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Binary Bacterial Toxins: Biochemistry, Biology, and Applications of Common *Clostridium* and *Bacillus* Proteins

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INTRODUCTION

Several proteins from gram-positive, spore-forming bacilli employ a synergistic binary mechanism for intoxicating eukaryotic cells; they include *Clostridium botulinum* C2 toxin, *Clostridium difficile* toxin (CDT), *Clostridium perfringens* iota (ι) toxin, *Clostridium spiroforme* toxin (CST), *Bacillus anthracis* edema and lethal toxins, as well as the *Bacillus cereus* vegetative insecticidal proteins (VIP). The protein components of these related toxins do not bind cells as a preformed "A-B" complex found in solution (Table 1), thus differing from many other bacterial binary toxins that engage cells as an intact "A-B" structure composed of single- or multiple-chain proteins (Table 2). *Bacillus cereus* and *Staphylococcus aureus* also produce other multiple-chain toxins composed of proteins not associated in solution; however, these pore-forming cytolysins remain on the cell surface and are devoid of enzymatic activity, thus differing from the *Clostridium* and *Bacillus* binary toxins described in this review (149a, 341a).

Intoxication by C2, CDT, CST, ι, VIP, or the *B. anthracis* edema and lethal toxins initially involves specific, receptor-mediated binding of "B" components to a targeted cell as monomers that form homoheptamers on the cell surface or as solution-generated heptamers (schematically depicted in Fig. 1). In either scenario, the "B" oligomers are generated only after proteolysis of "B" precursor molecules. The "B" heptamer-receptor complex then acts as a docking platform that subsequently translocates an enzymatic "A" component(s) into the cytosol via acidified endosomes. Once inside the cytosol, "A" components from this binary family can inhibit normal cell functions by (i) mono-ADP-ribosylation of G-actin, which induces cytoskeletal disarray and cell death; (ii) proteolysis of mitogen-activated protein kinase kinases (MAPKK), which inhibits cell signaling; or (iii) increasing intracellular levels of cyclic AMP (cAMP) that result in edema and immunosuppression. Not only are these toxins im-

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TABLE 1. *Clostridium* and *Bacillus* binary "A-B" toxins: an overview

Toxin and components	Gene location	Mol mass (kDa) (reference)	Enzymatic activity (reference)
C. perfringens ι toxin	Plasmid (140 kbp)		
Ia		45 (323)	ADP-ribosylation of actin (372)
Ib		94 precursor (323)	None
		81 activated (323)	None
C. spiroforme CST	Chromosome		
Sa		44 (147, 333)	ADP-ribosylation of actin (332)
Sb		92 precursor (147, 333)	None
		76 activated (147, 333)	None
C. difficile CDT	Chromosome		
CDTa		48 (324)	ADP-ribosylation of actin (334)
CDTb		99 precursor (324)	None
		75 activated (324)	None
C. botulinum C2 toxin	Chromosome		
C2I		49 (137)	ADP-ribosylation of actin (6, 310)
C2II		81 precursor (208)	None
		60 activated (48)	None
B. anthracis edema and lethal toxins	Plasmid pXO1 (182 kbp)		
EF		89 (360)	Adenylate cyclase (229)
LF		90 (62)	Zn^{2+} metalloprotease for MAPKK (107, 453)
PA		83 precursor (467)	None
		63 activated (467)	None
B. cereus VIP	Unknown		
VIP2		52[a]	ADP-ribosylation of actin (171)
VIP1		100[a]	None
		80[a]	None

[a] G. Warren, M. Koziol, M. A. Mullins, G. Nye, B. Carr, N. Desai, K. Kostichka, N. Duck, and J. J. Estruch, 1996, World Intellectual Property Organization patent application.

portant virulence factors representing effective vaccine targets for diseases like anthrax, but also they are useful biological tools for studying a myriad of cellular functions and delivering heterologous proteins into endosomal, as well as cytosolic, compartments. In light of heightened concerns involving *B. anthracis* and bioterrorism, this review provides a comprehensive glimpse at a family of related binary toxins produced by different *Clostridium* and *Bacillus* species. Important aspects of each binary toxin are highlighted, regarding biochemistry, genetics, proteolytic activation, structure, and function, as well as their applications in basic science and medicine.

BACTERIA: A RICH SOURCE OF BINARY TOXINS

The *Bacillus* and *Clostridium* genera represent ubiquitous bacilli commonly found throughout the world in soil, water, and gastrointestinal tracts of animals as well as humans. From an evolutionary perspective, the anaerobic clostridia (or their archaic relatives) probably represent some of the first bacteria on Earth, with perhaps closely related aerobic bacilli evolving thereafter during the genesis of an oxygenated atmosphere (127). Both genera grow in low-oxygen environments; however, the clostridia are better adapted for anaerobic life, with

TABLE 2. Examples of bacterial toxins produced as preformed "A-B" structures found in solution

Bacterium	Toxin (reference)	Structure (mol mass [kDa]/A:B ratio)
Clostridium botulinum	Botulinum neurotoxins A–G (332a)	Single protein (150/1:1)
Clostridium difficile	Toxins A and B (434a)	Single protein (~270–308/1:*X*)[a]
Clostridium novyi	Alpha toxin (182a)	Single protein (250/1:*X*)[a]
Clostridium sordellii	Hemorrhagic and lethal toxins (252a)	Single protein (~260–300/1:*X*)[a]
Clostridium tetani	Tetanus neurotoxin (332a)	Single protein (150/1:1)
Corynebacterium diphtheriae	Diphtheria toxin (330a)	Single protein (58/1:1)
Pseudomonas aeruginosa	Exotoxin A (330a)	Single protein (67/1:1)
Bordetella pertussis	Pertussis toxin (246a)	Multiple proteins (105/1:4)
Escherichia coli	Heat-labile enterotoxin (181)	Multiple proteins (85/1:5)
Shigella dysenteriae and other *Enterobacteriaceae*	Shiga and Shiga-like toxins (2)	Multiple proteins (~70/1:5)
Vibrio cholerae	Cholera toxin (181)	Multiple proteins (85/1:5)

[a] Repetitive "B" oligopeptide domains of different lengths are evident within the C terminus of the large clostridial toxins produced by *C. difficile*, *C. novyi*, and *C. sordellii*.

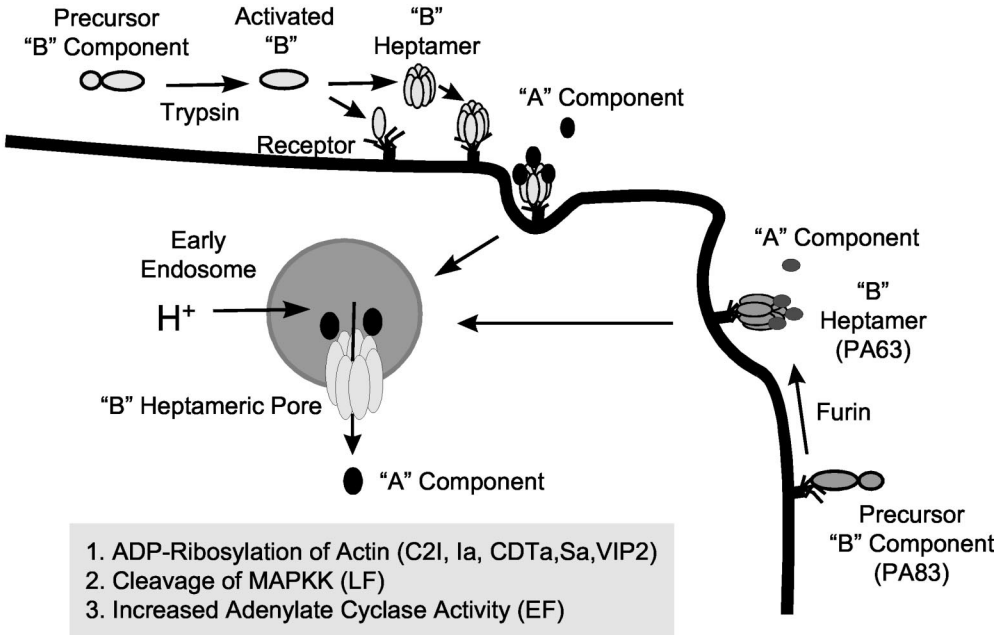


FIG. 1. Basic mechanisms of cell intoxication employed by *Clostridium* and *Bacillus* binary toxins. Cell-binding "B" precursors are first activated by proteolytic cleavage in solution or on the cell surface. The furin-based, cell-associated cleavage of *B. anthracis* PA83 is unique, since none of the other "B" precursors are activated after binding to a cell. Activated "B" components interact with a specific cell surface receptor(s) as either preformed, ring-shaped homoheptamers or monomers that subsequently form heptamers. An enzymatic "A" component(s) docks with the cell-bound "B" heptamer, and the receptor-holotoxin complex is then taken up via receptor-mediated endocytosis into early endosomes, which become acidified by vacuolar-type ATPases. An acidic environment is essential for translocating an "A" component(s) into the cytosol, since this induces a conformational change and subsequent insertion of the "B" heptamer into an endosomal membrane to form a channel. Although not clearly defined, it is likely that an "A" component(s) is transported into the cytosol through the "B" heptamer-induced channel.

various aerotolerance levels among different species. The recent sequencing of *B. anthracis* (349), *B. cereus* (186, 347a), and *C. perfringens* (385) genomes, as well as the deciphering of the toxin-encoding plasmid (pXO1) from *B. anthracis* (315), will inevitably reveal additional similarities between these, and other, microorganisms.

Compared to other bacteria, *Clostridium* and *Bacillus* species have developed unique mechanisms for survival within and outside of numerous host types, as evidenced by the various diseases frequently linked to their protein toxins and spores. *C. botulinum*, *C. difficile*, *C. perfringens*, and *C. spiroforme* are associated with a plethora of animal and human diseases and intoxications such as gas gangrene, food poisoning, antibiotic-associated diarrhea, pseudomembranous colitis, and/or enterotoxemia (52, 258, 411, 423). Maladies attributed to *B. anthracis* or *B. cereus* also occur in animals and/or humans, and they respectively include three forms of anthrax (cutaneous, intestinal, and inhalational) (38, 132, 172) and food poisoning (259). As described below, the binary toxins produced by *Clostridium* and *Bacillus* species are involved in diverse diseases, and this further accentuates the differences that also exist within this toxin family.

C. perfringens Iota Toxin

C. perfringens, also known as *Bacillus aerogenes capsulatus* or *Clostridium welchii* in older literature, was first discovered in 1892 by William Welch et al. and consists of five serotypes (A to E) classically based on the production of four lethal, dermonecrotic toxins (α, β, ε, and ι) neutralized by type-specific antiserum in mouse lethality and guinea pig dermonecrotic assays (182b, 258, 295, 457). Today, rapid genetic methods involving multiplex PCRs are more commonly used by many diagnostic laboratories for toxin typing of *C. perfringens* isolates (95, 96, 119, 150, 260, 412, 441, 480, 484). The binary ι toxin is produced exclusively by type E strains, implicated in sporadic diarrheic outbreaks among calves as well as lambs, and interestingly linked to a highly conserved, yet silent, enterotoxin gene localized on the same plasmid (45, 54, 178). Although *C. perfringens* ι toxin was initially described in 1940 by Bosworth (54), its binary nature was first elucidated in the mid-1980s by exploiting the fortuitous cross-reaction and neutralization of ι toxin with *C. spiroforme* antiserum (417, 421, 422). The two proteins that comprise ι toxin were designated iota a or Ia (slower moving) and iota b or Ib (faster moving), based on electrophoretic mobility in crossed immunoelectrophoresis. Ia and Ib are both nontoxic, as is the case for the individual components of all the *Clostridium* and *Bacillus* binary toxins described in this review, but when combined, they form a potent cytotoxin that is lethal to mice and dermonecrotic in guinea pigs (417, 421, 422). Later studies revealed that Ia is an ADP-ribosyltransferase (393) specific for actin (372) while Ib, which lacks any discernible enzymatic activity, binds to a cell surface protein and subse-

quently translocates Ia into the cytosol of a targeted cell (49, 356, 419).

C. spiroforme Toxin

Similar to the classic rod-shaped *C. perfringens* and enterically acting ι toxin, the distinctly coiled *C. spiroforme* also causes diarrheic deaths that are spontaneous or antibiotic induced in rabbits (52, 53, 70–73, 75, 183, 319, 320, 483), and perhaps in humans (25), via a binary ι-like toxin referred to as CST. Lagomorphs are most susceptible to *C. spiroforme*-induced diarrhea during stressful periods that include lactation, old age, weaning, and an altered diet (72). The Sa and Sb components of CST are respectively analogous to Ia and Ib of *C. perfringens* ι toxin, as first determined by crossed immunoelectrophoresis and neutralization studies with *C. perfringens* type E antiserum (52, 332, 333, 417). During the late 1970s, it was erroneously thought that *C. perfringens* type E represented the causative agent of various diarrheic outbreaks within rabbit colonies, since type E antiserum neutralized the in vitro cytotoxic effects of cecal contents from enterotoxemic animals (68, 70, 108, 202, 226, 319). However, *C. perfringens* type E was never isolated, and the real breakthrough came in 1982 when Carman and Borriello revealed a strong correlation between disease in rabbits and isolation of *C. spiroforme* (70), an organism not commonly associated with the normal intestinal flora (72). Overall, compared to the other bacteria and toxins portrayed in this review, *C. spiroforme* and particularly CST have received minimal attention and thus perhaps represent an area for future studies.

C. difficile Toxin

Closely related to the ι toxin and CST is the ι-like toxin produced by *C. difficile* (324, 334), consisting of CDTa and CDTb components that respectively share 80 and 82% amino acid sequence identity to *C. perfringens* Ia and Ib. In the United Kingdom and the United States, only 6 and 16% of all *C. difficile* isolates from hospitals and patients, respectively, contain both CDTa and CDTb genes (146a, 426), perhaps suggesting that this binary toxin is not essential for eliciting *C. difficile*-associated colitis, which is most commonly attributed to higher-molecular-weight proteins designated toxin A and toxin B (423). The protein components of CDT, CST, and ι toxin are interchangeable, thus generating biologically active chimeras that demonstrate conserved functionality among these clostridial species (166, 325, 333, 334, 417). Interestingly *C. difficile*, *C. perfringens*, and *C. spiroforme* are all associated with gastrointestinal diseases in humans as well as animals (52, 54, 63, 423), and the synthesis of common binary toxins with interchangeable protein components probably reveals a shared evolutionary path for these ubiquitous pathogens in a common niche.

