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Large clostridial cytotoxins

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Abstract The large clostridial cytotoxins are a family of structurally and functionally related exotoxins from *Clostridium difficile* (toxins A and B), *C. sordellii* (lethal and hemorrhagic toxin) and *C. novyi* (α-toxin). The exotoxins are major pathogenicity factors which in addition to their in vivo effects are cytotoxic to cultured cell lines causing reorganization of the cytoskeleton accompanied by morphological changes. The exotoxins are single-chain protein toxins, which are constructed of three domains: receptor-binding, translocation and catalytic domain. These domains reflect the self-mediated cell entry via receptor-mediated endocytosis, translocation into the cytoplasm, and execution of their cytotoxic activity by an inherent enzyme activity. Enzymatically, the toxins catalyze the transfer of a glucosyl moiety from UDP-glucose to the intracellular target proteins which are the Rho and Ras GTPases. The covalent attachment of the glucose moiety to a conserved threonine within the effector region of the GTPases renders the Rho-GTPases functionally inactive. Whereas the molecular mode of cytotoxic effects is fully understood, the mechanisms leading to inflammatory processes in the context of disease (e.g., antibiotic-associated pseudomembranous colitis caused by *Clostridium difficile*) are less clear.

Abbreviations *CDAD: Clostridium difficile*-associated diarrhea · *C3:* ADP-ribosyltransferase C3 from *Clostridium botulinum* · *LCT:* Large clostridial cytotoxin · *TcdA: Clostridium difficile* toxin A · *TcdB: Clostridium difficile* toxin B · *TcsL: Clostridium sordellii* lethal toxin · *TcsH: Clostridium sordellii* hemorrhagic toxin · *TcnA: Clostridium novyi* alpha-toxin

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Introduction

Large clostridial cytotoxins are a family of protein toxins put together on the basis of their cytotoxic activity, comparable enzyme activity, and same primary structure. Their cytotoxic activity is characterized by disaggregation of the actin cytoskeleton accompanied by cell rounding and formation of an arborized morphology. The family encompasses toxin A (TcdA) and toxin B (TcdB) from *Clostridium difficile*, the lethal (TcsL) and the hemorrhagic toxin (TcsH) from *Clostridium sordellii,* and the α-toxin (TcnA) from *Clostridium novyi.* The members are single-chain toxins with molecular masses of 250–308 kDa, autotransporters which mediate their own uptake into the target cells and exhibit transferase activity to covalently modify Rho- and Ras-GTPases. In contrast to their in vitro effects on the cytoskeleton of cultivated cells, the cytotoxins differ in their in vivo effects; they are major pathogenic factors which are causative for different diseases and clinical outcomes, respectively. Clinically most important is *C. difficile* which coproduces toxin A and toxin B, both causally involved in the antibiotic-associated diarrhea and the severe form, the pseudomembranous colitis (Bartlett 1994; Kelly and LaMont 1998; Kelly et al. 1994). Lethal toxin from *C. sordellii* is involved in diarrhea and enterotoxemia in domestic animals and in gas gangrene in human, whereas *C. novyi* α-toxin has been identified as causative agent for gas gangrene infections in human and animals (Hatheway 1990). The difference between comparable cytotoxic effects but different clinical features is likely based on the different organ targeting of the toxin-producing bacteria which colonize the gut or injured organs. Additional strain-specific pathogenic factors are most likely involved.

Because of their involvement in antibiotic-associated diarrhea and pseudomembranous colitis, toxins A and B from *C. difficile* are the best-characterized toxins of this family. The chief risk factor for pseudomembranous colitis is the exposure to antibiotics. Especially broad-spectrum antibiotics are thought to alter the normal microflora of the gut thereby allowing colonization and growth of *C. difficile*. The normal microflora seems to create an environment which is restrictive for *C. difficile* growth rather than to generate inhibitory factors (for reviews see Bartlett 2002; Kelly and LaMont 1998; Kelly et al. 1994; Mylonakis, Ryan et al. 2001; Stoddart and Wilcox 2002; Surawicz and McFarland 1999). In animal models, the toxins are able to induce all the symptoms of antibiotic-associated diarrhea and pseudomembranous colitis, i.e., secretory diarrhea, mucosal damage and inflammation of the mucosa (Lyerly et al. 1985; Triadafilopoulos et al. 1987, 1989). Based on these findings, both toxins have been classified as chief pathogenicity factors of *C. difficile*.

