Review

New insights into the mode of action of the actin ADP-ribosylating virulence factors *Salmonella enterica* SpvB and *Clostridium botulinum* C2 toxin

Holger Barth [a,*], Klaus Aktories [b]

[a] *Institut für Pharmakologie und Toxikologie, Universitätsklinikum Ulm, Germany*
[b] *Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, Germany*

A B S T R A C T

The C2 toxin from *Clostridium botulinum* represents the prototype of the family of binary actin-ADP-ribosylating toxins. These toxins covalently transfer ADP-ribose from nicotinamide adenine dinucleotide (NAD$^+$) onto arginine-177 of actin in the cytosol of eukaryotic cells resulting in depolymerization of actin filaments and cell rounding. The C2 toxin consists of two non-linked proteins, the enzyme component C2I and the binding and translocation component C2II, which delivers C2I into host cells. The ADP-ribosyltransferase SpvB from *Salmonella enterica* also modifies actin, but is delivered into the host cell cytosol from intracellular growing *Salmonella*, most likely via type-III-secretion. We characterized the mode of action of SpvB in comparison to C2 toxin *in vitro* and in intact cells. We identified arginine-177 as the target for SpvB-catalyzed mono-ADP-ribosylation of actin. To compare the cellular responses following modification of actin by SpvB or by the binary toxins without the influence of other *Salmonella* virulence factors, we constructed a cell-permeable fusion toxin to deliver the catalytic domain of SpvB (C/SpvB) into the cytosol of target cells. This review summarizes recent findings of research on the actin ADP-ribosylating toxins regarding their cellular uptake, molecular mode of action and the cellular consequences following ADP-ribosylation of actin.

Introduction

Mono-ADP-ribosylation of actin is an efficient mechanism by which a group of bacterial virulence factors directly attack the actin cytoskeleton of eukaryotic target cells. The first ADP-ribosylating toxins that had been discovered were the binary exotoxins from *Clostridia*, including *C. botulinum* C2 toxin (Aktories et al., 1986), *C. perfringens* iota toxin (Stiles and Wilkens, 1986; Vandekerckhove et al., 1987), *C. difficile* transferase CDT (Perelle et al., 1997a; Popoff et al., 1988), and *C. spiroforme* toxin (CST) (Popoff and Boquet, 1988). These toxins are enterotoxins and associated with gastrointestinal diseases of humans and animals (for review see Barth et al., 2004). Later, it became evident that the vegetative insecticidal proteins (VIPs) from *Bacillus cereus* are also binary actin ADP-ribosylating toxins (Han et al., 1999).

All binary actin ADP-ribosylating toxins are composed of two non-linked proteins: an enzyme component, in which ADP-ribosylates actin and a separate binding/translocation component,

which mediates the uptake of the enzyme component into the cytosol of target cells. We and others have discovered that the uptake of these toxins into cells requires a precisely coordinated sequence of events, which is best understood for the C2 toxin from *C. botulinum*. First, the proteolytically activated binding/translocation component binds to its receptor on the surface of target cells and forms complexes with the enzyme component (Barth et al., 2000; Kaiser et al., 2006). Following receptor-mediated endocytosis of the toxin complex, the binding/translocation component forms pores in the membranes of acidified endosomes (Barth et al., 2000). The pores are absolutely essential for the subsequent translocation of the enzyme components from the endosomal lumen into the cytosol (Blöcker et al., 2003b). We have demonstrated for the C2 toxin, that the enzyme component translocates in an unfolded conformation through the pores across endosomal membranes (Haug et al., 2003b) and that host cell factors, such as the chaperone Hsp90 and the protein folding helper enzyme cyclophilin A, facilitate membrane translocation of the enzyme component (Haug et al., 2003a; Kaiser et al., 2009). After translocation into the cytosol, the enzyme components of these toxins mono-ADP-ribosylate G-actin, i.e. they covalently transfer the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD$^+$) onto an arginine residue of actin (Aktories et al., 1986).

* Corresponding author at: Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Albert-Einstein-Allee 11, D-89081 Ulm, Germany.
Tel.: +49 731 500 65503; fax: +49 731 500 65502.
E-mail address: holger.barth@uni-ulm.de (H. Barth).
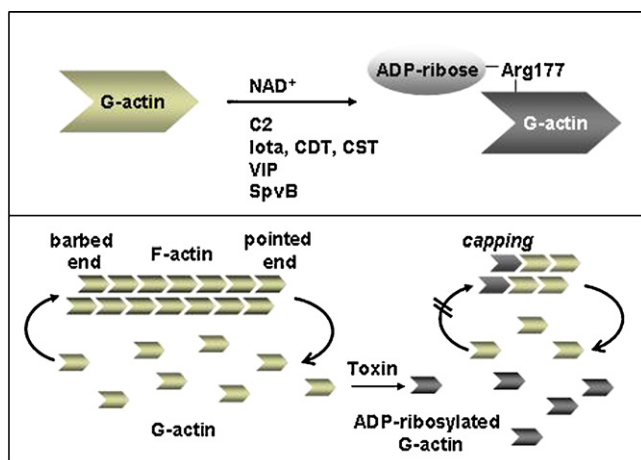
Fig. 1. Depiction of the mode of action of bacterial toxins and effectors, which mono-ADP-ribosylate actin at arginine-177. ADP-ribosylated G-actin binds to the barbed ends of F-actin, where it acts as a capping protein and prevents the further addition of unmodified G-actin molecules. The pointed ends are not affected. In consequence, actin polymerization is blocked and the filaments disassemble. C2, *C. botulinum* C2 toxin; CDT, *C. difficile* binary toxin; CST, *C. spiroforme* toxin; VIP, vegetative insecticidal protein; SpvB, *S. enterica* virulence factor.