C. botulinum C2 Toxin

C. botulinum, initially identified as *Bacillus botulinus*, was first described in 1895 by Emile van Ermengem following a food poisoning incident in Ellezelles, Belgium (97). Like *C. perfringens*, the neurotoxin types (A to G) of *C. botulinum* are classically determined by mouse lethality assays with toxin-specific antisera (182b, 389). However, the binary C2 enterotoxin produced by toxin types C and D is devoid of neurotoxicity but implicated in a fatal enteric disease of waterfowl, is cytopathic for many different cell types, and induces vascular permeability, necrotic-hemorrhagic lesions, and a lethal fluid accumulation in the lungs and intestinal tracts of various animals (113, 189, 191, 221, 276, 297, 298, 300, 302, 303, 305–308, 390). C2 toxin is synthesized by *C. botulinum* during sporulation (289) and incorporated into the spore coat (481), which is akin to the single-chain enterotoxin of *C. perfringens* or protective antigen from *B. anthracis* edema and lethal toxins (93, 130, 364, 371, 465, 466). Much of the pioneering work on characterizing the cell binding and translocation component (C2II), as well as the enzyme component (C2I), of C2 toxin was initiated by Ohishi and coworkers throughout the 1980s. Ohishi's laboratory observed, among many things, that (i) the biological effects of C2 toxin were the synergistic result of C2I plus C2II (297, 298) and (ii) C2II requires trypsin activation (designated C2IIa after proteolysis) for biological activity (299, 304, 309, 311). Simpson discovered in 1989 that C2IIa binding and subsequent entry of C2I into a targeted cell occurs by receptor-mediated endocytosis (392).

There are intriguing physical (molecular weight and epitopes) as well as functional (cytotoxicity) variations between C2I and C2II components produced by different *C. botulinum* strains (301, 307), which perhaps is not surprising from an evolutionary perspective. Similar structural and functional data are unfortunately lacking for the protein components of other *Clostridium* and *Bacillus* binary toxins, with one notable exception being the highly conserved protective antigen from *B. anthracis* (342). Finally, based on earlier reports that C2I possesses ADP-ribosyltransferase activity specific for arginine (391), the intracellular substrate of C2 toxin was subsequently identified in 1986 as actin (7, 310) and thus represents the discovery of a new family of bacterial ADP-ribosylating proteins that target the cytoskeleton.

B. anthracis Edema and Lethal Toxins, and *B. cereus* Vegetative Insecticidal Proteins

In contrast to *C. botulinum* or *C. perfringens*, phylogenetic relatives such as *B. anthracis* and *B. cereus* are not composed of serologically distinguishable toxin types, but they do produce binary toxins respectively involved in animal or human anthrax and insecticidal effects (98, 132, 171, 230, 259). Overall, there is remarkably limited variation among *B. anthracis* isolates throughout the world (203), and by some accounts *B. anthracis* is merely a genetic variant of *B. cereus* and *Bacillus thuringiensis*, possessing plasmids that encode unique toxins and a capsule (180, 186, 214, 315, 347a, 349). The natural disease elicited by *B. anthracis* occurs mainly in herbivores following spore transmission via ingestion, inhalation, an open skin wound, or even biting flies, but its nefarious use as a biological weapon was first associated with espionage during World War I (439). Subsequent military events before, during, and after World War II led to more vigorous research efforts by various countries, the discovery of promising therapies as well as vaccines, and a more comprehensive understanding of anthrax patho-

genesis particularly linked to the respiratory route and human disease (439).

The binary toxins produced by *B. anthracis* were the first multicomponent bacterial toxins ever described in the literature (408), and they consists of three synergistically acting proteins (413) now known as edema factor (EF), lethal factor (LF), and protective antigen (PA) (407). From a historical perspective, this discovery is quite fitting, since Robert Koch and Louis Pasteur initially used *B. anthracis* to prove profound scientific concepts involved in disease (Koch's Postulates in 1876) and immunology (Pasteur's vaccine studies in 1881). The PA molecule combines with EF and/or LF on the cell surface to respectively form edema and/or lethal toxins, which represent virulence factors that work synergistically toward bacterial survival and dissemination (77, 172, 204, 230, 279, 296, 321, 327, 335, 336). Unlike the protein components of CDT, CST, and ι toxin, those of C2 as well as the edema and lethal toxins do not form biologically active chimeras with other binary toxins (325, 418).

Relative to any other *Clostridium* or *Bacillus* binary toxin described in this review, much less information is available for the newly (1990s) discovered *B. cereus* VIP, composed of VIP1, a cell-binding component, and VIP2, an ADP-ribosyltransferase that targets actin (171). In addition to its insect-killing properties on Northern and Western corn rootworms via VIP, *B. cereus* is involved in human food poisoning (158a) and is considered a nonlethal intestinal symbiont of various soil-dwelling insects such as roaches, sow bugs, and termites that, when foraging, inadvertently ingest *Bacillus* as well as *Clostridium* spores (251). Future studies with specific antibodies and gene probes for the various components of binary toxins described in this review will probably yield new toxins produced by different species of *Bacillus*, *Clostridium*, and perhaps other bacterial genera.

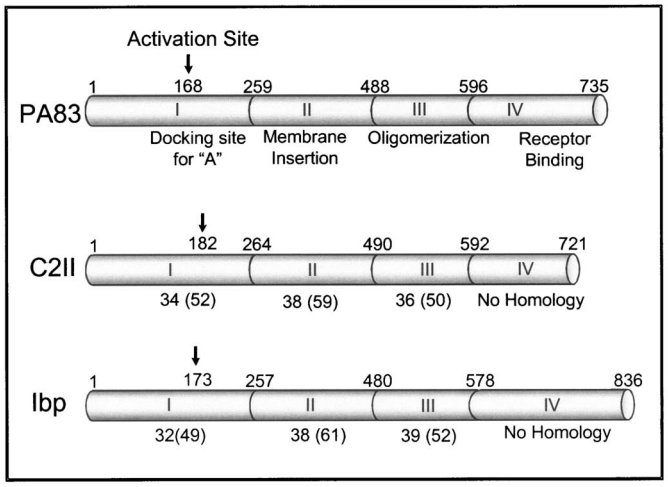
BIOCHEMISTRY, GENETICS, AND PROTEOLYTIC ACTIVATION

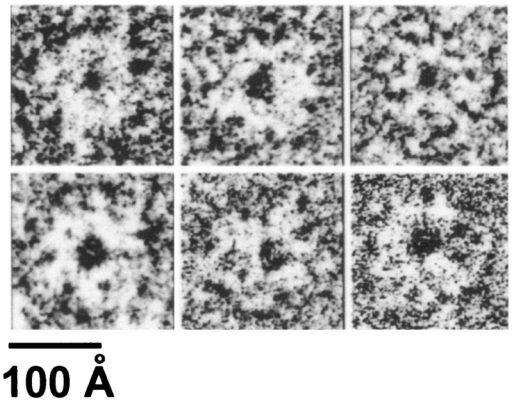
The protein components of *C. botulinum* C2 toxin (302), *C. difficile* CDT (324), *C. perfringens* ι toxin (422), *C. spiroforme* CST (333), *B. anthracis* edema and lethal toxins (408, 413), and *B. cereus* VIP (171) are produced as separate "A" and "B" molecules not associated in solution. Table 1 lists the gene locations, molecular weights, and enzymatic activities of these bacterial binary toxins. The isoelectric points of mature "A" components range from 5.2 (*C. perfringens* Ia) to a distinctly high 9.3 (*C. difficile* CDTa), while the "B" component range encompasses pH 4.5 (*C. difficile* CDTb) to 6.2 (*C. botulinum* C2II) (331). The cell-binding components are enzymatically inert (as ascertained by existing assays) and produced by the bacterium as precursor molecules, with isoelectric point shifts of less than 0.8 pH unit following activation by various serine-type proteases such as chymotrypsin, furin, or trypsin derived from the bacterium, host, or exogenous addition in vitro (122, 211, 325, 417). The resultant loss of an N-terminal peptide (~20 kDa) from a "B" precursor induces conformational changes that facilitate homoheptamerization, either in solution or on the cell surface, and subsequent docking with an "A" component(s). To further understand the various mechanisms for proteolytic activation of bacterial toxins, we recommend the comprehensive review by Gordon and Leppla (156).

Like other multicomponent toxins, the enzymatic and binding proteins of all *Clostridium* and *Bacillus* binary toxins are encoded by distinct genes that have a 27 to 31% G+C content (331). As an example, the genes for *B. anthracis* EF, LF, and PA are located on a large (182-kbp) plasmid called pXO1 (271, 312, 315) and span a 23-kbp region (230). Among the clostridia, "A"- and "B"-component genes are transcribed in the same orientation from a common operon consisting of an "A" gene located 40 to 50 nucleotides upstream of the "B" gene, with a known exception being the open reading frames for C2 toxin, which are separated by 247 nucleotides (137, 147, 208, 323, 331). There is also another significant difference at the genetic level since *C. botulinum* C2 toxin, *C. difficile* CDT, and *C. spiroforme* CST are chromosome encoded, in contrast to the plasmid-localized *C. perfringens* ι toxin. Each "A" and "B" component of the *Clostridium* and *Bacillus* binary toxins, but not those from C2, is respectively synthesized with a signal peptide consisting of 29 to 49 and 39 to 47 residues (331). These findings are consistent with proteins secreted during logarithmic growth; however, the C2 toxin is evident from sporulating *C. botulinum* only after sporangium lysis (289, 331). It was recently shown that an extracellular chaperone protein, PrsA, is important for efficient folding and secretion of PA83 from *Bacillus* species via a Sec-dependent route (477), but it is unknown if similar chaperones play any role with binary toxin components produced by the other bacilli.

Production of *B. anthracis* toxin is controlled by the positive regulatory gene *atxA*, also located on pXO1, via protein binding ~110 bp upstream of the ATG start codon, and its importance in pathogenesis is further evidenced by the observation that less virulent strains lack the *atxA* gene (94, 214). The 56-kDa protein encoded by *atxA* is unique, with little sequence homology to other known transcriptional activators (94). In addition to the genes for edema and lethal toxins, *atxA* regulates the expression of others on pXO1 (182), the pXO2 plasmid (93 kbp) that encodes the capsule (165), and the chromosome (56, 270). Bicarbonate, carbon dioxide, and temperature also represent environmental factors that regulate the synthesis of *B. anthracis* edema and lethal toxins, as well as capsule (214, 230, 270). Gene products or environmental factors that may affect the production of other bacterial binary toxins described in this review are currently unknown, although divalent cations seemingly play a role in CST synthesis via an undefined mechanism (74).

Comparisons of amino acid sequences among the *Clostridium* and *Bacillus* binary-toxin components reveal similar evolutionary paths, since they share (i) 80 to 85% identity within the ι-toxin family, which includes CDT, CST, and ι toxin, but the signal peptide sequences are less highly conserved (40 to 61% identity); (ii) 31 to 40% identity between C2 and ι-family toxins; (iii) 26 to 30% identity between PA and clostridial "B" components; and (iv) 29 to 31% identity between VIP and equivalent clostridial toxin components, which overall suggests that these toxin genes were derived from a common ancestor. Clearly, the "B" components are structurally conserved between *Clostridium* and *Bacillus* binary toxins (Fig. 2A), and over time these proteins have adapted to transporting unique





% Identity of Precursor "B" Proteins

	C2II	Iota b	CDT b	S b	PA
C2II	100	37	38	36	26
Iota b	37	100	81	82	28
CDT b	38	81	100	78	28
S b	36	82	78	100	28
PA	26	28	28	28	100

% Identity of Activated "B" Proteins

	C2II	Iota b	CDT b	S b	PA
C2II	100	38	40	38	27
Iota b	38	100	82	84	30
CDT b	40	82	100	81	30
S b	38	84	81	100	29
PA	27	30	30	29	100

% Identity of Cleaved Peptides

	C2II	Iota b	CDT b	S b	PA
C2II	100	36	36	34	24
Iota b	36	100	74	73	22
CDT b	36	74	100	67	18
S b	34	73	67	100	20
PA	24	22	18	20	100

FIG. 2. The cell-binding "B" proteins of *Clostridium* and *Bacillus* binary toxins are activated by serine-type proteases, share varying sequence homology, and form heptameric ring-like structures. (A) Proteolytic cleavage sites, domain functions, and amino acid lengths of PA83, C2II, and Ibp precursor molecules are shown on the left. Below C2II and Ibp are the percent identities (and in parentheses are the percent homologies) for the amino acid sequences from each domain relative to PA83. Percent sequence identities of the "B" precursors (top), activated "B" proteins (middle), and N-terminal peptides released after proteolysis of "B" precursors (bottom) are shown on the right. Sequences were found in either the DNA Data Bank of Japan (DDBJ) with accession number D88982 (C2II) or GenBank with accession numbers M22589 (PA), I40862 (Ib), X97969 (Sb), and L76081 (CDTb). Modified from reference 31 with permission. (B) C2IIa heptamers on lipid bilayers as detected by electron microscopy. Modified from reference 32 with permission.

"A" components into cells. For example, there are striking differences between "A" components such as *B. anthracis* EF (an adenylcyclase) and LF (a metalloprotease), which are quite distinct structurally and enzymatically from the ADP-ribosyltransferases within the C2 or ι toxin families. Although unproven, it is very plausible that the binary toxin genes originated from an ancestral form of clostridia and were horizontally transferred between *Bacillus* and *Clostridium* species via plasmids capable of inserting into the bacterial chromosome, as perhaps evidenced by the CDT, CST, and C2 toxin genes. However, with one known exception found in a *C. spiroforme* strain, insertion sequences do not commonly flank these toxin genes. This omission may indicate that genetic rearrangements and/or deletions occurred after successful transfer (331).