Since toxin A induces fluid accumulation in the ileum of animals and ileal explants, it was designated as enterotoxin. Toxin B, which does not possess direct toxic activity towards animal ileum (Lyerly et al. 1985; Triadafilopoulos et al. 1987), is about 100- to 1,000-fold more cytotoxic than toxin A to cultured cell lines and has therefore been named cytotoxin (Lyerly et al. 1982). The cytotoxic activity of toxins A and B differs only with respect to potency but not with respect to the cytotoxic feature. Both toxins induce shrinking and rounding of cultured cells, initially accompanied by formation of neurite-like retraction fibers. These retraction fibers disappear in the course of intoxication and the cells are completely rounded. In the terminal phase the cells partially detach. The morphological changes are accompanied by disaggregation of the actin cytoskeleton. The cell-spanning stress fibers disappear and the remainder of the actin filaments accumulates in the perinuclear space (Ciesielski-Treska et al. 1989; Fiorentini et al. 1989, 1990; Fiorenti-

ni and Thelestam 1991; Malorni et al. 1990; Siffert et al. 1993). The other members of the family of large clostridial cytotoxins, i.e., α-toxin, and lethal and hemorrhagic toxin, induce the same effects with some variations in detailed aspects (Bette et al. 1991; Ciesielski-Treska et al. 1991; Oksche et al. 1992; Popoff 1987).

Genetic organization of the toxins

The toxins of *C. difficile* (Barroso et al. 1990; Sauerborn and Von Eichel-Streiber and Sauerborn 1990; Von Eichel-Streiber et al. 1990, 1992a) and *C. sordellii* (Green et al. 1995) are encoded by the chromosomal DNA, whereas the gene of α-toxin from *C. novyi* (Hofmann et al. 1995; Schallehn et al. 1980) is phage-encoded. *C. difficile* toxins A and B are encoded by two separate genes which are part of a conserved chromosomal region called pathogenicity locus (PaLoc), having a size of 19.6 kb (Braun et al. 1996; Hammond and Johnson 1995). The PaLoc carries five genes *tcdA* to *E: tcdA* encodes for toxin A, *tcdB* for toxin B, *tcdD* (also known as *txeR*) for an RNA polymerase sigma factor required for the activation of toxin gene expression (Dupuy and Sonenshein 1998; Mani and Dupuy 2001) and *tcdE* for a holin-like pore-forming protein (Gründling et al. 2001; Hundsberger et al. 1997). The holin-like tcdE is postulated to permeabilize the clostridia during the stationary phase, thereby releasing toxins A and B (Mukherjee et al. 2002). This notion fits very well with the finding that the toxins do not possess a signal sequence which is in charge for a regulated export.

Although toxins A and B are usually coexpressed, variant toxinogenic strains exist which can be characterized by PCR ribotyping. The most-studied variant of *C. difficile* is a strain that only produces toxin B but not toxin A (A^-B^+) (Borriello et al. 1992; Depitre et al. 1993; Von Eichel-Streiber et al. 1995; Rupnik et al. 1998; Sambol et al. 2000). The basis for this is a deletion in the *tcdA* gene. In addition to toxins A and B, a third toxin, named CDT, is released by some strains (Geric et al. 2003; Perelle et al. 1997; Rupnik et al. 2003a). CDT is related to *Clostridium perfringens* iota toxin and is composed of two separate nonlinked components. The genes coding for both components are chromosomally located, but outside the PaLoc (Perelle et al. 1997; Popoff et al. 1988). The CDTb is the binding component which mediates the cell entry of the enzymatic component CDTa. CDTa exhibits ADP-ribosyltransferase activity to covalently modify cellular actin. ADP-ribosylated actin is incapable of polymerizing and thus results in complete destruction of the actin cytoskeleton. The role of CDT in pathogenesis has not been characterized yet.