The molecular consequences of the toxin-catalyzed ADP-ribosylation of actin were first investigated for C2 toxin, the prototype of this toxin family (Fig. 1). ADP-ribosylation at arginine-177 turns G-actin into a "capping protein", which binds to the barbed ends of actin filaments and thereby blocks the addition of non-modified G-actin to this end (Wegner and Aktories, 1988; Weigt et al., 1989). In contrast, ADP-ribosylated G-actin does not affect the pointed end of actin filaments. In consequence, this leads to an increase of the "critical concentration" of actin polymerization (Weigt et al., 1989) and thereby to a complete depolymerization of actin filaments resulting in cytoskeletal disruption and rounding up of cultured monolayer cells (Miyake and Ohishi, 1987; Ohishi et al., 1984; Wiegers et al., 1991; Aktories and Wegner, 1989). Recently, we have discovered that ADP-ribosylation of actin by C2, iota and CDT toxins resulted in caspase-dependent cell death (Heine et al., 2008; Hilger et al., 2009). Although the overall mode of action of all members of this toxin family is comparable, C2 toxin can be distinguished from the group of iota-like toxins, including iota, CDT, and CST. The iota-like toxins are very closely related and their components are interchangeable and form functional toxin complexes among each other but not with the components of C2 toxin (Gülke et al., 2001; Perelle et al., 1997b; Popoff, 1987). Another interesting difference is that iota toxin ADP-ribosylates β/γ-actin as well as α-actin, while C2 toxin only modifies β/γ-actin (Mauss et al., 1990; Ohishi, 2000; Schering et al., 1988).

In 2000, the SpvB protein from *Salmonella enterica* was identified as another actin-ADP-ribosylating virulence factor, which is not an exotoxin but delivered from intracellular-located *Salmonella* into the host cell cytosol, most likely via a type-III-secretion mechanism (Lesnick et al., 2001; Tezcan-Merdol et al., 2001). We performed a detailed biochemical analysis of the catalytic domain of the SpvB protein (C/SpvB) and identified arginine-177 as the modification site for C/SpvB in an actin homologue of a *Drosophila* indirect flight muscle protein (Hochmann et al., 2006). Later, it was confirmed by another laboratory that this is the modification site in actin (Margarit et al., 2006). Like iota toxin, C/SpvB ADP-ribosylated β/γ-actin as well as α-actin (Hochmann et al., 2006). Finally, we investigated the consequences following ADP-ribosylation of actin by C/SpvB in intact cells by a genetically engineered cell-permeable C/SpvB fusion toxin and compared the effects with the effects caused by the binary clostridial exotoxins (Pust et al., 2007). The

recent findings of this research as well as the new insights into the cellular uptake mechanism of binary actin ADP-ribosylating toxins are summarized in this review.

The C2 toxin from *Clostridium botulinum*

The C2 toxin is produced by *C. botulinum* types C and D and was isolated in 1980 by Ohishi and co-workers (Ohishi et al., 1980a), who demonstrated that the enzyme component C2I and the binding/translocation component C2II act synergistically to mediate the cytotoxic effect, i.e. cell rounding (Ohishi et al., 1984). In 1986, Aktories and co-workers identified actin as the eukaryotic substrate of C2 toxin thereby introducing the new family of actin ADP-ribosylating toxins (Aktories et al., 1986). Subsequently, it was shown that C2I and other members of this toxin family (e.g. C. perfringens iota toxin) ADP-ribosylate actin at arginine177 (Vandekerckhove et al., 1987, 1988).