B. anthracis PA83

The PA protein of *B. anthracis* edema and lethal toxins is unique and perhaps more versatile than other "B" components within the binary toxin family. For example, the PA precursor (PA83) is proteolytically activated by several routes into PA63 and these include (i) trypsin in buffer (294), (ii) an unidentified protease(s) in serum (66, 118), and (iii) ubiquitous cell surface proteases (furin or furin-like) which recognize a consensus sequence ($_{164}$RKKR$_{167}$) not present in the binding components from other binary toxins lacking any recognized mechanisms for cell surface activation (155, 211, 230). However, *C. spiroforme* Sb does have an RSAR site located 208 residues from the N terminus that perhaps reflects an evolutionary remnant of a furin cleavage site (147). Furin activation of bacterial toxins is rather common, as evidenced by the single-

chain ADP-ribosyltransferases such as *Corynebacterium diphtheriae* diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A, which enter cells by receptor-mediated endocytosis and subsequently inhibit protein synthesis (155, 156, 447).

Activation of PA83 on the cell surface is quite robust, since proteolysis occurs at 4°C or on chemically fixed cells (230). Studies with a furin-resistant variant of PA83 reveal that this molecule is not readily internalized into cells, suggesting that surface-associated PA83 is not "wasted" (i.e., internalized prior to proteolytic activation, oligomerization, and transport of EF and/or LF into the cytosol) (40). Additionally, the 20-kDa peptide generated after proteolysis of the PA83 precursor slows PA63 clearance from the cell surface (40), perhaps by preventing heptamer formation, localization into lipid rafts (1), and ultimately endocytosis, which further optimizes EF and LF docking opportunities. The 20-kDa peptide also does not form membrane channels like PA63 (215). To our knowledge, similar work with the 20-kDa precursor peptides from other binary toxins described in this review has not been reported in the literature. Following proteolysis, whether in solution or on the cell surface, PA63 readily assembles into sodium dodecyl sulfate (SDS)-resistant, hydrophobic homoheptamers that form pH-dependent (pH $\leq$7), ion-permeable channels in membranes obstructed by known channel blockers (39, 46, 245, 272, 274, 275, 294, 326). X-ray crystallography of the PA83 monomer reveals four distinct domains, and the PA63 heptamer, as first demonstrated by electron microscopy in 1994 by Milne et al. (275), forms a ringed structure 160 Å in diameter and 85 Å in length, with a central lumen diameter of 35 Å (326).

Another unique aspect of PA versus the cell-binding components of other binary toxins is that the PA63 heptamer provides a cell surface docking site for two different proteins, EF and LF (281, 282). Both EF and LF possess a common, N-terminal heptapeptide (VYYEIGK) that is integral for competitive docking interactions with PA63 (168, 219, 224, 229). This sequence does not appear in the enzymatic components of other binary toxins, further supporting previous experimental data showing that PA does not bind or internalize heterologous "A" molecules (325). In fact, the only known complementation of heterologous components that exists within the binary-toxin family occurs among the enterically acting *C. difficile* CDT, *C. perfringens* ι toxin, and *C. spiroforme* CST (324, 393, 417, 418).

C. botulinum C2II

Trypsin activation of the 81-kDa C2II precursor into C2IIa (60 kDa) occurs between K181 and A182 (48), generating stable C2IIa homoheptamers in solution (32). The C2IIa complex mediates biological effects on cells, in conjunction with an ADP-ribosyltransferase (C2I), that involve the formation of ion-permeable channels in lipid membranes (373). Although C2II precursor binds to cells, it is not readily activated by cell surface proteases like PA83 and it does not dock with C2I or facilitate cytotoxicity (276, 311). X-ray crystallography of C2II has not been reported to date, but electron microscopy of C2IIa oligomers on lipid bilayers reveals annular heptameric structures with an inner diameter of 20 to 40 Å and an outer diameter of 110 to 130 Å (Fig. 2B) (32). As described below, N-terminal residues 1 to 87 of C2I are intimately involved in docking with heptameric C2II on the cell surface (37).

C. perfringens Ibp and Ia

Helen Ross and colleagues first reported in 1949 that ι toxin requires proteolytic activation (362); however, it took 35 more years before additional clues revealed that the Ib precursor (designated Ibp) was the likely target (417). Following the separation of Ia and Ibp from early-log-phase (<10-h) cultures of *C. perfringens* type E, as done by DEAE ion-exchange chromatography, trypsin proteolysis of fractions containing Ibp or Ia markedly (i) increases enzyme-linked immunosorbent assay (ELISA) readings for Ibp, but not Ia, versus the same untreated fractions, thus suggesting a conformational shift in Ibp that unveils cryptic epitopes recognized by Ib-specific antibodies; and (ii) increases the guinea pig dermonecrotic activity of the Ibp fraction when combined with untreated Ia. It was subsequently discovered, after cloning and sequencing of the ι-toxin gene, that proteolytic activation of Ibp into Ib occurs at A211 (323), which then facilitates Ia docking (419), formation of voltage-dependent ion-permeable channels in membranes (213), and formation of SDS-stable heptamers on cell membranes (288, 420) and in solution (49, 288). However, Ib oligomers formed in solution are seemingly less stable and do not promote cytotoxicity compared to solution-generated oligomers of PA63 or C2IIa. Additionally, Ib heptamers generated in solution versus on the cell membrane do not induce K^+ release and are efficiently digested by pronase after binding to Vero cells at 37°C (288). Like C2II, which also lacks a furin cleavage site, Vero cell-bound Ibp is not activated over time (148) or with an excess of trypsin or chymotrypsin (420). To date, extensive proteolytic activation studies similar to those for C2II, Ib, and PA have not been conducted with *B. cereus* VIP1, *C. difficile* CDTb, or *C. spiroforme* Sb.

Other proteases such as pepsin, proteinase K, subtilisin, and thermolysin activate Ibp more efficiently in solution than trypsin does, and as more recently discovered, Ia is also proteolytically activated by these enzymes, with a resultant loss of 9 to 13 amino acids from the N terminus (148). It is still uncertain whether proteolysis of Ia increases (i) efficiency of docking to cell-bound Ib, (ii) efficiency of translocation into cells, and/or (iii) ADP-ribosyltransferase activity. Proteolytic activation of Ia is unique among the "A" components from binary toxins; however, upon further examination, they too may possess similar proteolysis patterns.

It is noteworthy that among another family of "A-B" toxins composed of heterologous proteins that form holotoxins in solution, like *Escherichia coli* heat-labile, *Shigella dysenteriae* Shiga, and *Vibrio cholerae* cholera enterotoxins, the enzymatic "A" components are also processed by serine-type proteases. However, following proteolysis, these "A" components form A_1 and A_2 subunits linked by a disulfide bond subsequently reduced by protein disulfide isomerase located in the endoplasmic reticulum (2, 140, 141, 181, 228, 250).

PROTEIN STRUCTURE AND FUNCTION

B. anthracis PA, LF, and EF

X-ray analysis of PA (326), LF (314), and EF (103) crystal structures has provided invaluable information, as described below, and these molecules naturally represent "templates" for the components of other *Clostridium* and *Bacillus* binary toxins not crystallized to date. The PA protein contains four distinct domains that are seemingly applicable for the "B" components (C2II, CDTb, Ib, Sb, and VIP1) of other binary toxins described in this review (Fig. 2A). Various studies reveal that domains 1 (N terminus), 2, 3, and 4 (C terminus) are respectively involved in docking to an enzyme component(s), channel formation in lipid membranes, oligomerization, and binding to a specific cell surface receptor(s). For instance, domains 1 (amino acids 1 to 259) and 4 (amino acids 597 to 735) of PA83 are respectively responsible for (i) docking with EF and/or LF and (ii) binding to the cell surface via protein receptors recently identified as variants 1 and 2 of tumor endothelium marker 8, as well as human capillary morphogenesis protein 2 (60, 64, 80, 86, 92, 243, 245, 326, 361, 379, 401, 449). There is relatively little amino acid homology of PA domains 1 and 4 to their equivalents from other *Clostridium* and *Bacillus* binary toxins, which is not surprising since these regions respectively afford a unique docking site for distinct "A" components and receptor-binding specificity (33, 48, 252, 325, 418). Domain 1 contains the proteolytic cleavage site (R_{167}) that subsequently triggers the release of a 20-kDa precursor peptide and formation of PA63 heptamers (211). The remaining segment of domain 1 (designated 1′ and consisting of residues 168 to 259) faces the channel lumen, unlike the peripherally located domain 4 (326), and is associated with two Ca^{2+} molecules (coordinated via residues D_{177} or D_{235} and D_{179}, D_{181}, E_{188}) that evidently preserve a PA63 structure necessary for proper folding, resistance to proteolytic degradation, heptamer formation, and docking with EF or LF (138, 166a). Currently, no one has reported an association of Ca^{2+} with the "B" components from other binary toxins; however, concerted investigations have not been conducted evidently. Although their role is still unclear, various divalent cations (Ca^{2+}, Co^{2+}, and/or Zn^{2+}) are required for Sa and Sb production in vitro by *C. spiroforme* (74).

Within domain 1′, residues R_{178}, K_{197}, R_{200}, F_{202}, L_{203}, P_{205}, I_{207}, I_{210}, and K_{214} have been identified by two different groups as being critical for the docking of EF and LF (80, 92). The maximum number of EF or LF molecules interacting with a PA63 heptamer has been controversial, since estimates range from seven (400) via nondenaturing gel electrophoresis to a more convincing three (92, 281, 282) when determined by multiple methods that include gel filtration chromatography, multiangle laser light scattering, and analysis of oligomer-deficient variants of PA63. Similar stoichiometric analysis of A-B interactions has not been done with the other binary toxins. Additionally, it is still not known if all enzyme molecules docked with a PA63 heptamer are efficiently translocated into the cytosol. It is possible that translocation of an EF or LF molecule induces conformational changes in the PA63 heptamer that either facilitate, or perhaps even inhibit, the subsequent passage of other "A" molecules.

The importance of domain 4, and particularly residues 671 to 721, in PA binding to cells was first described by Little et al. on the basis of epitope mapping with monoclonal antibodies (240, 242, 243). Subsequent mutagenesis efforts by other groups show that Y_{681}, N_{682}, D_{683}, and P_{686} represent key residues critical for PA binding to its receptor (61, 361, 449). Further evidence that an exposed loop (residues 703 to 722) is important for PA binding to cells was obtained by Brossier et al. (64) via deletions of 9 or 16 amino acids from this region. Other studies of domain 4 reveal that truncations of only 5 to 12 amino acids from the far C terminus of PA prevent binding to cells (401, 449), suggesting an important role in direct binding and/or conformational integrity of PA (230). Similar investigations with C2II and Ib also unveil a pattern of exquisite sensitivity regarding deletions within the C terminus and subsequent effects on biological activity (48, 252), thus demonstrating a conserved structural trait among "B" components from this binary-toxin family.

A comparison of amino acid sequences between PA, C2II, and Ib reveals 27 to 38% identity; localized primarily within central domains 2 (amino acids 259 to 487) and 3 (amino acids 488 and 596) (Fig. 2A), that respectively participate in channel formation/enzyme translocation and oligomerization of PA63 monomers (43, 46, 47, 205, 206, 208, 213, 283, 324, 326, 373, 381, 382). As previously described, PA83 is readily converted into PA63 and homoheptamers by serine-type proteases in solution or on cell membranes (66, 118, 245, 272, 275, 294, 326). After proteolysis at pH <7, domain 2 undergoes a conformational shift that stabilizes a PA63 heptamer which binds to cells and docks with the enzyme but is translocation defective (272). However, if PA63 heptamers are proteolytically generated and maintained at pH >8, they are less stable (as evidenced by their SDS solubility at room temperature) but represent a biologically active "prepore" that binds to cells, docks with EF/LF, and subsequently translocates EF/LF into the cytosol following endosomal acidification (272). Domain 2 contains a "Greek key" motif (residues 262 to 368) that unfolds to form a β-hairpin amphipathic loop (residues 302 to 325) which inserts into the membrane (357), thus promoting an acid-driven prepore-to-pore conversion (43, 167, 290). Although PA63 has little sequence homology to the alpha-hemolysin of *Staphylococcus aureus*, there are striking similarities in how these heptameric, pore-forming proteins produced by quite different bacteria insert into membranes (410). Further investigations of domain 2 identify PA residues D_{425} and F_{427}, which are conserved in C2II, CDTb, Ib, and Sb (147, 208, 323, 324), as critical for channel formation as well as translocation (381, 382). Another group has shown that alanine mutations of residues W_{346}, M_{350}, and L_{352} within domain 2 result in a PA63 heptamer unable to facilitate LF-induced cytotoxicity, perhaps because of dysfunctional membrane insertion and/or enzyme translocation (39). Analysis of PA63 crystals produced at pH 6 and 7.5 shows that residues 342 to 355 become exposed at the lower pH (326), thus promoting a more hydrophobic state and oligomerization (39, 215, 275). It is also within domains 2 and 3 that alanine mutations of Q_{277} (buried) and F_{554} (surface exposed) respectively increase the thermostability of wild-type PA, which might be useful in improving vaccine stability (396). Additional mutagenesis studies of a surface-exposed, hydrophobic "patch" in domain 3 reveal that alanine replacement of highly conserved residues F_{552}, F_{554}, I_{562}, L_{566}, or I_{574}, also

evident in the "B" components of other *Clostridium* and *Bacillus* binary toxins, equivocally results in an oligomer-defective molecule of PA63 (5, 206, 283). Except for a recent study by Blöcker et al. with *C. botulinum* C2II (47), there has been very little structure-function analysis within domains 2 and 3 of "B" components from the other binary toxins.

