1994; Bachmeyer et al., 2001; Blöcker et al., 2003). A cluster of hydrophobic and hydrophilic amino acids (303–331) within C2II may play a critical role in membrane insertion, along with E399 and F428 (Blöcker et al., 2003; Lang et al., 2008). Moreover, in acidic media, C2IIa forms pores in the cytoplasmic membranes of intact cells which translocate C2I directly into the cytosol. Such experiments mimic acidified endosomes where C2IIa heptamers form transmembrane pores to translocate C2I from the endosomal lumen, across the endosomal membrane, and into the cytosol.

As described by different groups for both *C. perfringens* Ib and *B. anthracis* PA, studies also reveal that the C-terminus of C2IIa facilitates binding to cell-surface receptor (Blöcker et al., 2000).

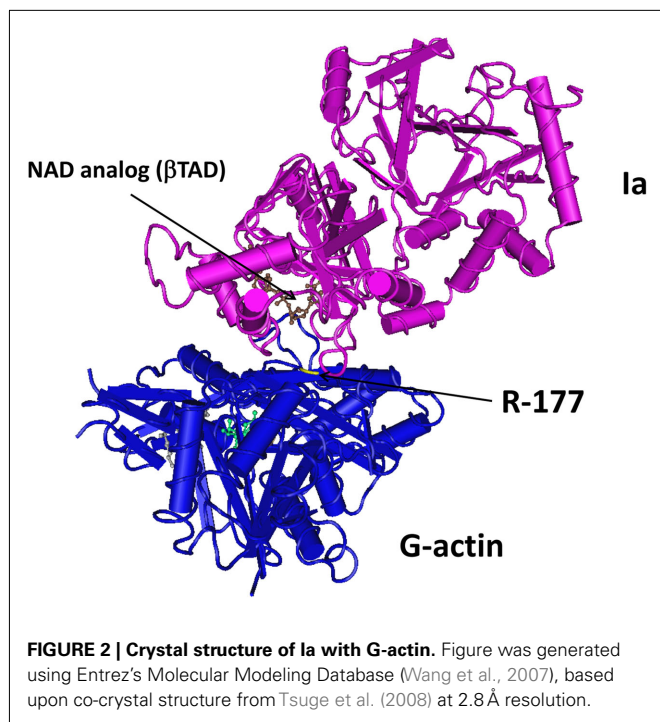
Antiserum specific for the C-terminus (domain 4; residues 592–721), but not domains 1 (residues 1–264) or 3 (residues 490–592), blocks C2IIa binding to cells. Antiserum against domain 4 neutralizes C2 cytotoxicity *in vitro* when preincubated with C2IIa, but this is not the case after C2IIa has bound the cell surface. As described for Ib Mabs binding to Ib (Marvaud et al., 2001; Stiles et al., 2002), neutralizing epitopes on C2IIa are perhaps sterically hindered after C2IIa-cell receptor interactions. Deletion studies targeting the N-terminus of C2II precursor show that loss of residues 1–181, normally cleaved upon proteolytic activation, impacts proper folding (Blöcker et al., 2000). The sequence similarities existing between PA, C2II, and Ib are primarily localized within central domains 2 and 3. For PA, these domains participate in oligomerization, channel formation, and enzyme translocation (Benson et al., 1998; Mogridge et al., 2001; Sellman et al., 2001). Except for one study with *C. botulinum* C2II (Blöcker et al., 2003), very little structure–function analysis has occurred within domains 2 and 3 of B components from the other clostridial binary toxins.