The pathophysiological role of C2 toxin is not completely understood so far, although the molecular consequences following ADP-ribosylation of actin have been investigated in detail. As a consequence of C2 toxin-induced depolymerization of actin filaments, adherent cells round up and epithelial and endothelial cells loose their barrier functions (Ohishi et al., 1980b; Ohishi, 1983a, 1983b; Wiegers et al., 1991). Therefore, C2 toxin is a potent enterotoxin that causes necrotic, hemorrhagic lesions in the intestinal wall (Ohishi and Odagiri, 1984). It was demonstrated that application of 2 pmole of purified C2 toxin kills mice, rats, guinea pigs, and chickens within 1 h (Simpson, 1982). The half lethal dose ($LD_{50, i.v.}$) of purified C2 toxin for mice is less than 50 fmole. However, C2 toxin does not cause the typical symptoms of botulism, i.e. flaccid paralysis (Simpson, 1982). All *C. botulinum* C and D strains that produce C2 toxin also produce the extremely potent neurotoxins, which cause botulism, and therefore the role of C2 toxin in disease is still not completely understood. Recently, it was demonstrated that the binary actin-ADP-ribosylating toxins from *Clostridia*, including C2 toxin, induce the formation of long microtubule-based protrusions, which form a dense meshwork on the surface of intestinal epithelial cells (Schwan et al., 2009). *Clostridia* adhere to these protrusions and the amount of bacteria attached to the cell layer is increased about 4–5 fold. Importantly, a comparable effect is observed in the large intestine of toxin-treated mice (Schwan et al., 2009). These new findings point to a possible role of actin ADP-ribosylating toxins in facilitating colonization of toxin producing clostridia.

The binding/transport component C2II mediates uptake of the toxin

The binding/translocation component C2II is a protein of 80 or 100 kDa, depending on the individual C2 toxin-producing strain of *C. botulinum* (Barth et al., 2000; Sterthoff et al., 2010). C2II requires proteolytic activation at lysine-181 within its N-terminal domain to gain its biological activity (Barth et al., 2000; Ohishi, 1987). The molecular consequences of C2II activation have been investigated in our laboratories in detail. A ~20 kDa peptide is removed from the N-terminal domain of C2II by trypsin *in vitro* (Barth et al., 2000). Under physiological conditions, this activation might occur within the gut by host and/or bacterial proteases. The activated protein forms ring-shaped heptamers (C2IIa), which mediate two essential steps during toxin uptake into eukaryotic cells (Barth et al., 2000) (Fig. 2). First, C2IIa enables binding of C2I to the cell surface and later, after internalization of the C2IIa/C2I complex, C2IIa mediates membrane translocation of C2I from endosomal vesicles into the host cell cytosol. We have demonstrated that complex formation between C2IIa and C2I can either occur prior to binding of C2IIa to the cell surface or C2I binds to already cell-bound C2IIa (Kaiser
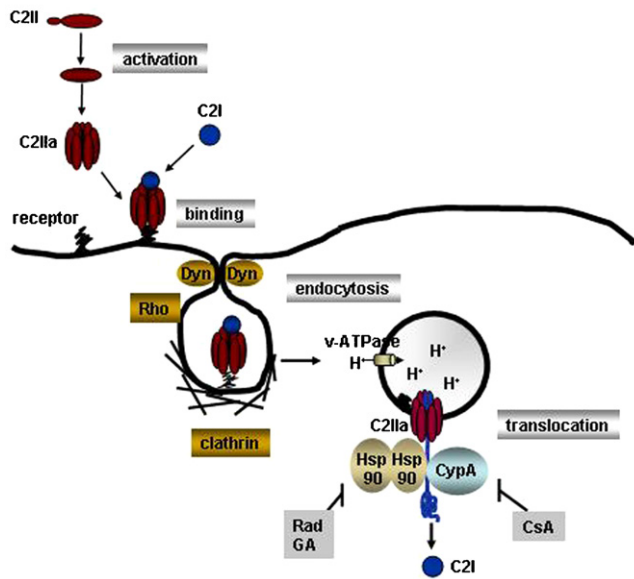
Fig. 2. Uptake of the binary C2 toxin from *C. botulinum* into mammalian cells. First, the binding/translocation component is proteolytically activated. The activated C2IIa heptamer binds to a carbohydrate receptor on the cell surface and assembles with the enzyme component C2I. The toxin complex is internalized via clathrin- and Rho-dependent endocytosis and reaches early endosomal compartments. Following acidification of the endosomal lumen, C2IIa inserts into the endosomal membrane and forms pores. Finally, C2I unfolds to translocate through the pores into the cytosol. This step is facilitated by the host cell factors Hsp90 and cyclophilin A. Dyn, dynamin; Rad, radicicol; CsA, cyclosporine A.

et al., 2006). C2IIa binds via its C-terminal domain to complex, hybrid carbohydrate structures, which were found on all eukaryotic cell types (Eckhardt et al., 2000). This might explain the sensitivity of all yet tested eukaryotic cell types towards C2 toxin. Receptor-mediated endocytosis of the toxin complex occurs via dynamin- and clathrin-dependent pathways and Rho was identified as a crucial factor for this process (Pust et al., 2010). Following endocytosis, the toxin complex is delivered to early endosomal vesicles (Barth et al., 2000). There, triggered by the acidic conditions in the lumen of early endosomes, C2IIa heptamers convert from their pre-pore to the pore conformation and insert as functional pores into the endosomal membranes. The pore formation is absolutely essential for the subsequent translocation of C2I from the endosomal lumen into the cytosol and our earlier results strongly suggested that C2I translocates through the lumen of the C2IIa pores across membranes (Schmid et al., 1994; Bachmeyer et al., 2001; Barth et al., 2000; Blöcker et al., 2003a, 2003b). The narrow inner diameter of 2–3 nm for the C2IIa pre-pore structure implies at least partial unfolding of the C2I protein ($\sim$50 kDa) because translocation of C2I in its native conformation would require a minimum pore diameter of more than 4 nm (Schleberger et al., 2006). However, translocation of C2I as an unfolded protein raised the questions how it becomes refolded in the cytosol and whether host cell factors, such as chaperones and/or protein folding helper enzymes might be involved in this process.