The crystal structure of LF, a Zn^{2+} metalloprotease specific for the N terminus of a conserved family of eukaryotic proteins (MAPKK) involved in cell signal transduction (106, 107, 210, 452, 453), was recently reported by Pannifer et al. (314). Upon entering a cell via PA, where macrophages have been considered the primary target for lethal toxin (131, 174) but recent work suggests that other cell types are adversely affected too (3, 208a, 278), LF binds to various MAPKK on the latter's C-terminal regulatory region and cleaves within a proline-rich, N-terminal site that subsequently inhibits MAPKK interaction with the substrate and enzymatic activity (83, 107, 452). Additionally, cleavage of MAPKK may also result in modification by ubiquitin and rapid degradation by proteasomes (433). However, the role that truncated MAPKK play in toxicity is still uncertain, as evidenced by their cleavage in macrophages resistant to lethal toxin-induced cell death (321, 459). Perhaps susceptibility to lethal toxin is dictated by a kinesin-like motor protein (Kif1C), since polymorphic forms of Kif1C evident in resistant, but not susceptible, murine macrophages result in protection via an unknown mechanism not involving toxin entry or processing (459). A PA-LF combination increases the permeability of macrophage membranes, depletes intracellular ATP levels, and ultimately causes cell lysis, but these effects are all readily prevented by reducing agents, amines, bestatin, monensin, or inhibitors of metallopeptidases and possibly caspases (131, 175, 176, 207, 208a, 268, 316, 335, 336, 433). In addition to the in vitro effects described above, mice devoid of macrophages are resistant to lethal-toxin-induced mortality (174).

Akin to a host's adverse reaction to endotoxin or bacterial superantigens (i.e., *S. aureus* enterotoxins), proinflammatory cytokines may also play a role in *B. anthracis* edema toxin and lethal-toxin activity, but this concept still remains controversial (116, 152a, 173, 174, 184, 197, 207, 217, 218, 269, 278, 321, 335, 336). An apparent augmentation of cytokine-induced damage involves lethal toxin and indirect repression of select nuclear hormone receptors for estrogen, glucocorticoid, and progesterone that normally provide a protective anti-inflammatory response for a host (460). In addition to cytokine-elicited damage by the *B. anthracis* toxins, a cytokine-independent hypoxia induced by lethal toxin causes terminal necrosis of the liver and metaphyseal bone marrow (278, 344). Overall, lethal toxin seemingly represents a defense mechanism employed by the bacterium to weaken the host immune system by eliminating or impairing the immunological responsiveness of major cell types (i.e., macrophages and dendritic cells) naturally involved in pathogen clearance (137a). However, as recently shown by Salles et al. (369), a low percentage of macrophages can adapt to and resist high concentrations of lethal toxin when initially exposed to a lower yet not uniformly lethal dose of toxin in vitro. It will be interesting to see if this phenomenon is also apparent in vivo and perhaps is linked to quorum sensing. Finally, *B. anthracis* spores use macrophages as a germination site (162, 163, 465, 466), and perhaps lethal toxin, as well as other less well defined virulence factors, may facilitate the survival and dissemination of *B. anthracis* throughout the host from this mobile, normally pathogen-hostile environment (97a, 162a, 321). Since the bacterium is not directly transmitted from an infected individual to another possible host, eventual killing of the host and subsequent deposition of spores into the soil logically appear to be important mechanisms for *B. anthracis* survival and dissemination.

Like PA, LF also contains four distinct domains. The N-terminal domain 1 (amino acids 1 to 267) is important for docking to PA (345), blocks PA63-induced channels (487), and shares a multi-α-helix bundle as well as β-sheet structure with domain 4, perhaps reflecting domain duplication (Fig. 3A). Although structural similarities exist between domains 1 and 4, there is very little sequence homology and there are no functional commonalities. As mentioned above, it is within domain 1 that LF and EF both contain a common VYYEIGK sequence important for docking to PA63 heptamers (168, 219). Monoclonal antibodies against LF prevent docking with cell-bound heptamers of PA63 and cross-react with EF, presumably via a shared epitope within domain 1 (241). In addition to the conserved VYYEIGK sequence, residues H_{35}, H_{42}, D_{187}, and F_{190} play an important role in the proper LF conformation necessary for docking to PA63 heptamers (19, 395).

Domain 2 (encompassing amino acids 263 to 297 and 385 to 550) of LF conformationally mimics the C-terminal catalytic domain of VIP2 from *B. cereus*, but a critical glutamic acid necessary for ADP-ribosyltransferase activity (a property lacking in LF) is replaced by lysine. However, there is very little (15%) amino acid sequence identity between domains 2 of VIP2 and LF, perhaps suggesting an evolutionary divergence (maybe convergence?) of similar genes within this family of bacterial binary toxins. Domain 3 (amino acids 303 to 382) forms an α-helical bundle embedded between the second and third helices of domain 2, shares a hydrophobic surface with domain 4, and provides substrate specificity (314). Domain 4 (amino acids 552 to 809) contains the enzymatic active site and an HEXXH motif ($_{686}$HEFGH$_{690}$) common among various Zn^{2+} metalloproteases (210, 345), including the lambda toxin of *C. perfringens* (193), which may activate Ia and/or Ib of ι toxin in vitro and in vivo (148).

The EF molecule, a Ca^{2+}/calmodulin-dependent adenylate cyclase (229) that shares structural homology (24% sequence identity) and epitopes with the *Bordetella pertussis*, but not mammalian, adenylate cyclases (158), has also been crystallized with and without calmodulin (103, 104). The N terminus of EF (or LF) docks with PA via an aforementioned VYYEIGK sequence (219, 223, 239), while residues 291 to 776 are sufficient for catalysis (Fig. 3A) (105). Residues 342 to 358 contain a motif (GXXXXGKT) commonly found in other ATP-binding proteins (136), including the *B. pertussis* adenylate cyclase that has 88% homology with EF in this region of 17 amino acids (223). Intriguingly, *C. difficile* CDTb, *C. perfringens* Ib, and *C. spiroforme* Sb also have a similar ATP-binding motif within the N terminus that appears dysfunctional and unnecessary for the biological activity of these common "B" components (147). The increased levels of intracellular cAMP induced by EF, which can be 1,000-fold higher than basal levels (230, 434), are nonlethal and transient, since the intracellular half-life of EF is ~2 h (229).

As eloquently described over 20 years ago, the activity of EF

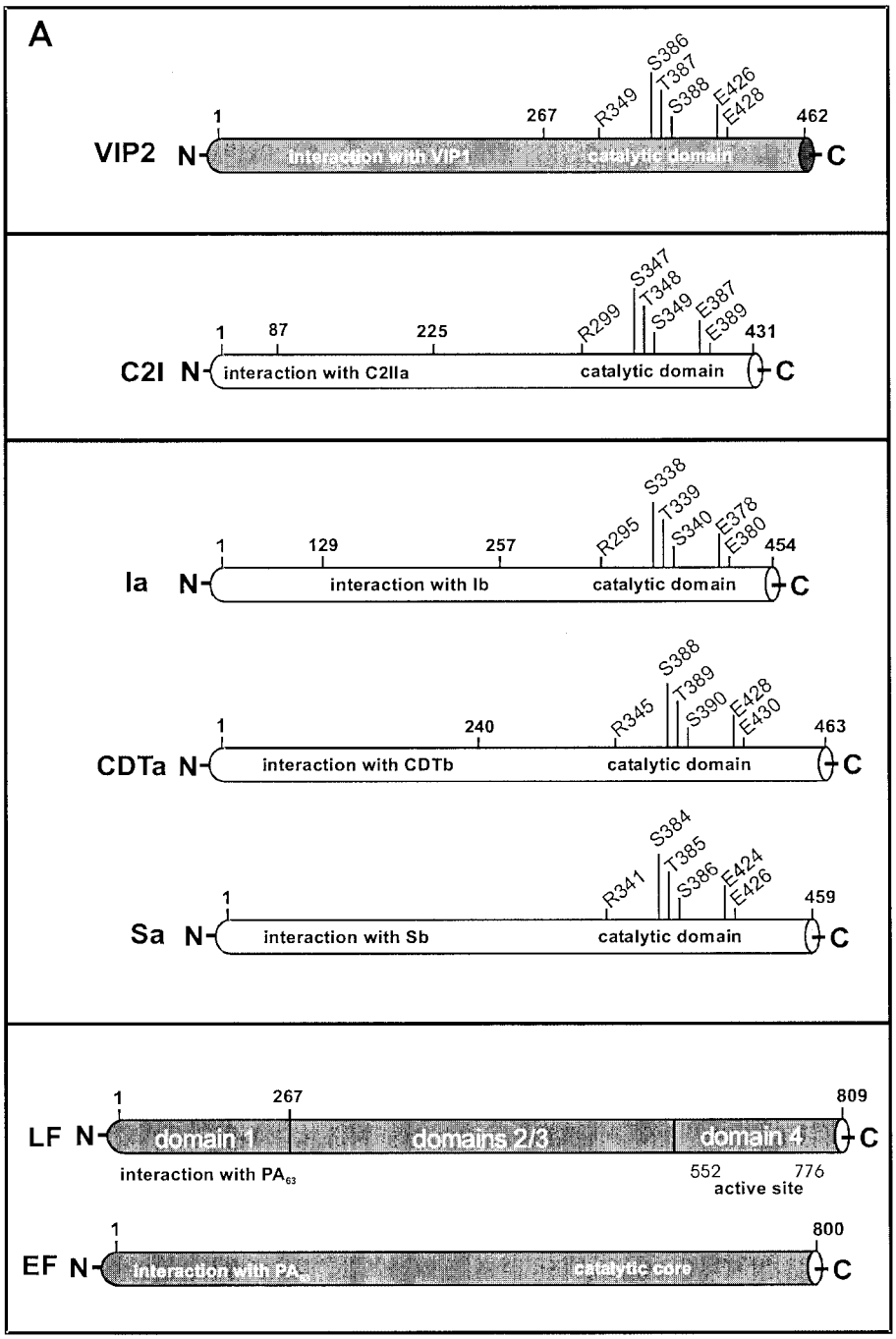


FIG. 3. (A) Enzymatic "A" proteins of *Clostridium* and *Bacillus* binary toxins with known catalytic sites and docking domains for "B" heptamers. (B) ADP-ribosylation of G-actin at R_{177} by C2I, according to the *B. cereus* VIP2 model as proposed by Han et al. (171). In the left-hand panel, the hydrophobic cleft of the C2I catalytic domain is depicted with bound NAD. Amino acids E_{387} to E_{389} stabilize an intermediate state before nucleophilic attack on G-actin R_{177}, thus yielding mono-ADP-ribosylated G-actin and nicotinamide (right-hand panel). The same mechanism is utilized by other ADP-ribosylating toxins (CDT, CST, and ι) that modify G-actin. Panel B modified from reference 31 with permission.

is intimately dependent on calmodulin (229), a conserved Ca^{2+}-sensing protein (16.5 kDa) that is found in all eukaryotic cells and binds to numerous proteins directly or indirectly involved in the cytoskeleton, ion flow, transcription, and vesicular trafficking (104). Calmodulin interactions with EF are dependent on Ca^{2+} levels, which, when too high, inhibit binding to EF and catalysis. Second, hydrophobic and hydrophilic residues within EF regions 501 to 540 (K_{525} in particular) and 616 to 798 induce a conformational shift in EF, after calmodulin binding, which exposes the catalytic core and H_{351} found in three globular domains encompassed by residues 294 to 622 (104, 223, 286, 384). Calmodulin interactions with EF also stabilize EF residues 579 to 591, which may also be important in ATP binding and catalysis. Once EF enters a cell, it triggers

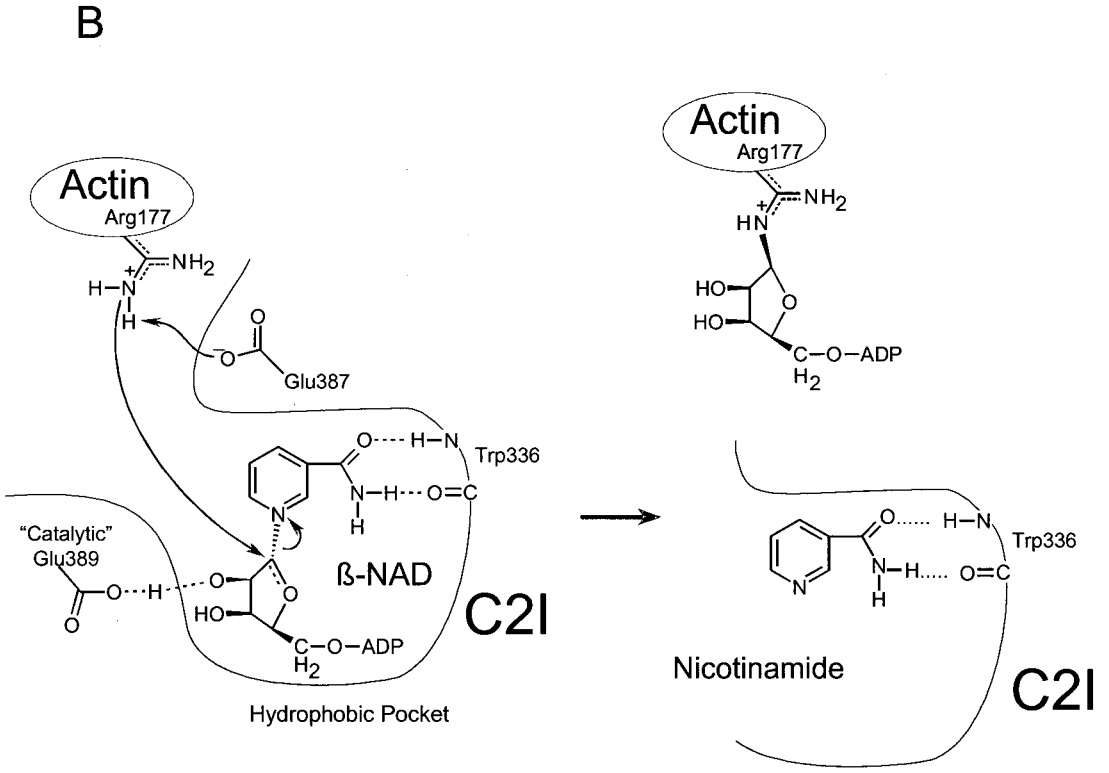


FIG. 3—*Continued.*

Ca^{2+} influx and subsequently elevates intracellular cAMP levels, as demonstrated in various cell types that include leukocytes (220). Increased cAMP levels in leukocytes can profoundly decrease the host immune response (57, 87) by inhibiting lymphocyte proliferation (405), phagocytosis (296), oxidative burst (479), and proinflammatory cytokine release (184); thus, EF seems to be another clever bacterial tool that enables *B. anthracis* to survive and flourish during an infection (220). The effects of EF are no doubt acting in concert with those of LF, the latter affecting MAPKK signaling pathways important in macrophage activation, nitric oxide production, and cytokine release (172, 279, 321, 335, 336). Additionally, the synergy between EF and LF can increase melanogenesis, thus generating perhaps the dark eschar commonly associated with cutaneous anthrax (216). The many complementary and conspiring roles that edema and lethal toxins play in *B. anthracis* survival via host impairment will become even more evident in the future, with time and well-crafted experiments.