STRUCTURE AND FUNCTION OF A COMPONENTS

Enzymatic components of iota, CDT, and C2 toxins consist of two comparable-sized domains of ~200 amino acids. The N-terminal domain of each is enzymatically inactive and serves as a docking site for complementary B component. Residues 1–87 of C2I mediate binding to C2II heptamers and translocation into the cytosol (Barth et al., 1998a, 2002a,b). Alignment of C2I with *Bacillus cereus* vegetative insecticidal protein 2 (VIP2), a related ADP-ribosyltransferase, reveals relatedness within amino acids 1–225 (C2I) and 60–275 (VIP2) that includes four exposed α -helices (Han et al., 1999). Active sites are located in the C-terminus of these enzymes, harboring conserved amino acids for catalysis. Mutation of the first glutamic acid in the EXE motif of C2I prevents ADP-ribosyltransferase, but not nicotinamide adenine dinucleotide (NAD)-glycohydrolase, activity while the second glutamic acid affects both (Barth et al., 1998b; Sakurai et al., 2003). An STS triad is also commonly located near the active site and promotes binding to NAD. These residues are conserved amongst various ADP-ribosyltransferases from prokaryotes and eukaryotes (Carroll and Collier, 1984; Jung et al., 1993; van Damme et al., 1996; Han et al., 1999; Sakurai et al., 2003).

Mutagenesis of Ia within the NAD binding cavity reveals that Y₂₄₆ and N₂₅₅ are important for ADP-ribosyltransferase, but not NAD-glycohydrolase, activity while Y₂₅₁ is involved in both (Sakurai et al., 2003). Enzymatic activity is inhibited by removing divalent cations associated with actin, but low temperature (0°C) remarkably decreases activity by only 50% versus that at 37°C (Just et al., 1990).

Crystallography studies with components of different clostridial binary toxins have been reported by various groups. Tsuge et al. (2003, 2008) revealed Ia interactions with actin at 2.8 Å resolution (Figure 2). Similar efforts by Sundriyal et al. (2009) show CDTa (1.85–2.25 Å resolution) at different pH (4.0, 8.5, 9.0) and complexed with NAD. C2I has also been resolved at 1.75 Å, and like CDTa, there are few conformational changes that occur with varying pH (Schleberger et al., 2006). This latter point is particularly pertinent since an acidic environment (endosome or extracellular fluid), with C2II-mediated channels, promotes C2I



translocation into the cytosol. Sequence identity of C2I with Ia (or CDTa) is only 40%. A similar duplicate domain structure exists for the related *B. cereus* VIP2 (Han et al., 1999). Like the clostridial binary toxins, VIP2 is transported into cells via a separate B component (VIP1) that forms oligomers and channels in lipid membranes (Leuber et al., 2006). Relative to the iota and C2 family toxins, less is known about VIPs produced by *Bacillus* species.

CELLULAR UPTAKE OF CLOSTRIDIAL BINARY TOXINS

To come within range of any intracellular substrate, and like other bacterial protein toxins, the clostridial and bacillus binary toxins must first bind to the exterior of a targeted cell (Figure 3). This is followed by internalization of the toxin complex and translocation of A component from acidified endosome into the host-cell's cytosol. These individual steps during cellular uptake are mediated by the multifunctional B components of binary toxins (Barth et al., 2004). Serine-type proteases activate B monomers that then form homoheptamers (most commonly reported arrangement) on the cell surface or in solution prior to cell contact. Binding of B components from clostridial binary toxins involves lipid rafts (Hale et al., 2004; Nagahama et al., 2004, 2009), with the B oligomer – receptor complex acting as a docking platform for A component(s). The receptors for Ib and C2IIa are distinct proteins, in which carbohydrates play an important role in binding of C2IIa but not Ib (Eckhardt et al., 2000; Stiles et al., 2000). In contrast, the cell-surface receptors for *B. anthracis* PA have been definitively identified as tumor endothelium marker (TEM) 8 (Bradley et al., 2001) and human capillary morphogenesis gene (CMG) 2 (Scobie et al., 2003). Lipid rafts also facilitate PA clustering and endocytosis (Abrami et al., 2003).