Preparation of large clostridial toxins

The toxins are purified in a classical biochemical procedure. The purification steps are: growth of the clostridia under anaerobic conditions, ammonium sulfate precipitation of proteins from the culture supernatant, and ion exchange chromatography of the resolubilized proteins to separate toxin A from toxin B. Toxin A is further purified by using a thyreoglobulin affinity purification step. An affinity purification for toxin B is not available and only a subsequent gel permeation chromatography will improve the purity of toxin B to some degree. Lethal toxin from *C. sordellii* is purified analogous to toxin B, but with low yield and poor purity (Just et al. 1997; Krivan and Wilkins 1987; Moos and Von

Eichel-Streiber 2000; Von Eichel-Streiber et al. 1987). The master way to solve the problem of purity is the expression of recombinant toxins. In case of toxin B, the application of recombinant toxin B purified from an *E. coli* expression system was reported, but properties and purity of the recombinant toxin have not been mentioned (Pfeifer et al. 2003). In contrast to the *E. coli* expression system, toxin A preparation was reported from a *Bacillus megaterium* system (Burger et al. 2003). Recombinant toxin A is enzymatically more active than classically purified toxin A and differences in biological activity suggest the presence of biologically active contaminations in classically purified toxin A (R. Gerhard and I. Just, unpublished data).

For long-term storage, the LCT should be frozen at −80°C in the presence of 20% of glycerol. However, even correct long-term storage in the range of more than 6 months results in continuous decrease in activity. Repeated thawing and freezing should be strictly avoided because of dramatic loss of activity. For short-term storage, the toxins are stable at 4°C for 1–2 weeks, when the concentration is above 100 µg/ml.

Structure of the toxins

All large clostridial cytotoxins possess the same primary structure (Fig. 1). They are single-chain proteins which seem to exist in a monomeric form. Currently, three domains are

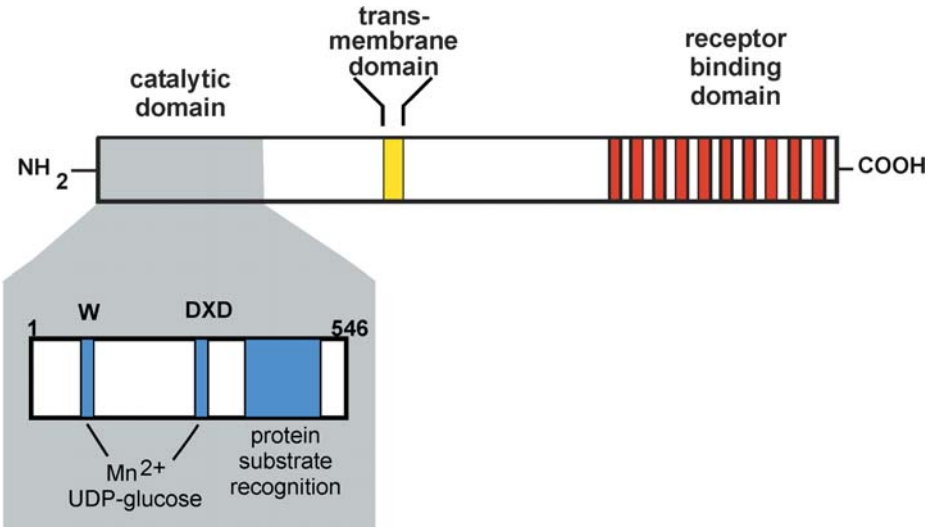

Fig. 1 Structure of the large clostridial toxins illustrated for toxin B. Toxin B is constructed of three functional domains. The receptor-binding domain is composed of repetitive oligopeptide elements commonly accepted as motif for binding to sugar structures. The possible multivalent binding to cell surface structures induces receptor-mediated endocytosis. A hydrophobic region exhibits a putative transmembrane domain which is thought to form a pore or channel, thereby allowing the catalytic domain to translocate into the cytoplasm. The catalytic domain executes the mono-glucosyltransferase activity to modify the Rho-GTPases. The first 546 residues of the N-terminus are the minimum size of the catalytic domain. The tryptophan-102 (*W*) and the D X D motif (residues 286–288) are involved in UDP-glucose cosubstrate binding through Mn^{2+} or Mg^{2+}. The C-terminal part (residues 408–468) of the catalytic domain covers the protein substrate recognition site.