C. botulinum C2II and C2I

In various ways, the C2 toxin is structurally similar to *B. anthracis* edema and lethal toxins, with a particular emphasis on the cell-binding proteins, PA and C2II (Fig. 2A). Studies reveal that the C terminus of C2II also facilitates receptor-mediated binding, since deletion of only seven C-terminal residues effectively prevents C2IIa interactions with cells (48). Antiserum specific for the C terminus (domain 4; residues 592 to 721) blocks C2IIa binding to cells as determined by Western blot analysis and cytotoxicity assays, unlike antisera toward domain 1 (residues 1 to 264) or 3 (residues 490 to 592) (48). Domain 4 antiserum neutralizes C2 cytotoxicity in vitro when preincubated with C2IIa, but not after C2IIa has bound to a cell, thus suggesting that neutralizing epitopes are sterically hindered after C2IIa-cell interactions. Deletion studies focused on the N terminus of C2II reveal that this region (residues 1 to 181), lost after proteolytic activation of the C2II precursor, may be important for proper folding of the molecule (48). Recent mutagenesis efforts with C2IIa in a conserved region encompassing amino acids 303 to 331 of domain 2, putatively involved in membrane insertion and channel formation, show that voltage gating but not chloroquine binding or translocation of C2I into the cytosol is lost following an $E_{307}K$ mutation (47).

For the C2I molecule, residues 1 to 87 mediate binding to C2IIa heptamers and translocation across the cytoplasmic membrane (Fig. 3A) (37). Alignment of C2I with VIP2 from *B. cereus* reveals that amino acids 1 to 225 of C2I correspond to the N domain of VIP2 (residues 60 to 275) containing α-helices 1 to 4 (residues 1 to 87 in C2I and 60 to 133 in VIP2). Residues 12 to 29 of C2I are akin to the first α-helical structure encompassing residues 71 to 85 in VIP2, while the other N-terminal helices are also exposed on the protein surface (171). Further analysis of VIP2 crystals shows two structurally homologous domains possessing similar folding patterns, a result most probably generated by gene duplication. X-ray crystallography of other bacterial ADP-ribosyltransferases such as *B. pertussis* pertussis toxin (415), *C. diphtheriae* diphtheria toxin (82), *E. coli* heat-labile enterotoxin (404), *P. aeruginosa* exotoxin A (235), and VIP2 (171) reveals that within the C terminus there are (i) two antiparallel β-sheets flanked by a pair of α-helices and (ii) a highly conserved catalytic domain contain-

ing an $_{387}EXE_{389}$ motif found in prokaryotic as well as eukaryotic ADP-ribosyltransferases (444). However, the sequence homology of these toxins within the C terminus is low. Further studies of the EXE motif of *C. botulinum* C2I show that an $E_{387}Q$ mutation prevents ADP-ribosyltransferase, but not NAD-glycohydrolase, activity while the same alteration of E_{389} inhibits both (36). These results are similar to those derived from mutagenesis of *P. aeruginosa* exoenzyme S, an ADP-ribosyltransferase specific for Ras GTPases (347, 358).

C. perfringens Ib and Ia

Like PA and C2II, the importance of various domains for Ib activity has been ascertained via deletion mutagenesis and antibody studies (252, 420). Similar to PA and C2II, truncation of 10 amino acids from the C terminus (domain 4) abrogates Ib binding to Vero cells, and Ib peptides containing ≥200 C-terminal residues represent competitive inhibitors of ι cytotoxicity in vitro (252). Deletion of 27 N-terminal Ib residues within domain 1′ prevents Ia docking and intoxication, but there is little effect on Ib binding to cells as this truncated domain is an effective competitor of ι toxin (252). In this same study, three monoclonal antibodies against a common N-terminal epitope within residues 28 to 66 had no effect on Ib binding or cytotoxicity. It is possible that these immunoreagents do not occupy the Ib site necessary for Ia docking; alternatively, Ib oligomerization and/or docking of Ia may readily displace these antibodies. In contrast, two monoclonal antibodies that recognize unique Ib epitopes within C-terminal residues 632 to 655 afford protection against ι cytotoxicity via two different mechanisms. One of these antibodies prevents Ib binding to cells, as determined by flow cytometry (252) and Western blot analysis (420), while the other has no effect on Ib binding but efficiently prevents Ib oligomerization on the cell surface. Results for the latter C-terminal binding antibody further demonstrate the importance of Ib oligomerization on biological activity of ι toxin, as do studies with Ibp, a molecule that remains as a cell-bound monomer (419, 420). Similar to the polyclonal antibody studies with domain 4 of C2II (48), monoclonal antibodies that recognize the same domain of Ib do not bind or afford any in vitro protection toward cell-bound Ib and Ia (252, 420). All of the Ib monoclonal antibodies recognize Ibp or *C. spiroforme* Sb in an ELISA and Western blot analysis, but none cross-react with *B. anthracis* PA (252). Surprisingly, C2II is also recognized in an ELISA by one of the monoclonal antibodies that prevent Ib interactions with cells, but it does not neutralize C2 cytotoxicity. Such a finding is intriguing, since C2II and Ib bind unique receptors via their C terminus and share little sequence homology within this epitope (48, 135, 252). To complement the studies done with PA and enhance our understanding of ι toxin (46, 205, 206, 283, 381, 382, 399), future investigations focused on domains 2 and 3 of Ib should be done to more clearly delineate residues required for oligomerization and channel formation.

Although no one has published the crystal structure of Ib, that of Ia has been recently reported by Tsuge et al. (438). Analysis of Ia reveals two domains that have conformational, but little sequence, similarity (Fig. 3A). The catalytic C domains of Ia (residues 211 to 413) and VIP2 (residues 266 to 461) (171) are also quite homologous, with 40% sequence identity and a similar distribution of surface charges. However, one obvious difference between Ia and VIP2 is the spatial orientation of the first glutamic acid found within the conserved catalytic motif, EXE. Like C2I (36), the initial glutamic acid within the $_{378}EXE_{380}$ motif of Ia is important for ADP-ribosyltransferase, but not NAD-glycohydrolase, activity (287). Further analysis of Ia by site-directed mutagenesis or mass spectrometry of cyanogen bromide/trypsin-generated peptides reveals that C-terminal residues R_{295} and E_{380}, which are conserved among various ADP-ribosyltransferases (76, 196, 368, 432), are also important for Ia catalysis (287, 323, 444) (Fig. 3A). An additional motif, $_{338}STS_{340}$, found in Ia is also commonly located near the active site of many other ADP-ribosyltransferases. Although the ADP-ribosyltransferases of *C. botulinum* (C2I) or *C. difficile* (CDTa) have not been crystallized to date, structure-function studies show that the same amino acids are also necessary for enzymatic activity (36, 166). Extensive mutagenesis studies of Ia that focus on the NAD-binding cavity reveal that Y_{246} and N_{255} are important for ADP-ribosyltransferase, but not NAD-glycohydrolase, activity, unlike Y_{251} involvement in both (287). All ADP-ribosyltransferases within the binary toxin family (C2I, CDTa, Ia, Sa, and VIP2) target globular (G)-actin, which is a common and remarkably conserved protein found throughout nature and plays a pivotal role in the cytoskeleton and intracellular trafficking of all eukaryotic cells (84, 85, 110, 351, 470).

In contrast to the C-terminal similarities, the N-terminal domains of Ia (residues 1 to 210) and VIP2 (60 to 265) have only 20% sequence identity, dissimilar surface charges, and different conformations, as further evidenced by Ia possessing an additional α-helix (residues 61 to 66). Relative to "A" components of other binary toxins, the Ib docking region on Ia is more centrally located within the N-terminal domain (residues 129 to 257) (253) than C2I residues 1 to 87, needed for binding to C2II (37); LF residues 1 to 254, needed for interactions with PA (21); or CDTa residues 1 to 240, needed for docking to CDTb (166) (Fig. 3A). Overall, these data probably reflect evolutionary variation among the "A" and "B" proteins comprising these related *Clostridium* and *Bacillus* binary toxins.

ADP-Ribosylation: a Common Enzymatic Method Used by Various Bacterial Toxins

The basic mechanism of ADP-ribosylation employed by C2, CDT, CST, ι, and VIP toxins is remarkably well conserved by diverse bacteria from many different genera. All known ADP-ribosylating toxins use NAD, a ubiquitous molecule for reduction-oxidation reactions in eukaryotic and prokaryotic cells, as a source of ADP-ribose. There are at least four bacterial groups of ADP-ribosylating toxins based on the intracellular targets: (i) elongation factor 2 (modified by *C. diphtheriae* diphtheria toxin and *P. aeruginosa* exotoxin A via an N- and C-terminal active site, respectively); (ii) heterotrimeric G-proteins (modified by *B. pertussis* pertussis toxin, *E. coli* heat-labile enterotoxin, and *V. cholerae* cholera toxin via an N-terminal active site); (iii) Rho and Ras GTPases (modified by *C. botulinum* C3 exoenzyme and *P. aeruginosa* exoenzyme S via a C-terminal active site); and (iv) G-actin. Members of this last group include *B. cereus* VIP (171), *C. botulinum* C2 toxin (7, 350); and the ι-toxin family represented by *C. difficile* CDT

TABLE 3. Use of C2, CST, and ι toxins as tools to determine various cellular processes involving the actin cytoskeleton[a]

Effects (toxin)	Reference(s)
Activation of neutrophils and peptide receptor dynamics (C2)	16, 159, 292, 293, 468
Inhibited pseudopod formation, phagocytosis, and adhesion to laminin of murine macrophages (C2)	195
Decreased actin mRNA levels by increasing G-actin concentrations in rat hepatocytes (C2)	353, 354
Decreased expression of various proteins by 3T3-A31 mouse embryo cells (C2)	406
Decreased esterification of cholesterol by mouse peritoneal macrophages (C2)	431
Inhibited muscle contraction of guinea pig ileum but not rabbit aorta (C2)	256
Inhibited histamine release from rat peritoneal mast cells (C2)	55
Activation and apoptosis of human B lymphocytes (C2)	114, 262–266
Increased noradrenaline release from rat PC12 adrenal pheochromocytoma cells (C2)	254
Decreased catecholamine release from bovine chromaffin cells (C2)	142
Decreased degranulation and serotonin release from nonadherent, rat peritoneal mast cells; increased degranulation of adherent rat 2H3-hm1 basophils (C2)	339, 472
Decreased exocytosis of insulin in hamster HIT-T15 pancreatic islet cells (C2)	234
Increased exocytosis of serotonin from rat 2H3-hm1 basophils (C2)	340, 341
Increased steroid release from murine Y-1 adrenocortical cells (C2)	89, 485
Increased intracellular Ca^{2+} movement in rat 2H3-hm1 leukemic basophils (C2)	99
Inhibited insulin-regulated gene expression in rat L6 skeletal myoblasts (C2)	437
Decreased retrograde transport from Golgi to endoplasmic reticulum in rat kidney and human cervix (HeLa) cells (C2)	442, 443
Inhibited glucose transport in mouse 3T3-L1 adipocytes (C2)	374
Inhibited actin expression in mouse embryocytes (3T3-A31) and rat hepatocytes (C2)	44, 351, 352
Delayed human cervix (HeLa) cell cycling into mitosis (C2)	34
Decreased metastasis and invasion of murine BW5147 lymphoma (C2)	450
Increased endothelial permeability in various species (C2)	113, 115, 377, 387, 429
Increased exocytosis but decreased endocytosis in rat melanothrophs (CST)	84, 85
Decreased norepinephrine release from human SH-SY5Y neuroblastoma cells (ι)	454
Decreased integrity of intercellular junctions between polarized human adenocarcinoma colon (CaCo-2) cells (ι)	356

[a] Modified from reference 31 with permission.

(334), *C. perfringens* ι toxin (322, 445), and *C. spiroforme* CST (332, 394). All actin-modifying toxins have a C-terminally located active site (Fig. 3A).

The actin–ADP-ribosylating toxins can be subdivided into two groups: (i) C2 toxin, which exclusively mono-ADP-ribosylates at R_{177} the isoforms of β/γ-nonmuscle, as well as γ-smooth muscle, G-actin (6, 199, 201, 310, 446) (Fig. 3B), and (ii) the ι-like toxins, which mono-ADP-ribosylate R_{177} of all G-actin isoforms, including α-actin of skeletal muscle (255, 445). The "A" components of CDTa, Ia, and Sa have an LKDKE sequence between N-terminal residues 10 to 19 that is commonly associated with the binding of actin (331, 338). The C2I molecule has a different actin-binding sequence and location, $_{44}$LKTKE$_{48}$, which may also explain its unique substrate specificity. The enzymatic activity of ι toxin is inhibited by EDTA chelation of divalent cations associated with actin, but low temperature (0°C) decreases activity only 50% compared with that at 37°C (198). It has also been shown that enzymatic components of C2 and ι toxins, in the presence of nicotinamide excess, remove the ADP-ribosyl moiety from modified actin; however, C2I does not displace ADP-ribose from Ia-modified actin of skeletal muscle (198). Filamentous (F)-actin does not represent a substrate target. However, ADP-ribosylation of G-actin inhibits monomer assembly into F-actin strands (6, 7), which leads to decreasing F-actin, but increasing G-actin, concentrations within a cell (12, 461, 463). An actin-gelsolin complex, in which gelsolin facilitates actin nucleation and subsequent polymerization, is also modified by ι as well as C2 toxins that block additional nucleation activity (30, 201, 462, 476). Additionally, the ι and C2 toxins ADP-ribosylate G-actin complexed with ATPase, which results in an increased exchange, but decreased hydrolysis, of ATP (145, 146). From the perspective of bacterial survival, disruption of a eukaryotic cytoskeleton and reduction of ATP hydrolysis can prevent phagocytosis (16), intracellular trafficking, and ultimately induce cell death with subsequent release of valuable nutrients. From a scientist's perspective, toxins that modify actin have become invaluable tools for studying the cytoskeleton and numerous cell processes such as endothelium permeability, exocytosis and endocytosis, leukocyte activation, migration, etc. (Table 3). Previous reviews of this topic are acknowledged and recommended for further reading (9, 10, 13, 15, 88, 300).