To further understand the binding and oligomerization of clostridial binary toxins on cells, the potential role played by lipid rafts has been explored by different research groups. Lipid rafts are dynamic, cholesterol-rich, detergent-insoluble (at 4°C) regions on cell membranes that popularly serve as portals for invasive bacteria, viruses, and toxins (Vieira et al., 2010). It has been shown that *C. perfringens* Ib localizes into these membrane microdomains on Vero cells (Hale et al., 2004; Nagahama et al., 2004). The Ibp molecule, which binds to cells but does not promote iota toxicity, is not associated with lipid rafts on the cell surface. This finding suggests that the receptor for iota toxin exists outside of lipid rafts, but is perhaps “dragged” into these microdomains after binding to Ib. Protein composition of Ib-containing lipid rafts from Vero cells has been determined by proteomics (Blonder et al., 2005). Recent work by Schwan et al. (2011) suggests that lipid rafts also play a role in CDT intoxication, which includes unique microtubule-based extensions from intoxicated cells that promote *C. difficile* adherence. This same group (Papatheodorou et al., 2011) has recently revealed a rather obscure protein, lipolysis-stimulated lipoprotein receptor (LSR), as a receptor for CDTb and Ib. LSR is a type I transmembrane protein involved in uptake of lipoproteins, but has never been described as a receptor for any bacterial toxin. Such a finding excitingly paves the way for further understanding the uptake mechanisms of the iota-family toxins, which could lead to unique toxin-targeting therapies.

In addition to Ib, receptor-binding studies have also been reported for precursor and proteolytically activated forms of C2II (Ohishi and Miyake, 1985). C2IIa has unique hemagglutinating properties competitively inhibited by various carbohydrates such as *N*-acetylgalactosamine, *N*-acetylglucosamine, L-fucose, galactose, or mannose (Sugii and Kozaki, 1990). Trypsin or pronase treatment of human erythrocytes prevents C2II-induced hemagglutination, suggesting a glycoprotein of unknown identity. Furthermore, Fritz et al. (1995) revealed that chemically mutagenized CHO cells do not bind C2IIa. These cells are devoid of *N*-acetylglucosaminyltransferase I which facilitates formation of asparagine-linked complex and hybrid carbohydrates (Eckhardt et al., 2000). These cells are still susceptible to iota toxin; therefore, demonstrating that C2IIa and Ib recognize different receptors. C2, like iota and the *B. anthracis* binary toxins, uses lipid rafts for binding and entry into cells (Nagahama et al., 2009). C2 toxin effectively intoxicates all tested vertebrate cells (Ohishi et al., 1984; Sugii and Kozaki, 1990; Eckhardt et al., 2000), but the receptor for Ib is not as ubiquitous (Stiles et al., 2000).

The Ib receptor is resistant to various proteases, but not pronase. Rather extensive pretreatment of cells with lectins or glycosidases does not affect Ib binding, thus suggesting that carbohydrates play no role (Stiles et al., 2000). Experiments with polarized CaCo-2 (human colon) cells show that Ib receptor is namely localized upon the basolateral membrane (Blöcker et al., 2001; Richard et al., 2002). Additionally, Ib crosses a CaCo-2 cell monolayer at 37°C (but not 4°C) from the apical or basolateral surface independent of Ia (Richard et al., 2002). Ib that has traveled across a monolayer can internalize Ia on this distal surface, even when Ia is added 3 h after Ib.