assigned which reflect cell entry and biological activity. At the C-terminus, the receptor-binding domain is responsible for binding to and the uptake into the target cell. The intermediate part harbors the so-called transmembrane domain responsible for translocation of the toxin from the endosomes to cytoplasm. Finally, the N-terminally located catalytic domain exhibits mono-glucosyltransferase activity responsible for the modification of the intracellular target proteins. Only α-toxin possesses *N*-acetyl-glucosamine-transferase activity.

Receptor binding domain: The C-terminally located binding domain covers up to one third (in the case of toxin A) of the LCT molecule. Based on the primary sequence, it exhibits characteristic features which are described as repetitive peptide elements called combined repetitive oligopetides (CROPs) (Von Eichel-Streiber 1993; Von Eichel-Streiber et al. 1996). These CROPs consist of 20–22 or 50 amino acids. Most evident is the repetitive structure in the toxin A molecule which possesses 38 of these CROPs. The repeating units show homology with the carbohydrate-binding regions of glycosyltransferases from *Streptococcus mutans* (Von Eichel-Streiber and Sauerborn 1990; Von Eichel-Streiber et al. 1992b). This homology was the first hint that especially toxin A binds to cellular carbohydrate structures.

However, current pattern and motif scans using InterProScan (http://www.ebi. ac.uk/InterProScan/ EMBL-EBI), SMART (http://smart.embl-heidelberg.de/ EMBL) or Motif Scan (http://hits.isb-sib.ch/cgi-bin/PFSCAN/ ISREC) result in the proposals of putative cell wall binding repeats (CW). A CW repeat is 20 amino acid residues long and contains conserved aromatic residues and glycines. These repeats in multiple tandem copies are suggested to be responsible for the specific recognition of choline-containing cell-walls described for choline-binding proteins from *Streptococcus pneumonia* (Garcia et al. 1998; Rosenow et al. 1997). Similar but longer repeats were found in glucosyltransferases and glucan binding proteins from streptococci as mentioned above. A hint that the assignment of the C-terminal part as putative choline-binding may be of functional relevance is the finding that toxin A binds to nontoxicogenic *C. difficile* strains, thereby mediating binding of the clostridia to cells (P. Borriello, personal communication).

It is now generally accepted that the repetitive C-terminal region of the toxins is the receptor-binding domain. Four findings strongly argue for this notion:

1. The modular organization of this region (Von Eichel-Streiber et al. 1992b; Wren 1991).
2. The homology with the carbohydrate binding domain of bacterial proteins (Von Eichel-Streiber and Sauerborn 1990; Von Eichel-Streiber et al. 1992b).
3. The inhibition of the cytotoxic activity of toxin A by a monoclonal antibody recognizing an epitope of this domain (Frey and Wilkins 1992; Lyerly et al. 1986) and by a polyclonal antibody which selectively recognizes the C-terminal part of the toxin (Genth et al. 2000).
4. The sole receptor-binding domain functionally competes with the full-length toxin to delay the onset of cytotoxicity (Frisch et al. 2003).

Translocation domain: Based on secondary structure prediction, amino acid residues from about 1,030 to 1,100 are postulated to form a putative transmembrane-spanning region which is proposed to form a channel, thereby mediating the translocation of the toxin into the cytosol. However, the function of this putative domain has not been yet proven and it is only theoretically assigned.