Host cell chaperones facilitate membrane translocation of C2I

In 2003, we demonstrated that chaperone activity of the heat shock protein Hsp90 was crucial for the uptake of C2I into the cytosol of cultured epithelial cells, in particular in translocation of C2I across the membranes of early endosomal vesicles (Haug et al., 2003a). Pharmacological inhibition of Hsp90 activity by either radicicol or geldanamycin protected cells from intoxication by C2 toxin. Later, we found comparable results for the iota and CDT

toxins (Haug et al., 2004), implying a common Hsp90-dependent membrane translocation mechanism among this toxin family.

Recently, we reported that cyclophilin A, a peptidyl prolyl *cis/trans* isomerase (PPIase) that accelerates the *cis/trans* isomerization of peptide bonds after proline residues, facilitates membrane translocation of C2I (Kaiser et al., 2009). Treatment of cells with cyclosporin A (CsA), a specific pharmacological inhibitor of cyclophilins, prevented the uptake of C2I into the cytosol. Importantly, CsA had no influence on the ADP-ribosyltransferase activity of C2I or the early steps of toxin uptake, such as receptor binding, endocytosis and pore formation. CsA, as well as a specific antibody against cyclophilin A inhibited the release of active C2I ADP-ribosyltransferase from partially purified early endosomal vesicles into the cytosol *in vitro* indicating that cyclophilin A is decisively involved in membrane translocation of C2I. Moreover, the combination of CsA and radicicol showed a synergistic protective effect on cells challenged with C2 toxin (Kaiser et al., 2009), implying that cyclophilin A and Hsp90 act in a concerted manner to facilitate translocation of C2I across endosomal membranes (Fig. 2).

We demonstrated that C2I interacts with Hsp90 and cyclophilin A in intact mammalian cells and pull down experiments revealed that this interaction occurs via the N-terminal domain of C2I (Kaiser et al., 2009). This finding is plausible, because we have discovered earlier that the N-terminal domain of C2I interacts with C2IIa and mediates the translocation of the C2I protein through the C2IIa pore (Barth et al., 1998a, 2002). The new observation that cyclophilin A facilitates membrane translocation of C2 toxin is the first report that host cell PPIases are involved in cellular uptake of any bacterial toxin.

The ADP-ribosyltransferase C2I

The enzyme component C2I consists of two functionally different domains (Fig. 3). The N-terminal domain (C2IN, amino acid residues 1–225) is enzymatic inactive and interacts with C2IIa to mediate the uptake of C2I into the cytosol (Barth et al., 1998a, 2002). Therefore, C2IN was exploited as an adaptor for the efficient C2IIa-mediated transport of "foreign" cargo proteins into various eukaryotic cell types (Barth et al., 1998a). Recombinant C2 fusion toxins have been used as cell-permeable *Molecular Trojan Horses* in cell biology and experimental pharmacology to investigate vari-
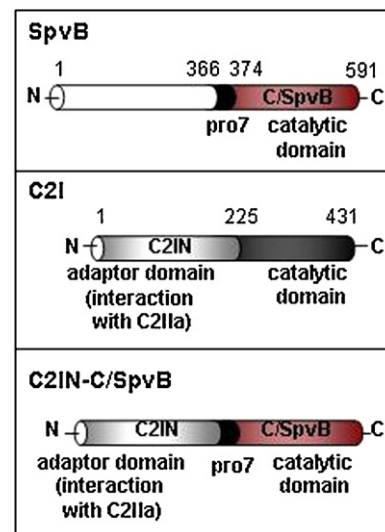
Fig. 3. Domain structures of *S. enterica* SpvB, *C. botulinum* C2I and the recombinant C2IN-C/SpvB fusion toxin. The C2IN domain of C2IN-C/SpvB interacts with the C2IIa binding/translocation component of the *C. botulinum* C2 toxin and mediates the transport of C2IN-C/SpvB into the cytosol of eukaryotic cells via the C2 toxin uptake mechanism. The C2IN and C/SpvB domains are connected by 7 proline residues.

ous cellular functions in intact cells and tissues (Barth et al., 2004; Barth and Stiles, 2008). The C-terminal part of C2I harbors the ADP-ribosyltransferase domain, which contains the *catalytic* amino acid residues glutamate-387, glutamate-389, serine-384, and arginine-299 (Barth et al., 1998b; Schleberger et al., 2006). These residues are essential for enzyme activity and highly conserved among the various bacterial mono-ADP-ribosyltransferases. After its uptake into the cytosol, C2I catalyzes the mono-ADP-ribosylation of G-actin, leading to a complete depolymerization of actin filaments and the cellular consequences following ADP-ribosylation of actin are described later in this article.