Western blot experiments reveal that Ib rapidly binds to cells at 37°C and forms a large (>200 kDa) complex within 1 min

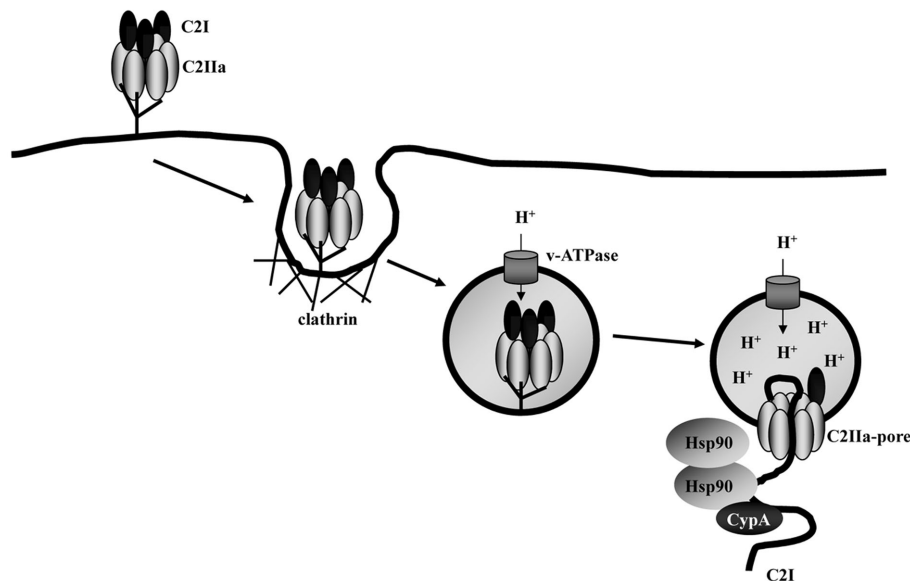


FIGURE 3 | Model for the cellular uptake of C2 toxin from

***C. botulinum*.** The C2IIa/C2I toxin complex binds to a receptor on the cell surface and is internalized via clathrin-dependent receptor-mediated endocytosis. Acidic conditions in the lumen of early endosomes trigger

membrane insertion and pore formation by C2IIa. C2I translocates in an unfolded conformation through the C2IIa pores across endosomal membranes into the cytosol. Hsp90 and cyclophilin A (CypA) facilitate translocation.

(Nagahama et al., 2002; Stiles et al., 2002). This complex, which does not form at 4°C, remains for at least 6 h and thus promotes Ia docking opportunities that generate holotoxin. Rapid binding of Ib followed by surface-sustained availability for Ia makes sense for any clostridial binary toxin.

Beyond cell-based studies, Sakurai and Kobayashi (1995) discovered that when Ib is injected intradermally into guinea pigs, Ia (injected intraperitoneally) can “find” Ib and cause localized dermonecrosis. Perhaps this “homing” characteristic of Ia can be exploited in future experiments from a medicinal perspective. Similar discoveries have been reported for the C2 toxin in both mice and rats (Simpson, 1982).

Following receptor-mediated endocytosis of the clostridial binary toxins, which can occur via clathrin-dependent and -independent mechanisms (Pust et al., 2010; Gibert et al., 2011), the A components of iota, CDT, and C2 toxins cross the endosomal membrane into the cytosol (Barth et al., 2000; Blöcker et al., 2001; Kaiser et al., 2011). This step is mediated by transmembrane pores formed by the B components and can be blocked by a macrolide antibiotic, bafilomycin A, which inhibits vacuolar-type ATPases that acidify the endosomal lumen. This suggests that acidic conditions are crucial for translocating A components from endosomes into the cytosol. Low pH evidently induces conformational changes within the B complex, promoting insertion into membranes and subsequent translocation of A components through pores into the cytosol. This process is also artificially induced from the cell surface into the cytosol by simply lowering media pH (Barth et al., 2000; Blöcker et al., 2001). There are unique pH requirements for translocating iota and C2 toxins, as iota requires a lower pH (≤ 5.0) versus C2 (≤ 5.5). The biochemical reasons for this difference are not known. Conversion of B.

anthracis PA heptamer from a pre-pore to pore state, when bound to CMG2 receptor, is also pH driven and controlled by the receptor (Lacy et al., 2004). Perhaps the unique receptors recognized by C2 and iota toxins play similar roles during pH-induced translocation.