Catalytic domain: Except for α-toxin all other large clostridial cytotoxins exhibit glucosyltransferase activity to glucosylate intracellular target proteins. They use the nucleotide sugar UDP-glucose as cosubstrate and transfer the glucose moiety to the target protein, the Rho-GTPases. α-toxin uses UDP-*N*-acetyl-glucosamine, thus, catalyzing glcNAcation of the Rho-GTPases. The minimal size of the functional catalytic domain spans the first 546 amino acids of the N-terminal part for toxin B and lethal toxin (Hofmann et al. 1997, 1998), whereas the catalytic domain of toxin A covers 659 residues (Faust et al. 1998). The essential structural element for enzyme activity is the so-called D-X-D motif, an amino acid sequence composed of aspartic acid-any amino acid-aspartic acid (Busch et al. 1998). This D-X-D motif, which is conserved in all clostridial cytotoxins, is present in various pro- and eukaryotic glycosyltransferases. UDP-glucose binding within the catalytic cleft of several eukaryotic glycosyltransferases is based on divalent metal ion-dependent coordination which structurally requires two adjacent aspartic acids, the D-X-D motif (Wiggins and Munro 1998). None of the aspartic acids, however, is identified to directly act as a catalytic amino acid (Boix et al. 2002). From the 3D structures of rabbit *N*-acetylglucosaminyltransferase and bovine beta1,4-galactosyltransferase, it can be deduced that one aspartate directly interacts with Mn^{2+} to further mediate UDP binding (Boeggeman and Quasb 2002; Unligil and Rini 2000; Unligil et al. 2000).

Mutational exchange of one of the aspartic acid residues results in a more than 1,000-fold decrease in transferase activity. Furthermore, mutant toxin loses its ability to be labeled with azido-UDP-glucose and decreased transferase activity cannot be rescued by an excess of manganese (Busch et al. 1998). In addition to the D-X-D motif, tryptophan-102 is also involved in binding of the cosubstrate UDP-glucose, and mutational exchange results in a dramatic decrease in transferase activity (Busch et al. 2000). These findings are important, not only for the understanding of the catalytic mechanism, but also as a basis for the generation of enzyme-deficient toxins (Fig. 1).

Cellular uptake

Toxins A and B are intracellularly acting cytotoxins which get access to their target cells via receptor-mediated endocytosis (Falnes and Sandvig 2000). So far, all tested cell lines are affected by toxins A and B, although they differ in sensitivity. Toxin B usually is up to 1,000-fold more potent than toxin A (Lyerly et al. 1982). Colonic and pancreas cell lines are much more sensitive to toxin A than nongastrointestinal tract cell lines (Kushnaryov et al. 1992). Cells of colonic source are comparably sensitive for both toxins or even more sensitive for toxin A (Hecht et al. 1992; Torres et al. 1992); however, human primary colonocytes are more sensitive to toxin B (Peppelenbosch et al. 1995). Cultured cell lines are reported to show saturable binding for toxins A and B and the difference in maximal binding capacity for each toxin indicates (Chaves-Olarte et al. 1997) different receptor entities.

Carbohydrate structures are thought to be an essential element for binding of toxin A to the cell membrane. Treatment of cells with glycosidases, N-glycosylation inhibitor tunicamycin, or proteases reduce but do not abolish toxin A effects on cells (Pothoulakis et al. 1996b, 1991; Smith et al. 1997). Furthermore, direct binding of toxin A to the terminal carbohydrate structure Galα1–3Galβ1–4GlcNAc was shown (Krivan et al. 1986; Tucker and Wilkins 1991). This structure, however, is absent in humans (Larsen et al. 1990). It

was demonstrated that toxin A also binds to GalNAcβ1–3Galβ1–4GlcNAc which is present in humans (Karlsson 1995; Teneberg et al. 1996). The membranous sucrase-isomaltase glycoprotein was identified as functional toxin A receptor in rabbit ileal brush border, but this receptor is not expressed in many toxin-sensitive tissues (e.g., human colon) (Pothoulakis et al. 1996b). It is conceivable that toxin A recruits several similar but not identical receptors. A structural feature of the receptor such as a carbohydrate may be of general importance but may not be the exclusive binding structure.