All family members of binary actin ADP-ribosylating toxins modify G-actin at arginine-177. However, there are differences among these toxins concerning their substrate specificity towards the actin isoforms. C2I modifies β/γ non-muscle G-actin and γ-smooth muscle actin but is about 400-fold less active towards α-actin while the iota-like toxins do not distinguish between the actin isoforms (Mauss et al., 1990; Schering et al., 1988). It has been suggested that the amino acid residue 361 of C2I and Ia is crucial for the modification of the individual actin isoforms (Schleberger et al., 2006). C2I contains serine at this position and the crystal structure revealed that this side-chain adopts a defined conformation and forms a salt-bridge with the diphosphate of NAD⁺. Ia contains an arginine residue at position 361 and the exchange of serine to arginine in C2I resulted in a "iota a-like" C2IS361R protein, which showed increased ADP-ribosyltransferase activity towards all actin isoforms and modified α-actin (Schleberger et al., 2006).

The virulence factor SpvB from *Salmonella enterica*

Besides the binary exotoxins, the virulence factor SpvB (*Salmonella* plasmid virulence B) from *Salmonella enterica* was identified as a new member of bacterial actin-ADP-ribosylating enzymes (Guiney and Lesnick, 2005; Lesnick et al., 2001; Otto et al., 2000; Tezcan-Merdol et al., 2001). *S. enterica* is a Gram-negative, food-borne pathogen, which causes diseases ranging from mild gastroenteritis to severe systemic infections in humans. An infection by *Salmonella* is associated with its intracellular growth in macrophages, where the bacteria grow and replicate in so-called *Salmonella*-containing vacuoles (SCV). Following replication, the bacteria are released from these vesicles and induce cell death of infected macrophages. The intracellular pathogenesis and therefore the virulence of *Salmonella* depend on the presence of the *Salmonella* pathogenicity island 2 (SPI2), which encodes for several effectors and for a type-III secretion system on the one hand and on the spv-plasmid, which contains the *Salmonella plasmid virulence* (spv) gene cluster on the other hand (Fang et al., 1991; Grob and Guiney, 1996; Guiney et al., 1995). The latter contains the spvB gene, which encodes for the ADP-ribosyltransferase SpvB, an essential factor for intracellular growth of *Salmonella* and therefore an essential virulence factor (for review see Guiney and Lesnick, 2005). In contrast to the exotoxins, the SpvB protein must be delivered from intracellular growing bacteria into the host cell cytosol and it was supposed that transport of SpvB into the cytosol occurs via the SPI2-encoded type-III-secretion system (Lesnick et al., 2001).

In 2000, the spvB gene-product of the *Salmonella enterica* virulence plasmid (spv) was identified as a mono-ADP-ribosyltransferase based on sequence homology to known ADP-ribosyltransferases (Tezcan-Merdol et al., 2001). The C-terminal part of SpvB (C/SpvB, amino acid residues 375–591, see Fig. 3) contains the ADP-ribosyltransferase domain (amino acid residues 390–591). This domain shares homology to the enzyme components of the binary actin-ADP-ribosylating exotoxins and harbors the highly conserved *catalytic* amino acid residues found among bacterial mono-ADP-ribosyltransferases including the biglutamic acid motif, which is characteristic for arginine-specific bacterial

mono-ADP-ribosyltransferases (Lesnick et al., 2001). In 2006 Margarit et al. solved the crystal structure of the ADP-ribosyltransferase domain of SpvB and found that SpvB shows key similarities to earlier characterized ADP-ribosyltransferases (Margarit et al., 2006). The active site residues are located similarly to equivalent residues of other enzymes of this family but the catalytic domain of SpvB differs from other actin-modifying ADP-ribosyltransferases by a 30 amino acid insertion in a helical subdomain (residues 415–445) and by its molecular surface.

Lesnick et al. (2001) expressed SpvB in CHO cells and showed by phalloidin staining of F-actin that the ADP-ribosylating activity of SpvB depolymerizes actin filaments in these cells. Furthermore, SpvB wild type but not the E538/540D mutant, which lacks ADP-ribosyltransferase activity, was essential for virulence in mice after infection with the respective *Salmonella* strain (Lesnick et al., 2001). The C-terminal domain of SpvB ADP-ribosylates a ∼40 kDa target protein in lysates of Jurkat cells (Otto et al., 2000) and Tezcan-Merdol et al. (2001) reported that the catalytic domain of SpvB as well as the full length SpvB protein ADP-ribosylates actin in macrophage-like cells and in epithelial cells. *In vitro*, the catalytic domain of SpvB prevents actin polymerization. Epithelial cells, which are infected with *S. enterica* (strain SH9325) show morphological alterations and the content of F-actin in these cells is decreased (Tezcan-Merdol et al., 2001). Prompted by these findings, we performed a detailed biochemical analysis of the catalytic domain of SpvB and identified arginine-177 as the modification site for C/SpvB in the recombinant actin isoform Act(88F) from *Drosophila* indirect flight muscle (Hochmann et al., 2006). Later, another laboratory confirmed that this is the modification site in actin, too (Margarit et al., 2006). Like iota toxin, C/SpvB ADP-ribosylated β/γ-actin as well as α-actin (Hochmann et al., 2006).