Furthermore, entry of iota toxin from the endosome into the cytosol of Vero cells differs from C2 toxin as per chloroquine, monensin, nigericin, and ammonium chloride inhibition (Gibert et al., 2007). Besides preventing endosomal acidification, chloroquine also physically blocks the C2IIa-induced pore thereby stopping C2I translocation (Schmid et al., 1994; Blöcker et al., 2003). Monensin, like nigericin, exchanges monovalent cations for protons that abolish the endosomal pH gradient. Because of inherent alkalinity, ammonium chloride increases pH within endosomes. The biological activity of iota toxin on Vero cells is not inhibited by monensin alone; however, a combination of monensin and valinomycin (a potassium ionophore) proves partially inhibitory, and there is a distinct decrement of iota-toxin activity with monensin plus bafilomycin A. Based upon these results, requirements of Ia entry from the endosome mimic those previously described for fibroblast growth factor (Wesche et al., 2006). Altogether, a pH gradient between the endosome and cytosol are required for translocating Ia (from early to late endosomes) and C2I (from early endosomes), but Ia also requires a membrane potential. Most likely, following translocation of A component into the cytosol, the B heptamers of clostridial binary toxins remain attached to the endosomal membrane and undergo lysosomal degradation (Ohishi and Yanagimoto, 1992; Richard et al., 2002). It is also possible, yet less likely, that B heptamers recycle back onto the cell-surface following release of A into the cytosol.

For C2 toxin it has been shown that translocation requires partial unfolding of the A component, C2I (Haug et al., 2003b). It can

be expected that A components from the other clostridial binary toxins also unfold into a “molten globule” to translocate through B heptameric pores within the endosomal membrane. Perhaps this occurs in a ratchet-type mechanism similar to the *B. anthracis* lethal factor (LF) via an N- to C-terminal direction, through the B (PA) pore, into the cytosol (Zhang et al., 2004; Krantz et al., 2006).

Recent studies with the C2, CDT, and iota toxins reveal that pH-dependent membrane translocation and/or refolding of the A components is facilitated by host-cell factors including the chaperone heat-shock protein 90 (Hsp90), and cyclophilin A, a peptidyl-prolyl *cis/trans*-isomerase (PPIase) (Haug et al., 2003a, 2004; Kaiser et al., 2009, 2011). PPIases are helper enzymes that catalyze slow protein-folding reactions (Fischer et al., 1989; Schmid, 1993). Treatment of cultured cells with specific pharmacological inhibitors of Hsp90 (geldanamycin and radicicol) or cyclophilin A (cyclosporine A) significantly delay the C2-, CDT-, and iota-induced rounding of cells. Moreover, these inhibitors prevent uptake of A components into the cytosol but do not influence other aspects of toxin uptake or enzyme activity. Inhibition of the chaperone and PPIase activities of Hsp90 and cyclophilin A respectively prevent translocation of A components into the cytosol, thus trapping them in the endosomes. The A components directly interact with purified Hsp90 and cyclophilin A proteins *in vitro* (Kaiser et al., 2009, 2011). Although the data support a common Hsp90/cyclophilin A-dependent translocation for clostridial binary toxins, the precise molecular mechanisms underlying the interaction between these host-cell factors and A components is not known and requires further investigation. Interestingly, Hsp90 is a conserved ATPase present in all eukaryotic cells and often complexed with other proteins, including PPIases (Wandinger et al., 2008). In conjunction with other heat-shock proteins, Hsp90 regulates trafficking of “client” proteins into the cytosol and assists various cell functions that include signaling (Pratt and Toft, 2003; Zuehlke and Johnson, 2010). The translocation process used by clostridial binary toxins is akin to that exploited by another ADP-ribosyltransferase from *Corynebacterium diphtheriae*, diphtheria toxin, involving a cytosolic complex of Hsp90 and thioredoxin reductase (Ratts et al., 2003). The latter might cleave the disulfide bond between A and B chains of diphtheria toxin, in which such reduction-based activation exists for other single-chain proteins like *C. tetani* tetanus toxin and *C. botulinum* neurotoxin A (Kistner and Habermann, 1992). However, such a cystine bond does not exist between AB components for clostridial and bacillus binary toxins.