CELL ENTRY AND INTOXICATION

"B" Binding to the Cell

To reach their intracellular substrate, and like many other bacterial protein toxins, the *Clostridium* and *Bacillus* binary toxins must first bind to a targeted cell via a receptor(s) as either a preformed or cell surface-generated homoheptameric "B" complex. Recent work in discovering cell surface receptors for these toxins has been quite fruitful, especially with *B. anthracis* PA. The PA receptor is a ubiquitous protein (117) first identified as tumor endothelium marker (TEM) 8 (variant 2), which consists of 368 amino acids and a von Willebrand factor A domain (60) commonly found on many integrins used as ligand-binding sites (61, 473). The role played by TEM 8 in normal cells is unknown; however, its expression in tumor cells can be relatively high (414). More recent discoveries show that variant 1 of TEM 8 (245) and a ubiquitous protein expressed by the human capillary morphogenesis gene 2 (CMG2) (379) also function as PA receptors. There is 40% amino acid sequence identity between TEM 8 (variant 2) and CMG2, which also have similar molecular weights and von Willebrand factor A domains containing an embedded metal ion-dependent ad-

hesin site important for PA and probably natural but yet unknown ligand interactions (61, 379). The high-affinity binding of PA to receptor, as represented by a 10^{-9} M association constant (117), is ionically (Mg^{2+} or Mn^{2+}, not Ca^{2+}) mediated via a carboxylate group provided by D_{683} on PA (61, 361). It has become clearer that PA can bind to various proteins possessing evolutionarily conserved, metal ion-containing domains on the cell surface (473), and perhaps further work done with the "B" components of other binary toxins will also yield exciting results that may include a common (or not so common) family of proteins exploited as cell surface receptors.

To further understand the binding and oligomerization properties of "B" components on cell surfaces, recent studies have delved into the potential role played by lipid rafts. Lipid rafts are cholesterol-rich, detergent-insoluble (at 4°C) "structures" or "microdomains" located on the outer cell membrane that inadvertently serve as dispersed attachment, entry, and sometimes exit sites cleverly pirated by various bacteria, viruses, and toxins (124, 177, 225, 277, 388). As recently reported, lipid rafts and a clathrin-dependent process promote the oligomerization as well as internalization of PA; however, the PA receptor is not initially raft associated (1). The assembly of PA63 into lipid rafts is cholesterol dependent and interrupted by β-methylcyclodextrin, a compound that depletes cell membranes of cholesterol (1). Via an ill-defined process mimicking ligand-dependent clustering of B-cell receptors into rafts (81), activated PA63 "forces" receptor localization into lipid rafts that subsequently generates PA63 heptamers and entry of EF and LF into the cytosol by acidified endosomes and cation-selective channels (46, 123, 157, 274). It is likely, but not definitively proven, that EF and LF travel into the cytosol through the lumen of a PA63-induced channel; however, translocated EF probably remains associated with the endosomal membrane (164). PA83, which does not form heptamers or ion channels, does not bind EF or LF, and does not facilitate any recognized toxicity, also shares these same attributes with other precursor molecules such as C2II and Ibp (46, 213, 288, 373, 420). Although little work has been done with lipid rafts and the other *Clostridium* or *Bacillus* binary toxins, recent results do suggest that *C. perfringens* Ib, but not Ibp, localizes into these membrane microdomains on Vero cells that are sensitive to ι toxin (169a).

In addition to PA, receptor-binding studies have also been done rather extensively with C2II. The C2II precursor and proteolytically activated C2IIa bind to cells (304); however, only C2IIa has hemagglutinating properties with human as well as animal erythrocytes, which is a process competitively inhibited by various sugars such as *N*-acetylgalactosamine, *N*-acetylglucosamine, L-fucose, galactose, and mannose (428). This study also shows that trypsin or pronase pretreatment of human erythrocytes prevents C2IIa-induced hemagglutination, thus suggesting that the receptor for C2II/C2IIa is a glycoprotein. Further revelations regarding the C2II receptor were provided by Fritz et al. (135) via chemical mutagenesis of CHO cells (designated RK14) that subsequently do not bind C2IIa because they lack the *N*-acetylglucosaminyltransferase I activity necessary for forming asparagine-linked carbohydrates (109). The altered gene contains a premature stop codon for W_{96}. From these experiments, it can be concluded that the receptor for C2IIa contains a complex or hybrid carbohydrate structure. In contrast, the RK14 cells are still susceptible to ι toxin, and this finding further demonstrates that C2IIa and Ib recognize unique receptors. C2IIa, like PA63 and Ib, also forms voltage-dependent channels in lipid membranes (26, 26a, 47, 50, 373), and a conserved pattern of hydrophobic and hydrophilic amino acids within C2II residues 303 to 331, a region also found in PA residues 325 to 356 of domain 2, may also play a critical role in C2IIa insertion into the membrane (47).

In contrast to C2IIa, which binds and facilitates C2I-mediated cytotoxicity in all tested vertebrate cells (31, 109, 276, 305, 428), the receptor for Ib is not as ubiquitous (419). The Ib receptor is a protein resistant to various proteases but not to pronase, as determined by flow cytometry (419) and subsequent Western blot experiments (420). Pretreatment of cells with various lectins or glycosidases does not affect Ib binding, suggesting that the receptor (or part of it) is not a carbohydrate and thus further distinguishing it from that for C2II (135). By using polarized CaCo-2 (human colon) cells, Blöcker et al. (49) discovered that the Ib receptor is localized primarily on the basolateral membrane, akin to that for PA on another human colon cell line (T84) (41). Richard et al. (356) revealed that Ib traverses CaCo-2 cells from either the apical or basolateral surface and internalizes Ia found on the distal side, even when Ia is added 3 h after Ib. In this latter study, addition of ι-toxin-neutralizing antiserum or Ib-specific monoclonal antibodies with Ia to the cell surface distal to Ib also did not affect ι cytotoxicity. Western blot experiments conducted by two different groups reveal that Ib rapidly binds to cells at 37°C and forms a large complex (>200 kDa) in less than 1 min that is evident for at least 2 h (288, 420). This is intriguing when compared to earlier work by Sakurai and Kobayashi (367) showing that Ia injected intravenously into mice 2 h after Ib administration causes death, suggesting that Ib is available for Ia docking on the cell surface over an extended period. If neutralizing ι toxin antiserum toward Ib is given only 5 min after an Ib injection, mice are not protected against Ia-induced death, and this reveals that Ib perhaps binds to cells very rapidly in vivo. Finally, in this study it was also discovered that when Ib is injected intradermally into guinea pigs and Ia is given intraperitoneally, a dermonecrotic lesion forms at the Ib injection site. Clearly, Ia is able to "find" distantly located Ib that is bound to the cell surface, and perhaps this characteristic can be exploited in future experiments involving ι toxin as a protein shuttle. Similar discoveries were also reported by Simpson (390) for C2 toxin in mice and rats. To date, receptor-binding studies for *B. cereus* VIP1, *C. difficile* CDTb, or *C. spiroforme* Sb are lacking in the literature.

"A" Docking to Cell-Bound "B" and Internalization

As described earlier in this review, N-terminal domains from the "A" and "B" components of each *Clostridium* and *Bacillus* binary toxin are intimately involved in docking to each other on the cell. After binding to a surface receptor, there are two major pathways for uptake of bacterial protein toxins that act intracellularly. One path involves retrograde routing through the Golgi apparatus and endoplasmic reticulum, as demonstrated by *S. dysenteriae* Shiga (370) and *V. cholerae* cholera (313) toxins. This method of cytosolic entry is inhibited by brefeldin A, a fungal macrolide that causes protein accumula-

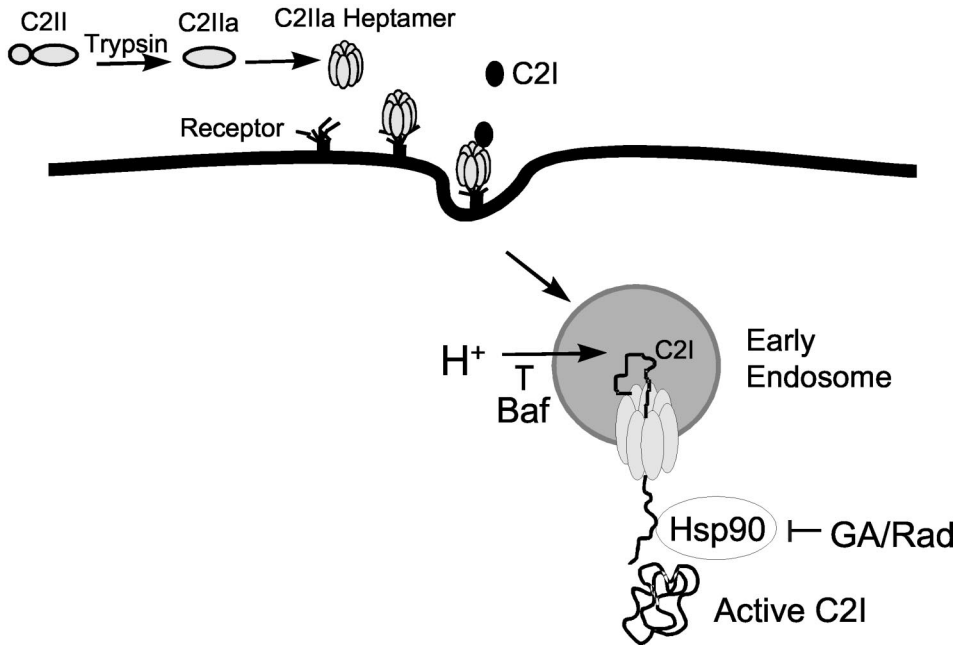


FIG. 4. Depiction of C2 intoxication via Hsp90, a process that is required for entry of C2I into the cytosol from acidified endosomes (179). A similar pathway is also used by ι toxin and CDT for intoxicating cells (178a). Translocation of C2I, CDTa, or Ia from the endosome requires an acidic pH, a process blocked by bafilomycin A (Baf) via specific inhibition of vacuolar-type ATPases located in the endosomal membrane. C2I partially unfolds during translocation across the endosomal membrane via Hsp90, a process specifically targeted by geldanamycin (GA) or radicicol (Rad), which results in trapping of C2I within the endosome.

tion within the endoplasmic reticulum (78). The second intracellular route exploited by bacterial toxins involves translocation from acidified early endosomes into the cytosol, like that employed by single-chain *C. diphtheriae* diphtheria toxin (248) as well as the multiple-chain *B. anthracis* edema and lethal toxins (131). Subsequent transport of vesicles from early to late endosomes involves microtubules that are readily depolymerized by nocodazole, a chemically synthesized molecule that inhibits trafficking into late endosomes (32, 49, 160, 365). Since neither brefeldin A nor nocodazole influences the biological activity of C2 or ι on cells, these toxins are like the edema and lethal toxins regarding transport from early endosomes into the cytosol. However, translocation of the C2, ι, or edema and lethal toxins across the endosomal membrane is blocked by a macrolide antibiotic, bafilomycin A, which inhibits vacuolar-type ATPases responsible for acidification of the endosomal compartment (Fig. 4) (32, 49, 469). A decreased pH probably induces conformational changes and membrane insertion of the heptameric "B" component, followed by translocation of the "A" component(s) across the endosomal membrane, which is also mimicked on the cell surface by simply lowering the pH of the medium (32, 49, 50, 131, 157, 247, 267, 272, 392, 400). However, there are unique pH requirements for binary toxins such as C2 and ι, since the latter requires a lower pH (≤5.0) than the former (≤5.5) for direct cytosolic entry through the cell membrane. Currently, it is not clear if "B" heptamers of these binary toxins enter the cytosol with the "A" components or remain attached to the endosomal membrane, possibly recycling to the cell surface (311, 356).

Recent results with C2, CDT, and ι toxins reveal that the "A" components traverse the endosomal membrane via heat shock protein 90 (Hsp90) and an ill-defined mechanism (Fig. 4) (178a, 179). Hsp90 is a highly conserved ATPase that is abundantly produced by all eukaryotic cells, and in conjunction with other heat shock proteins it plays an essential housekeeping role by regulating a myriad of proteins associated with cell signaling (337). Specific inhibitors of Hsp90, such as geldanamycin or radicicol, effectively delay C2-, CDT-, or ι-induced cytotoxicity in various cell types by inhibiting, respectively, C2I, CDTa, or Ia entry into the cytosol. Interestingly, cytosolic entry of *B. anthracis* lethal toxin differs in that it is not affected by Hsp90 inhibitors (179), thereby suggesting distinct endosome-to-cytosol translocation mechanisms for "A" components of this binary toxin family.

It is plausible, but not definitively proven, that the enzyme components from all *Clostridium* and *Bacillus* binary toxins unfold and thread through toxin-generated channels in the membrane, perhaps entering the cytosol like that proposed for LF or the ADP-ribosyltransferase of the single-chain diphtheria toxin (120, 471). The recent work by Ratts et al. (348) shows that Hsp90 and thioredoxin reductase, found in a cytosolic complex, are both required to transport *C. diphtheriae* diphtheria toxin from the endosome. Additionally, geldanamycin and radicicol are both necessary for inhibiting diphtheria cytotoxicity, whereas with CDT, C2, or ι toxins, either drug alone inhibits cytotoxicity with no evident additive or synergistic effects. It is unknown whether thioredoxin reductase cleaves the disulfide bond between the "A" and "B" chains of diphtheria toxin, although this is quite possible, since it does occur with *C. tetani* tetanus toxin and *C. botulinum* neurotoxin A (209). However, disulfide bonds and reductive activation have never been

described for any of the *Clostridium* and *Bacillus* binary toxins described in this review.