The function of the N-terminal domain of SpvB is not known so far. Interestingly, this domain shares homology to the secretory protein TcaC from *Photorhabdus luminescens*, an insect pathogen (Otto et al., 2000). The N- and the C-terminal domains of SpvB are connected by 7 proline residues and our results obtained with a C2 toxin-derived C/SpvB fusion toxin suggest that the proline residues are involved in translocating the protein into the cytosol (Pust et al., 2007). It should be mentioned that *Photorhabdus luminescens* produces an actin ADP-ribosylating toxin, called *photox*, which possesses an ADP-ribosyltransferase domain similar to the catalytic domain of SpvB and binary actin modifying toxins (Visschedyk et al., 2010). Also this toxin modifies actin in arginine-177.

ADP-ribosylation of actin by binary clostridial toxins and SpvB

Seminal studies on the molecular mode of action and cellular consequences following ADP-ribosylation of actin by C2 toxin were done by Aktories and co-workers in the late eighties. They found that C2I catalyzes the covalent transfer of the ADP-ribose moiety from NAD⁺ to arginine-177 of G-actin (Aktories et al., 1986). Later, it became evident that all other toxins of this family behave similarly although they differ in some aspects as described before in detail. Prompted by the finding that the virulence factor SpvB from *S. enterica* ADP-ribosylates actin at arginine-177 (Hochmann et al., 2006), we compared the functional consequences of ADP-ribosylation of actin induced by a cell-permeable SpvB fusion toxin and by C2 toxin in intact cells (Pust et al., 2007).

Molecular consequences following ADP-ribosylation of actin at arginine-177

ADP-ribosylation of actin in arginine-177 inhibits actin polymerization (Aktories et al., 1986; Reuner et al., 1987). Subsequently,

it was discovered that mono-ADP-ribosylation at arginine-177 turns G-actin into a "capping protein", which can bind to the barbed ends of F-actin but then prevents the addition of further non-modified G-actin molecules to this end of the filament, as depicted in Fig. 1 (Wegner and Aktories, 1988; Weigt et al., 1989). ADP-ribosylated G-actin does not affect the pointed end of filaments. In consequence, this mode of action increases the critical concentration for actin polymerization and probably results in a complete depolymerization of actin filaments (Weigt et al., 1989). Quite early Holmes et al. (1990) suggested on the basis of the crystal structure of actin and F-actin modeling that ADP-ribosylation of actin in arginine-177 must cause steric clashes in actin filaments. This view was supported recently by Margarit et al. (2006) showing that the ADP-ribosylation of actin by SpvB at arginine-177 did not result in major conformational changes but leads to a severe steric clash and results in disruption of the contact sites, which hold the actin filaments together. In agreement with this hypothesis is also the finding that G- but not F-actin is modified by the toxins (Aktories et al., 1986). ADP-ribosylation by C2 toxin and by SpvB inhibits the intrinsic ATPase activity of actin (Geipel et al., 1989, 1990; Margarit et al., 2006). Moreover, ADP-ribosylated actin shows an altered interaction with certain actin binding proteins, such as gelsolin, in comparison to unmodified actin. ADP-ribosylation results in a reduced nucleation activity of the actin–gelsolin complex (Just et al., 1993; Wegner et al., 1994; Wille et al., 1992). Finally, the toxin-mediated effects result in breakdown of the actin cytoskeleton and adherent cells round up within 2–3 h of incubation with the toxins.

ADP-ribosylation of actin by C2 toxin and iota-like toxins results in delayed caspase-dependent cell death

Although the immediate consequences following ADP-ribosylation of actin are well understood, the long-term responses of cultured epithelial cells following treatment with such toxins and their final fate were not investigated in detail before. Therefore, we have addressed whether cells, which have been treated with either binary actin ADP-ribosylating toxins or with SpvB, recover by reconstitution of new actin filaments or whether the observed cytopathic action of the toxins results in cell death.

Cells, which have been treated with the binary C2 or iota-like toxins stayed round even though the toxins were removed from the medium after their uptake into the cells (Heine et al., 2008; Hilger et al., 2009). When we analyzed the ADP-ribosylation status of actin in those cells, we only found ADP-ribosylated actin and could not detect unmodified actin within up to 3 days. Thus, the cytopathic effects of C2 and iota-like toxins were not reversible. Interestingly, toxin-catalyzed ADP-ribosylation was not a signal for an accelerated degradation of actin in the tested mammalian cell lines, including epithelial cells, fibroblasts and macrophages (Heine et al., 2008; Hilger et al., 2009) which is an interesting difference to the situation in some non-vertebrate cells, such as amoeba, where ADP-ribosylation at arginine-177 triggers an enhanced proteolytic degradation of actin (Tezcan-Merdol et al., 2005). However, the reason for this remarkable difference is not known so far.