In contrast to the clostridial binary toxins, cytosolic entry of *B. anthracis* lethal toxin is not affected by Hsp90 inhibitors (Haug et al., 2003a; Zornetta et al., 2010; Dmochewicz et al., 2011). This latter result further suggests differences in translocating clostridial and bacillus binary toxins. Moreover, Hsp90 might be generally selective for bacterial ADP-ribosyltransferases like the cholera toxin of *Vibrio cholerae* (Taylor et al., 2010), which is structurally distinct from the binary toxins described in this current review.

New knowledge about the molecular mechanisms underlying cellular uptake of binary clostridial toxins can provide useful therapeutic targets against these toxins. For example, targeting of CDT could perhaps diminish some of the enteric ill-effects of epidemic (CDT-producing) strains of *C. difficile*. Examples of novel

therapeutics might include derivatives of chloroquine (Bachmeyer et al., 2001) or methyl- β -cyclodextrin (Nestorovich et al., 2011), which interfere with pore formation by B components and subsequent translocation of A components. Another possibility is the targeted pharmacological inhibition of individual host-cell factors that translocate A components, such as cyclophilin A (Barth, 2011).

A more comprehensive understanding of how clostridial binary toxins enter cells can also aid their potential use as medicinal shuttles. This latter aspect is particularly interesting since fragments of the C2 (Barth et al., 1998a, 2002b; Pust et al., 2007; Fahrner et al., 2010a,b) and iota (Marvaud et al., 2002) toxins have been successfully employed as “Molecular Trojan Horses” to deliver foreign proteins (e.g., enzymes) into the cytosol of various mammalian cell types without causing damage during entry. Because this approach enables targeted manipulation of living cells, recombinant fusion toxins do not only represent valuable tools for cell biology and experimental pharmacology, but also potentially attractive therapeutics (Barth and Stiles, 2008).

ADP-RIBOSYLATION OF ACTIN... A PATHOGEN'S SURGICAL STRIKE UPON THE CYTOSKELETON

Mono-ADP-ribosylation of host proteins is a common mechanism employed by diverse, pathogenic bacteria via the actions of protein toxins (Massignani et al., 2006). All of these toxins use eukaryotic-provided NAD, a ubiquitous molecule necessary for energy metabolism, as a source of ADP-ribose to alter the function of critical eukaryotic proteins necessary for life.

There are four groups of ADP-ribosylating toxins based upon their intracellular targets: (1) elongation factor two (EF2) modified by *C. diphtheriae* diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A via an N- and C-terminal active site, respectively; (2) heterotrimeric G-proteins targeted by *Bordetella pertussis* pertussis toxin, *Escherichia coli* heat labile enterotoxin, and *V. cholerae* cholera toxin by way of N-terminal active sites; (3) Rho and Ras GTPases modified by *C. botulinum* C3 exoenzyme and *P. aeruginosa* exoenzyme S through C-terminal active sites; and (4) G-actin (Holbourn et al., 2006; Massignani et al., 2006). All actin-modifying toxins have a C-terminal active site and are designated as type IV ADP-ribosyltransferases. Although sequence homologies may be low between different ADP-ribosyl transferases (i.e., prokaryotic and eukaryotic), topography of the enzymatic cleft and catalytic residues remains quite conserved (Tsuge et al., 2008).