Inevitably, these recent discoveries with Hsp90 and its role on the entry of toxic ADP-ribosyltransferases into the cytosol, along with further investigation including other binary bacterial toxins, will likely elucidate other factors involved in the cytosolic entry of proteins from the endosome. Such studies might also lead to more efficient use of bacterial toxins as protein shuttles, in particular delving into the types of molecules transported into the cytosol of targeted cells from the endosome. Finally, results from these endeavors will also probably provide new targets for potential therapeutics against bacterial toxins that traffic through the endosome.

BACTERIAL BINARY TOXINS: VERSATILE PROTEIN SHUTTLES, VACCINE TARGETS, AND THERAPEUTICS

Protein Shuttles

Historically, the single-chain exotoxin A of *P. aeruginosa* and *C. diphtheriae* diphtheria toxin have each received much attention regarding their use as vehicles for heterologous proteins (317). As described in this section, the *Clostridium* and *Bacillus* binary toxins have also provided excellent tools for shuttling heterologous proteins, and even DNA, into cells. Again, the pioneering work on, and subsequent knowledge gleaned from, *B. anthracis* toxins has resulted in important advances in this field. For example, there have been many studies employing LF (amino acids 1 to 254) in which LF^{1-254} can be fused to either the N or C terminus of a heterologous protein (20–23, 230, 231, 261, 273, 383) and subsequently internalized by a targeted cell via heptameric PA63. However, fusions consisting of LF residues 1 to 198 or deletion of just 40 N-terminal residues from the wild-type molecule (without a fusion protein) are not cytotoxic (21), further revealing an important role played by the N terminus of LF in docking and/or translocation.

The immune system represents just one potential target for chimeras generated from *B. anthracis* lethal toxin. Recombinantly linked or disulfide-linked LF chimeras can induce cytotoxic T-cell responses in vivo and/or in vitro via the delivery of major histocompatibility complex class I epitopes originating from viruses (i.e., influenza virus, human immunodeficiency virus, and hepatitis C virus) or bacteria (*Listeria monocytogenes*) (27–29, 69, 101, 153, 154, 284, 486). An exciting variation on stimulating immunity involves the anticancer capabilities of lethal toxin (129, 216). Like previous studies with a diphtheria toxin chimera containing the C fragment of *C. tetani* tetanus toxin for specific targeting of neurons (128), it is also possible to direct PA toward unique surface receptors, as evidenced by fusing a c-Myc epitope consisting of 10 amino acids to the C terminus of PA, which subsequently binds to (and kills with LF) a hybridoma line expressing c-Myc antibody (233, 448).

Another novel twist on using *B. anthracis* lethal toxin as an antitumor compound is the exploitation of specific proteolysis by urokinase plasminogen activator, a surface protein commonly associated with malignant cells (244, 246). By replacing the furin cleavage site (RKKR) of PA83 with SGRSA, a recognition site for urokinase plasminogen activator, there is decreased toxicity and broad antitumor effects in mice when combined with the use of LF^{1-254} fused to the enzymatic domain of *P. aeruginosa* exotoxin A.

In addition to shuttling proteins into cells, DNA encoding luciferase or green fluorescent protein has been successfully transported into COS-1 cells by PA and an LF^{1-254} fusion containing a 16-kDa DNA-binding domain from Gal 4, a yeast transcription factor (144). In the future, this may represent an attractive alternative to the use of viral vectors, which are more common in gene therapy today (100, 194). Overall, the numerous shuttle experiments that have been done with *B. anthracis* lethal toxin represent "trend-setting" uses that foretell a bright future for *Clostridium* and *Bacillus* binary toxins. However, another important question that remains unanswered involves fusion constructs of any binary toxin and the maximum size of heterologous proteins transported into the cytosol. Of course size but also an ability to "unfold," "thread," and properly "refold" within the cytosol probably dictate the ability of any given protein to translocate into the cytosol as a biologically active form. For the most part, many aspects of getting heterologous proteins into targeted cells via any *Clostridium* or *Bacillus* binary toxin remain largely unexplored by the different laboratories working in this field.

As with any protein-based system used for targeted delivery of protein and/or DNA into cells in vivo, host-developed antibodies to the protein constituents could diminish subsequent efficacy. For instance, it has been reported that a PA-LF combination elicits higher antibody titers toward LF than does LF alone (65, 249, 328). Mahlandt et al. (249) first suggested in 1966 an additive immune response to LF with PA, and Pezard et al. (328) later reported that *B. anthracis* strains expressing both PA and LF (or EF) induced LF (or EF)-specific antibodies in mice, yet strains expressing LF (or EF) alone elicited very little specific antibody. Finally, vaccine studies show that spores from recombinant *B. anthracis* strains that express PA and LF^{1-254} fused to the 50-kDa C fragment of tetanus toxin result in higher-titer neutralizing antibodies toward tetanus toxin than do spores from a PA-deficient strain (65). To date, though, there have been no in vivo studies addressing specific antibodies elicited toward any *Clostridium* or *Bacillus* binary toxin when used as a shuttle system.

In light of these serological data from various groups, the potential problem of developing specific antibodies to lethal toxin-based shuttle proteins after repeated exposure might be partly circumvented, since recent evidence suggests that LF^{1-254} conjugates of green fluorescent protein or human immunodeficiency virus peptides can enter cells without PA and subsequently elicit a major histocompatibility complex class I-induced response from $CD8^+$ T cells (222). The entry of LF^{1-254} into cells without PA clearly represents a major paradigm shift for the *Clostridium* and *Bacillus* binary toxins; however, LF can evidently enter cells through a pinocytotic route, albeit less efficiently than the PA63-facilitated translocation that may also be needed for proper trafficking of LF toward MAPKK before degradation (222, 402).

Similar to *B. anthracis* LF, the noncatalytic N-terminal domain (residues 1 to 225) of *C. botulinum* C2I (designated as $C2I^{1-225}$) is also important for docking with its "B" component (C2IIa) and has been engineered as a fusion protein (31, 33) with C3 or C3-like ADP-ribosyltransferases produced by *B. cereus*, *C. botulinum*, *C. limosum*, and *S. aureus* (14, 200, 427,

474, 475). ADP-ribosyltransferases of the C3 and C3-like family are each ~23 kDa, have 35 to 75% amino acid sequence identity among themselves but only 23% identity to "A" components from the ι-toxin family, and inactivate small GTPases (Rho A, B, and C) through mono-ADP-ribosylation of N_{41} (8, 11, 363, 380). The recently determined crystal structure of *C. botulinum* C3 exoenzyme reveals conformational similarities to the C-terminal domain of C2I containing the active site (170). Curiously, C3 and C3-like exoenzymes may enter cells via a very speculative and inefficient pinocytosis, cytolysin-mediated transport, or ill-defined secretion mechanism (247a), since neither binding nor transport components are seemingly produced by any of the host bacteria. Due to poor cell penetration, the C3 and C3-like proteins are ideal for shuttle experiments since high concentrations (>10 μg/ml) must be applied to cells for an extended period (up to 24 h) to elicit any cytotoxic effects. However, when a $C2I^{1–225}$-C3 construct is added at low concentrations (100 ng/ml) with C2IIa to various primary isolated or continuously cultured cell monolayers for 3 h, nearly all are found to possess the typical "C3 morphology" within 60 min, as evidenced by cell contraction and neurite-like protrusions (33). Analysis of time-versus-concentration curves suggests that the potency of the $C2I^{1–225}$-C3 fusion toxin (with C2IIa) is 300-fold higher than that of C3 toxin plus C2IIa. Upon removal of fusion toxin from the medium, cytopathic effects are reversed within hours via reorganized stress fibers and actin cytoskeleton, thus leading to normal morphology attributed to newly synthesized Rho protein (33, 35). In contrast, recovery from $C2I^{1–225}$-C3 intoxication is not observed if cells are treated with inhibitors of either protein synthesis (cycloheximide or puromycin) or proteasomes (lactacystin) (35). Biochemical analysis of lysates from cells incubated with the C3 fusion toxin reveals that Rho A, B, and C, but not other Rho types or actin, are modified with ADP-ribose (33). C3 can be fused to either the N or C terminus of $C2I^{1–225}$ and efficiently delivered into cells like wild-type C2I (31, 37), even if $C2I^{1–225}$ is in the midst of a 75-kDa fusion protein (glutathione transferase–$C2I^{1–225}$-C3).

Further investigation has shown that the minimal C2I fragment facilitating C3 transport into cells consists of residues 1 to 87, which is as effective as the larger $C2I^{1–225}$ construct (37). The $C2I^{1–87}$ fragment represents a minimal part of C2I needed for not only translocation but also competitive binding with C2I for C2IIa. The N terminus of C2I is important for docking with C2IIa, since deletion of just 30 amino acids from the $C2I^{1–225}$-C3 fusion results in loss of cytotoxicity (with C2IIa) due to poor translocation and not inhibited binding to cell-associated C2IIa (37). Clearly, the C2 shuttle system is quite versatile for translocating heterologous proteins into cells and studying various cell functions, as further illustrated in Table 4.

In addition to the *C. botulinum* C2 or *B. anthracis* lethal toxins, a recent study with *C. perfringens* ι toxin reveals that it, too, acts as an effective shuttle for heterologous proteins (253). It was shown that the minimal Ia fragment necessary for efficient transport of *C. botulinum* C3 into Vero cells, via Ib, is the catalytically inactive $Ia^{129–257}$. Linkage of C3 to the C terminus of Ia peptides of different sizes is most effective on cells, since all N-terminally linked conjugates when incubated with Ib are not cytotoxic. However, each N- or C-linked C3 conjugate of Ia is enzymatically active in vitro, as evidenced by modification of a 21-kDa protein (Rho) in bovine brain homogenates; therefore, C3 enzymatic activity is not greatly altered in any fusion construct. Additionally, most of the N-linked conjugates effectively dock to Ib on the cell surface, suggesting either that C3-Ia conjugates may be inefficiently translocated or that upon entering the cytosol, the enzyme may not adopt a biologically active conformation. These results differ from those for the lethal and C2 toxin systems, in which either the N- or C-terminal linkage of LF or C2I fragments to heterologous proteins, respectively, results in PA- or C2II-facilitated transport of biologically active proteins into cells (22, 23, 31, 37, 273). Although various in vitro studies have employed chimeric C2 and ι toxins as shuttle systems, results from in vivo studies are clearly lacking in the literature and represent the next logical step for future studies.

TABLE 4. C2-C3 fusion toxins as tools for studying the role of Rho GTPases in various cell types[a]

Effects on:	Reference
Aquaporin 2 translocation from rat renal cells	212
Anion channel activity in bovine endothelial cells	291
Cyclooxygenase-2 gene expression in rat renal cells	169
Secretion of von Willebrand factor from human umbilical vein cells	451
IL-1[b]-stimulated synthesis of IL-2 in murine EL-4 thymoma cells	102
Survival of rat cerebellar neurons	236
Uptake and degradation of lipoproteins by mouse J774 macrophages	366
Transendothelial migration and adhesion of human monocytes	424
Growth cone collapse of chicken retinal cells	456

[a] Modified from reference 31 with permission.
[b] IL, interleukin.

Vaccine Targets

Vaccines represent a historically powerful means of controlling various diseases, anthrax being one of the first. The initial vaccine study for anthrax was publicly conducted by Louis Pasteur and colleagues in the spring of 1881 at a farm near Pouilly-le-Fort, located ~30 miles southeast of Paris. The inoculum was live, temperature-attenuated *B. anthracis* that subsequently afforded protection in sheep (318, 436). Following such an achievement, this vaccine was widely accepted by the veterinary community and used throughout the world for ~50 years in cattle and sheep until the discovery of the Sterne strain, which lacks the pXO2 plasmid (capsule encoding) but not the pXO1 plasmid (toxin encoding) (134, 416). It was recognized only 20 years ago that the *B. anthracis* inoculum used in the Pasteur vaccine was probably cured of pXO1 plasmid after growth at an elevated temperature (271).

Today, 120 years after Pasteur's pioneering efforts, we are still pursuing *B. anthracis* and better methods of controlling anthrax by appropriate vaccines and therapeutics (134, 381, 399). Unlike the studies conducted during the Koch-Pasteur era, which focused primarily on disease in farm animals, current endeavors are primarily aimed at anthrax in humans from natural and, even more importantly, unnatural (i.e., biological warfare and bioterrorism) sources (134). The vaccine target of greatest interest has been the PA molecule (24, 90, 121, 134, 143, 161, 185, 187, 188, 190, 213a, 227, 237, 329, 354a, 398,

473a), ergo its name from work initially presented by Gladstone (151) and subsequently by others (59, 440, 478). Among numerous *B. anthracis* strains tested ($n = 26$), the PA molecule is evidently quite conserved at the gene and amino acid levels (342) and thus provides an appropriate target for developing broadly efficacious "second-generation" vaccines for humans (121). Antibodies toward PA protect against lethal intoxication and/or infection by *B. anthracis* spores, which contain PA in their coat that can be exploited by the immune system via PA-specific antibodies that enhance spore uptake, germination, and killing by macrophages (465, 466).

The current anthrax vaccine for humans in the United States, known as anthrax vaccine adsorbed (AVA) or BioThrax, was approved by the Food and Drug Administration in 1970. This vaccine is far from optimal, since it consists of formalin-treated culture filtrate from a nonproteolytic, toxin-positive/capsule-negative strain (V770-NP1-R) of *B. anthracis* (133, 477) in aluminum hydroxide adjuvant that is administered via six subcutaneous injections over 18 months, with an annual boost (330). Standardization of AVA has also been a problem, since the PA content is not measured in each vaccine batch and a universally accepted assay has not been established to quantitate PA specific-antibodies among vaccinees (232, 329, 354a). A slight variation of AVA, employing Sterne strain 34F2 and an aluminum phosphate adjuvant, was approved in 1979 for human use throughout the United Kingdom (134, 477). To diminish the time course and number of injections and avoid potential adverse reactions in humans, various studies aimed at developing a second-generation vaccine demonstrate that a simplified inoculum composed of purified, recombinant PA alone is efficacious when given mucosally, parenterally, or transcutaneously to animals (58, 125, 143, 254a, 398).