Cells, which have been treated with either C2 or iota-like toxins stayed viable for up to 24 h although cells were round within 2–3 h of toxin treatment (Heine et al., 2008; Hilger et al., 2009). However, C2 toxin-treatment resulted in a cell cycle arrest at the G2-mitosis-boundary, as discovered earlier for synchronized HeLa cells and the key inducers of mitotic entry, p34^{cdc2} kinase and Cdc25-C phosphatase, were not activated in those cells (Barth et al., 1999). More recently, we have reported that a portion of the C2 toxin-treated cells died after about 15–24 h (Heine et al., 2008). This delayed cell death was accompanied by typical hallmarks of apoptosis, including annexin V-staining and cleavage of poly-ADP-ribosyltransferase-1 (PARP-1), an indication of caspase-3 activation. By using specific pharmacological caspase inhibitors, we demonstrated that caspases 8 and 9 were activated in C2 toxin-treated cells (Heine et al., 2008).

The application of the binding/translocation C2IIa together with an enzymatic inactive C2I protein was not cytotoxic, indicating that the ADP-ribosyltransferase activity of C2I was absolutely essential to induce cell death (Hilger et al., 2009). These results implied that the degradation of the actin cytoskeleton might be crucial for induction of cell death, however, the molecular mechanisms connecting the toxin-mediated depolymerization of F-actin and the activation of caspases are not known so far. Treatment of epithelial cells (Vero) with iota toxin or CDT resulted in delayed caspase-dependent cell death, likewise (Hilger et al., 2009). Thus, the iota-like toxins and C2 toxin behave comparably regarding their non-reversible cytopathic mode of action and toxin-mediated cell death.

The long-lived nature of the enzyme components in the host cell cytosol correlates with the ability of actin-ADP-ribosylating toxins to induce cell death

To investigate the consequences following ADP-ribosylation of actin by SpvB in intact mammalian cells, we constructed a recombinant fusion toxin in which the catalytic domain C/SpvB was fused to the C2IN adaptor domain from *C. botulinum* C2I to exploit the binary C2 system for delivery of C/SpvB into the cytosol of target cells (Pust et al., 2007). Treatment of various mammalian cell lines with C2IN-C/SpvB plus C2IIa resulted in the expected depolymerization of actin filaments and cell rounding. The C2IIa-dependent uptake of the C2IN-C/SpvB fusion toxin was blocked by bafilomycin A1 as well as by radicicol and cyclosporine A. This finding implied that this recombinant toxin also translocates from acidified endosomes into the cytosol and that Hsp90 and cyclophilin are crucial for its uptake, just as discovered for C2I. Interestingly, the efficiency of the C2IIa-mediated uptake of C2IN-C/SpvB into the host cell cytosol correlated with the presence and length of the proline linker, which connects the C2IN and C/SpvB domains and, thus, the proline residues might be crucial for membrane translocation of the protein (Pust et al., 2007).

Although C2IN-C/SpvB is taken up into the cytosol of cultured cells just like C2I and induces comparable immediate cytopathic effects, the long term responses of cells treated with C2IN-C/SpvB on the one hand or with C2 or iota-like toxins on the other hand were different and completely unexpected. Most important, the cytopathic effect induced by treatment of cells with C2IN-C/SpvB and C2IIa was transient and cells recovered from intoxication (Hilger et al., 2009). Once intoxicated cells regained a flat morphology and stayed viable for several days. However, those cells failed to divide, resulting in enlarged bi- and multinuclear cells. Along with cell recovery, unmodified actin was detectable in those cells after a while, although the total amount of actin was completely ADP-ribosylated immediately after intoxication by C2IN-C/SpvB (Hilger et al., 2009).