Pathogen disruption of the eukaryotic cytoskeleton through actin can alter many vital processes, including: (1) vesicle trafficking; (2) phagocytosis; (3) migration; (4) epithelial barrier formation and binding to extracellular matrix; as well as (5) signaling (Aktories et al., 2011). Ultimately, these cumulative events induce cell death with subsequent release of valuable, intracellular nutrients for the pathogen and other microbes within that microenvironment. Furthermore, bacterial toxins that modify actin have become invaluable tools for studying the cytoskeleton and numerous cell processes.

Actin is a conserved protein (~42 kDa in monomeric G form) found throughout nature, playing a pivotal role in filament (F-actin) formation essential for cytoskeleton development and cellular processes (Wertman and Drubin, 1992; Aktories et al.,

2011). Mono-ADP-ribosylation of G-actin inhibits monomer assembly into F-actin strands (Aktories et al., 1986, 2011), via steric hindrance of hydrophobic loop interactions between G-actin molecules (Figure 4). Ultimately modified G-actin does not bind F-actin strands, decreasing concentrations of the latter, while concomitantly increasing the G-actin pool inside a cell (Aktories et al., 1989). In addition to the actin–gelsolin complex, both the iota and C2 toxins modify G-actin complexed with ATPase that results in increased exchange, but decreased hydrolysis, of ATP (Geipel et al., 1989). F-actin does not represent a direct target for any clostridial binary toxins.

There are six actin isoforms in birds and mammals, depending upon tissue type, and include: α -skeletal; α -cardiac; α and γ smooth muscle; as well as β and γ cytoplasmic (Perrin and Ervasti, 2010; Aktories et al., 2011). Interestingly, bacteria also contain a cytoskeletal matrix consisting of actin homologs (MamK, MreB, ParM, etc.) that vary between species (Cabeen and Jacobs-Wagner, 2010). Like eukaryotes, the cytoskeleton of a bacterium plays major life-sustaining functions that include division, shape, protein localization, and DNA segregation. The targeting of prokaryotic actin, similar to the iota and C2 toxins that modify eukaryotic actin, could perhaps lead to novel anti-infectives (Vollmer, 2006). We are unaware of any bacterial toxin that modifies bacterial homologs of actin. Along these lines, an indole compound inhibits growth of efflux-deficient *P. aeruginosa* by binding to the ATP-binding site on MreB (Robertson et al., 2007).

The clostridial binary toxins form two obvious groups based upon actin substrates. The *C. botulinum* C2 toxin only modifies R₁₇₇ of β/γ -non-muscle, as well as γ -smooth muscle, G-actin (Aktories et al., 1986; Ohishi and Tsuyama, 1986; Vandekerckhove et al., 1988). In contrast, the iota-toxin family is less discriminating and modifies all known G-actin isoforms (Mauss et al., 1990). The enzymatic CDTa, Ia, and Sa components each possess a LKDKE sequence, important for binding to G-actin, within the N-terminus (Popoff, 2000). However, the C2I molecule has a unique actin-binding sequence (LKTKE) and location that might help explain distinct substrate specificity.

Uematsu et al. (2007) have shown that actin disassembly by C2 toxin induces microtubule assembly and polarization of human leukemic cell lines. More recent studies by Schwan et al. (2009,

2011) reveal that treatment of gastric epithelial cells with CDT, iota, or C2 toxin induces microtubule protrusions from the membrane that promote adherence and colonization of *C. difficile* to the colonic mucosa. These protrusions are most likely dependent on cholesterol- and sphingolipid-rich microdomains of the cytoplasmic membrane (Schwan et al., 2011). This concept introduces a novel twist to pathogen–cell interactions, elicited by clostridial binary toxins.