The veterinary vaccine is quite different from that used in humans, since it consists of *B. anthracis* spores (Sterne strain) that do not readily elicit PA antibodies (in guinea pigs), yet it affords better protection after one injection than that achieved after three injections of the acellular vaccine (440). It is perhaps because of this heightened immune response to spores that certain investigators are now exploring the use of PA and formaldehyde-inactivated, recombinantly detoxified spores of the Sterne strain as a potential second-generation vaccine for humans (63a).

Experimentally, various studies have been done to identify novel methods of vaccination against anthrax. For instance, parenteral vaccinations with DNA encoding PA, LF, or both have proven effective against a lethal toxin challenge in mice (343). Subcutaneous or intramuscular inoculations of mice with replication-deficient vectors, composed of Venezuelan equine encephalitis virus or adenovirus and the PA83 gene, also afford protection against a subcutaneous or intravenous challenge with *B. anthracis* spores or lethal toxin, respectively (227, 432a). Another report by Garmory et al. (139) also shows that mice immunized orally or intravenously with PA-expressing *Salmonella enterica* develop PA-specific antibodies and protection against an intraperitoneal challenge with *B. anthracis* spores. It is logical that antibodies against PA, particularly those targeting the C terminus, will prove efficacious, since this region binds to cell surface receptors (401, 449) and contains immunodominant epitopes that protect against intoxication as well as *B. anthracis* infection (126, 243, 257).

In addition to using PA, Sterne spores, or a combination of the two as vaccine targets, a dually active anthrax vaccine has been reported recently by two different groups (355, 376). These novel conjugate vaccines consist of PA plus poly-γ-D-glutamic acid (PGA) capsule that, when used in mice, effectively elicit toxin-neutralizing antibodies and complement-mediated killing of *B. anthracis*. Relative to PA and certainly PGA alone, conjugates containing both PA and PGA elicit higher antibody titers toward each of these antigens, with a 10- to 20-fold molar excess of decameric PGA representing an optimal immunogen (355, 376). Perhaps the concept of dually active anthrax vaccines represents a future form of prophylaxis toward not only *B. anthracis*, but also other pathogens that possess multiple virulence factors. It is clear that various groups are pursuing novel vaccines against anthrax, which bodes well for future prophylaxis methods that should entail less time, lower cost, and reduced reactogenicity compared with the current methods of protection.

Compared to the *B. anthracis* toxins, much less vaccine work has been done with other *Clostridium* and *Bacillus* binary toxins described in this review. However, various antibody studies with Ib of *C. perfringens* ι and C2IIa of *C. botulinum* C2 toxins show that their C termini, like that for PA63 (240, 243), contain toxin-neutralizing epitopes (48, 252). Studies by Sirard et al. (403) also reveal that the Sterne vaccine against anthrax can be recombinantly modified to express Ibp of ι toxin and then subsequently used to prophylactically protect mice against a lethal CST or ι toxin challenge. This brings up an interesting possibility regarding the use of this commonly accepted, spore-based vaccine as a vehicle for eliciting protective immunity against various bacteria, viruses, and toxins of veterinary and human importance (63a).

In addition to the studies with Sterne spores, there have been efforts by Ellis et al. (111) to develop veterinary vaccines against *C. spiroforme*. This work shows that a formalin toxoid of *C. spiroforme* culture supernatant containing CST elicits active immunity in weaning rabbits against a toxin challenge; however, passive immunity was not transferred by does to their young. Protection was evident after only one subcutaneous injection, but two injections elicited even better results. Unfortunately, it is unknown how effective this vaccine is toward a *C. spiroforme* challenge since these experiments were not done and/or not reported in this publication.

Therapeutics

The area of research into therapy of disease due to *Clostridium* and *Bacillus* binary toxins has historically received very little effort, but the deliberate anthrax attacks within the United States have dramatically elevated the stakes, attention, and resources now focused upon rapidly developing therapies for two major virulence factors of *B. anthracis:* the edema and lethal toxins (51, 79, 425). Antibiotics such as ciprofloxacin, doxycycline, and penicillin are indeed generally effective in vivo against *B. anthracis* (17, 38, 67, 201a, 346, 430); however, rapid diagnosis of disease as well as timely and lengthy administration of antibiotics are both critical for patients and make the difference between life and death for those suffering from

inhalational forms of anthrax (122a, 192, 346). Perhaps the recent discovery of bacteriolytic phage enzymes specific for *B. anthracis* will afford, in the future, an additional "magic bullet" for clinicians, especially if antibiotic-resistant strains are encountered (149, 378).

To increase the survival rates among patients with inhalational anthrax, which insidiously begins as a seemingly innocuous cough and flu-like malady that can rapidly progress to shock, respiratory failure, and death, adjunct therapy directed toward edema and/or lethal toxins will probably provide a pivotal advantage (67, 344, 425). With either of these toxins, PA represents a central figure for *B. anthracis* survival in the host, as demonstrated by in vitro and in vivo antibody studies (42, 112, 133, 134, 213a, 238, 354a, 466) or inhibition of lethal toxin-induced cytotoxicity in vitro via a soluble receptor fragment (60). Logically, the "B" component of any bacterial binary toxin represents a prime therapeutic or vaccine target, since it is theoretically easier to neutralize a toxin before it enters a cell. Antibodies to PA, when administered promptly in vivo, do indeed provide an effective immunotherapeutic after the onset of a *B. anthracis* infection (213a, 238, 354a). Additionally, it was shown years ago that a recombinant PA83 lacking the furin cleavage site, and thus unable to form heptamers or dock with EF or LF, protects rats against a subsequent dose of lethal toxin via competition with wild-type PA for receptor (397). Therapeutic targeting of PA has also been advocated with a PA32 fragment (composed of domains 3 and 4) that prevents the binding of wild-type PA to cells (86).

More recently, various laboratories have developed novel means of inhibiting *B. anthracis* toxins involving a dominant-negative strategy via PA. Mutations of K_{397}, D_{425}, and/or F_{427} within domain 2 (4, 381, 382, 482), swapping of residues 302 to 325 (2β2–2β3 loop) from domain 2 with the *C. perfringens* Ib equivalent (399), and cysteine replacement of each amino acid in PA63 (285a) have generated translocation-defective molecules of PA that still oligomerize and dock with EF and/or LF. Only one dominant-negative PA molecule in a heptamer, otherwise composed of wild-type PA63, impressively protects cells in vitro against lethal toxin. These forms of PA also effectively prevent lethal toxin activity in vivo upon concomitant injection. In essence, such PA variants adsorb EF and LF out of the circulation, and future studies should ascertain whether this type of therapy represents a viable adjunct in vivo, with antibiotics, against anthrax. Additionally, the inhibition of LF docking to a PA63 heptamer and subsequent cytotoxicity has also been accomplished by biopanning a phage display library (285). By employing this technique, a dodecapeptide was discovered that proved most efficacious toward lethal toxin in vitro and in vivo when bound to a polyacrylamide backbone.

To complement the above protein- and peptide-based therapies, low-molecular-weight inhibitors of edema and lethal toxins have also been studied recently by various university, government, and company laboratories that are focusing on the inherent enzymatic activities of EF and LF (91, 280, 313a, 409, 435). Computational analyses of databases containing low-molecular-weight compounds, along with the known crystal structures of EF, LF, and PA, provide a strong starting point for drug discovery directed toward inhibiting toxin binding to cell surface receptors as well as enzymatic activity (152, 409). Ironically, it was from such a database for antitumor drugs that LF activity was first ascertained to mimic an existing compound that adversely affects the MAPKK pathway (107, 464).

Once computational analysis of databases has been done and potential inhibitors have been identified, a necessary component of discovering any toxin therapy among a myriad of candidates is the development and subsequent use of simple, accurate, and high-throughput assays like those recently demonstrated for LF involving fluor-tagged peptide substrates (91, 359, 435). As an example, a plate-based assay described by Cummings et al. (91) employs a coumarin fluor and quencher separated by 17 amino acids containing an LF cleavage site that, upon proteolysis, enables the fluor to subsequently emit a detectable signal. In many ways, this type of assay mimics the same format as that for in vitro proteolysis studies with *C. botulinum* neurotoxins A, B, and F (18, 375).

Recent efforts to competitively inhibit EF through a unique catalytic site involving an ATP binding region not shared with eukaryotic cyclases also has potential benefits against other pathogen-produced adenylate cyclases, like those from *B. pertussis*, *P. aeruginosa*, and *Yersinia pestis* (409). Soelaiman et al. (409) show that certain quinazoline compounds competitively inhibit the binding of ATP to the catalytic site of EF and also protect mouse adrenocortical cells in vitro when added before edema toxin. Unfortunately, "therapeutic-like" experiments were either not done or not reported with drug administered after toxin exposure. Additionally, it is well established that edema toxin requires Ca^{2+} for activity (229); however, somewhat surprising is that inhibitors of intracellular Ca^{2+} (i.e., dantrolene or cyclosporine A) also prevent lethal toxin activity on murine macrophages in vitro, which probably involves increased permeability of cell membranes via a Ca^{2+}-dependent phospholipase A_2-based mechanism (386). Unfortunately, these compounds are nonspecific and will likely affect many other Ca^{2+}-dependent processes necessary for normal cell functions. Finally, the most promising drug to date against edema toxin is perhaps one already clinically approved for treating chronic hepatitis B infections via inhibition of viral DNA polymerase (384a). The cellular metabolite of adefovir dipivoxil, an acyclic nucleotide phosphonate that binds to EF with 10,000-fold-higher affinity than ATP, clearly protects various cell types in vitro from the effects of edema toxin.

For the other *Clostridium* and *Bacillus* binary toxins described in this review, specific inhibition by newly discovered compounds has not been studied because these toxins are not commonly associated with human disease and do not directly represent a bioterrorism threat. However, it is known that peptide-based inhibitors of ι toxin, composed of domain 4 from Ib, prevent the binding of wild-type Ib to cells and subsequent Ia-induced cytotoxicity (252). Similar work has not been done with the C2, CDT, CST, or VIP binary toxins. Clearly, there is much work to be done regarding therapeutics towards *Clostridium* and *Bacillus* binary toxins, and the toxins from *B. anthracis* represent an immediate focal point as well as global concern. Upon considering the numerous structural commonalities within this binary-toxin family, protein-based inhibitors seem like one of many largely unexplored and logical paths into the future.

CONCLUSIONS

As presented in this review, the binary toxins from various *Bacillus* and *Clostridium* species possess unique and versatile characteristics that are useful for (i) delivering proteins into cells, (ii) discerning the role of actin in various cellular functions, (iii) providing vaccine targets, and (iv) developing recombinantly modified therapeutics. From a historical perspective, the discovery of *C. perfringens* ι toxin in 1940 was the first for any *Clostridium* or *Bacillus* binary toxin (54). However, in 1956 the multicomponent structure of *B. anthracis* toxins was initially reported and thus represents the first binary description for any of these toxins (407, 408). Subsequently, the multicomponent nature of *C. botulinum* C2 toxin, *C. perfringens* ι toxin, *C. spiroforme* CST, *B. cereus* VIP, and *C. difficile* CDT was elucidated in 1980 (302), 1986 (421, 422), 1988 (332), 1992 (171), and 1997 (324), respectively. Therefore, the edema and lethal toxins of *B. anthracis* represent a "standard" for this binary family that is further evidenced by the number of publications written, and information determined, over time. For example, determination of the crystal structures of each *B. anthracis* toxin component (EF [103], LF [314], and PA [326]) and specific identification of the cell surface receptors for PA (60, 61, 245, 379) represent exciting discoveries currently lacking for the other binary toxins. Notable exceptions, however, include the crystal structures of Ia (438) and VIP2 (171).

As with any family, there are intriguing similarities but also differences among its members that become clearer over time and further study. The "B" precursors of *Clostridium* and *Bacillus* binary toxins described in this review are all proteolytically activated by serine-type proteases in solution, but the PA83 molecule from *B. anthracis* is also uniquely processed on a targeted cell by surface-associated furin or furin-like proteins. The distinct versatility of PA63 heptamers is also apparent with the transport of different enzymatic moieties (EF and LF) into a cell, whereas "B" heptamers from the other binary toxins transport only one type of enzyme, an ADP-ribosyltransferase specific for G-actin. In contrast to the uniform requirement for proteolytic activation of "B" components, Ia of ι toxin is the only "A" component in this binary family that is activated by proteases. The evolutionary relationships that exist among *Clostridium* and *Bacillus* binary toxins are made more evident by the generation of biologically active chimeras within the ι toxin family via interchangeable "A" and "B" molecules, which does not occur with any of the C2 or *B. anthracis* edema toxin and lethal toxin components.

Looking into the future, domain swapping between *Clostridium* and *Bacillus* binary toxins beyond what Singh et al. (399) initially described for PA and Ib could be an eventual reality that yields novel protein-based therapeutics targeting the *B. anthracis* edema and lethal toxins. Further exploration that employs gene probes and specific toxin antibodies will also probably unveil new binary toxins produced by other bacteria and perhaps those from different genera. Such discoveries could reveal additional clues to the evolutionary tracts taken by these binary toxins and of course by the bacteria that produce them. Besides the extensive pathogenesis work done with edema and lethal toxins of *B. anthracis*, it still remains a mystery whether the other *Clostridium* and *Bacillus* binary toxins afford any unique advantage(s) for their bacterial hosts. Again, this is a reflection of the literature not yet available for the other binary toxins.

Finally, in our opinion, it is evident that the bacterial toxins described in this review possess additional untapped promise for the future. A knowledge-based understanding of the past will hopefully spawn additional creative efforts, by various groups, involving the *Clostridium* and *Bacillus* binary toxins.

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