The re-appearance of non-ADP-ribosylated actin was prevented when protein neo-synthesis was blocked in the cells by cycloheximide (Hilger et al., 2009). This observation indicated that after a certain time period the newly synthesized actin was no more modified by C2IN-C/SpvB, implying that there was no more ADP-ribosyltransferase activity in the cytosol at that time point. Consistent with this finding, cells did not recover when fresh C2IN-C/SpvB was frequently applied to the cells in combination with the C2IIa transport component (Hilger et al., 2009). Under such conditions, cells stayed round and died after a further incubation period, just as observed after treatment with the clostridial actin ADP-ribosylating toxins. Moreover, no unmodified actin was detectable in those cells. Consistently, apoptotic cell death was induced when
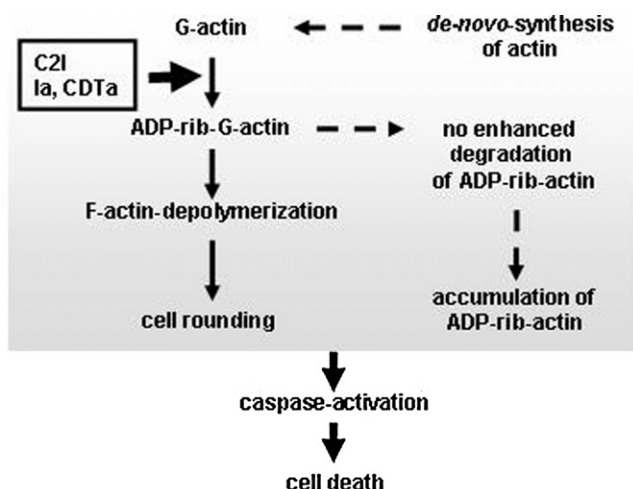
Fig. 4. Model of the cytotoxic effects caused by clostridial actin-ADP-ribosylating toxins. In the cytosol of eukaryotic target cells, the enzyme components of C2 toxin (C2I), iota toxin (Ia) and *C. difficile* transferase (CDTa) mono-ADP-ribosylate actin. This covalent modification results in rapid depolymerization of actin filaments and cell rounding within about 3 h after toxin application. Because the ADP-ribosyltransferases are stable in the cytosol for up to 24–48 h, any newly synthesized actin becomes ADP-ribosylated in the cytosol which prevents the reconstitution of F-actin and the recovery of rounded cells. ADP-ribosylation does not lead to an enhanced degradation of actin and therefore the summary of these events results in an accumulation of ADP-ribosylated actin in the cytosol. After about 18–24 h, apoptotic events become detectable in toxin-treated epithelial cells.

SpvB was permanently expressed in the cytosol of macrophage-like cells, as reported by Kurita et al. (2003).

Taken together, our results obtained with the recombinant C2IN-C/SpvB fusion toxin revealed that complete, yet transient, ADP-ribosylation of actin in the host cell cytosol mediated the same immediate cytopathic effects as observed after intoxication by C2 or iota-like toxins but did not lead to comparable long-term responses of intoxicated cells, including cell death (Hilger et al., 2009). In conclusion, these findings were a strong hint that the enzyme components of the binary actin ADP-ribosylating toxins persist for a relatively long time period as enzyme active ADP-ribosyltransferases in the cytosol, which might be the reason for the non-reversible cytotoxic effect of extremely low amounts of C2 and iota toxins, while the C2IN-C/SpvB fusion toxin lost its enzyme activity much more rapidly in the host cell cytosol.

When we monitored C2I, Ia and C2IN-C/SpvB in the cytosol of intoxicated epithelial cells, enzymatic active C2I and Ia, but not C2IN-C/SpvB ADP-ribosyltransferases were indeed detectable for at least 48 h (Hilger et al., 2009). Moreover, both C2I and Ia proteins, but not C2I-C/SpvB were detectable for up to 48 h in the cytosol by Western blot analysis, indicating that C2I and Ia were rather stable and not extensively degraded in the host cell cytosol. When degradation of the C2IN-C/SpvB fusion toxin was prevented by a pharmacological proteasome inhibitor, cells did not recover from intoxication and consistently no unmodified actin re-appeared.

Conclusions

Finally, it is important to stress that there is no discrepancy between our results obtained with the C/SpvB fusion toxin and earlier reports that the SpvB-catalyzed ADP-ribosylation of actin resulted in apoptosis of human macrophages after about 18–24 h after infection with *Salmonella*. In our studies, we have used the recombinant C2IN-C/SpvB fusion protein as a tool to unravel the impact of actin-ADP-ribosylation upon apoptosis. However, from our experiments, no conclusions can be drawn concerning the mode of action for full-length SpvB in cells, because SpvB translo-

cates from intracellular *Salmonella* into the cytosol while the C/SpvB fusion toxin was internalized by the C2 toxin-specific uptake mechanism. Therefore, the amounts, the intracellular localization and the degradation of the C/SpvB fusion toxin and the full length SpvB in the host cell cytosol are likely not comparable.

We have demonstrated the long-lived nature of the C2I and Ia proteins in the cytosol of mammalian cells and in combination with our observations with the C/SpvB fusion toxin, we concluded that this remarkable stability of the C2 and iota-like toxins might be crucial for the observation that toxin-treated cells never recovered and finally died. According to our current model (Fig. 4), the long-lived nature of clostridial ADP-ribosyltransferases in the cytosol leads to ADP-ribosylation of any newly synthesized G-actin which prevents reconstitution of actin filaments and recovery of intoxicated cells. Permanent ADP-ribosylation of actin by persistent, enzymatically active ADP-ribosyltransferases is most likely essential for the toxin-mediated, delayed cell death, induced by the binary actin ADP-ribosylating exotoxins from *Clostridia*.

Finally, it is not clear whether the data obtained with cultured cells mirrors the situation in animals but the new knowledge on toxin-induced cell death might explain the hemorrhagic lesions in the intestinal wall of C2 toxin-treated animals and might contribute towards better understanding of the role of CDT in *C. difficile*-associated diseases, such as *pseudomembranous colitis*.

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