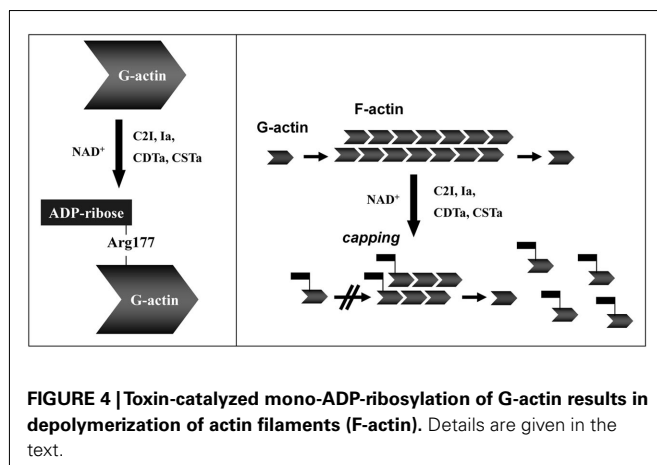
Furthermore, ADP-ribosylation of actin by C2 toxin arrests cell cycling at the G2/M boundary (Barth et al., 1999). Treatment with either the C2 or iota toxins results in delayed caspase-dependent death ~20 h after toxin application (Heine et al., 2008; Hilger et al., 2009). It is clear that toxins, like those produced by clostridia and which specifically modify actin, have become invaluable tools for studying cell biology and experimental pharmacology. There is much more to be learned from the ways these toxins work on cells.

PEERING INTO THE FUTURE VIA A PORTAL OF THE PAST

Discovery of *C. perfringens* iota toxin in 1940 by Bosworth was the first for any clostridial binary toxin. It was not until 1956 that the multi-component structure of *B. anthracis* toxins was initially reported, thus representing the first binary description for any bacterial toxin (Smith, 2002). The passing of three more decades eventually revealed the multi-component nature of various clostridial binary toxins described in this review. Many different laboratories led to these discoveries, with different toxins, from different clostridia.

The B heptamers from clostridial binary toxins shuttle one type of enzyme, a mono-ADP-ribosyltransferase specific for G-actin, into cells. This paradigm diverges with the *B. anthracis* PA, which transports lethal (LF) and edema (EF) factors possessing different enzymatic properties. Additionally, recent findings by Kronhardt et al. (2011) show that PA can also bind and subsequently transport *C. botulinum* C2I into cells. The efficiency of C2I transport by PA was at least 50-fold less than C2IIa. Furthermore, EF and LF bind to C2IIa oligomers in lipid bilayers but are not transported into cells. Within the iota family, enzyme is transported by heterologous B components from other clostridial species. To date, the *C. botulinum* C2 toxin is still distinct amongst the clostridial binary toxins in that C2IIa exclusively transports C2I. An ability of these B components to transport another protein into a cell makes them natural shuttles that can perhaps, with further study, transport medicinal molecules into cells. Crossing of a medicinal molecule through a cell membrane into the cytosol can be a daunting challenge, but the aforementioned *Clostridium* and *Bacillus* binary toxins have naturally solved this problem for rather large (i.e., protein) cargo. An important discovery for better understanding the mode of action of any bacterial toxin involves specific receptor identification. Very recent work by Papatheodorou et al. (2011), in which LSR serves as a receptor for Ib and CDTb, opens up an exciting realm for future research with the iota-family toxins.

It is our opinion that genetic analysis of other species (genera perhaps?) will yield more binary toxin-like producers, as evidenced by a PCR-based study showing *C. novyi* type A strains containing the *C. botulinum* C2I and/or C2II genes (Heffron and



Poxton, 2007). Additionally, some strains of *B. cereus* associated with lethal pneumonia in humans possess the anthrax toxin genes (Hoffmaster et al., 2004). Furthermore, one of these isolates produce a novel ADP-ribosyltransferase called certhrax which shares 34% identity with the catalytic region of CDTa (Hoffmaster et al., 2006; Fieldhouse et al., 2010). Certhrax also possesses 31% identity with LF, but lacks protease activity. Such discoveries reveal a dispersed genetic template for binary toxins that is, to date, more prevalent in clostridia. Evidently binary toxin “successes” of the

past, and those today, promote further success of various bacterial pathogens into the future.

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