Research on toxin A interaction with its receptor has been done so far by studying mere binding of the toxin or toxin fragments based on the notion that saturable binding is identical to specific binding (Chaves-Olarte et al. 1997; Krivan et al. 1986; Pothoulakis et al. 1996a, b; Rolfe 1991; Smith et al. 1997; Tucker and Wilkins 1991). If the toxins possess the suggested properties of lectins, it cannot be excluded that the toxins bind to several structurally related carbohydrates in a saturable manner. Only one of them is the specific receptor that mediates endocytosis of the toxins. This notion is supported by the findings that toxin A binds to carbohydrate structures not existing in humans (Krivan et al. 1986; Larsen et al. 1990; Pothoulakis et al. 1996b; Tucker and Wilkins 1991) and to immunoglobulin and nonimmunoglobulin components of milk (Rolfe and Song 1995) and thyreoglobulin (Krivan and Wilkins 1987).

A competition study tested the functional binding of toxin A, i.e., its binding to intact cells followed by cellular uptake and cytotoxic effects (Frisch et al. 2003). In this study only the complete receptor binding domain of toxin A was found to compete with the holotoxin A and delayed the onset of cytotoxic effects. Surprisingly, half part of the receptor binding domain covering about 19 of 38 repetitive elements was completely incapable of binding to the toxin receptor and competing with the holotoxin. This finding, together with the result that the intermediary part of toxin A contributes indirectly to receptor binding, does not argue against multiple receptor-binding subunits, but supports the view that at least the correct assembly and the correct folding of all CROPs are prerequisite for receptor binding. Alternatively, it is conceivable that toxin A interacts with a single receptor molecule, which means that despite the repetitive structure only one unique cell receptor is recognized. The latter notion is supported by the finding that a monoclonal antibody that recognizes only two small epitopes in the 109 kDa receptor-binding domain is able to block receptor binding and toxic effects (Frey and Wilkins 1992; Lyerly et al. 1986). In addition to competition, the receptor binding domain itself was able to induce its endocytosis, a finding supporting the view that this domain is fully functional in binding and mediating cell entry (Frisch et al. 2003). Whereas toxin A receptor is partially characterized, nothing is known about the toxin B receptor, except that it is different from that of toxin A.

After binding to carbohydrate membrane structures, toxin internalization is induced by receptor-mediated endocytosis, but the mechanism which initiates endocytosis is unclear. It was shown by electron microscopy that internalization of toxin A takes place via clathrin-coated pits (Kushnaryov and Sedmark 1989; Von Eichel-Streiber et al. 1991). However, at those times alternative pathways such as rafts and caveolae have not been identified. The endocytosed vesicles are processed to endosomes where vesicular-ATPase-triggered acidification takes place. This step of uptake is generally accepted, because inhibition of acidification by applying different methods results in inhibition of cytotoxic effects and thus in protection of the cell (Alfano et al. 1993; Florin and Thelestam 1983, 1986; Henriques et al. 1987). Furthermore, inhibition of cytotoxic effects by alkalization can be

circumvented by short-term acidification of the extracellular medium allowing the toxin to directly translocate through the plasma membrane. The translocation step has been proposed to take place in analogy to the cell entry of the well-characterized diphtheria toxin (Kaul et al. 1996); the low pH in the endosomes induces refolding of the toxin resulting in the insertion of the transmembrane domain into the endosomal membrane followed by translocation of the catalytic domain. This hypothesis is now substantiated by the finding that artificial acidification (pH 5) resulted in an increase in hydrophobicity of toxin B which is reversible (Qa'Dan et al. 2000). The increased hydrophobicity allows the toxins to interact with the endosomal membrane to form a pore which mediates translocation. In fact, pH-dependent pore-formation of toxin B and lethal toxin was nicely demonstrated by the $^{86}Rb^{+}$-release from intact cells (Barth et al. 2001). This channel-forming property was assigned to the N-terminally truncated form of toxin B; the catalytic domain was dispensable. PH-dependent channel formation is a general feature of toxin B, because it can be reproduced in an artificial lipid bilayer model (Barth et al. 2001). Surprisingly, toxin A was unable to induce pores.

That only the catalytic domain and not the complete holotoxin B is translocated to the cytoplasm has recently been demonstrated in an elegant study applying N-terminally tagged recombinant toxin B (Pfeifer et al. 2003). The cleaved catalytic domain was found in the cytoplasm but not the holotoxin or the toxin fragment covering residues 547–2366, which have only been detected at membranes. The protease involved and the step where proteolytic cleavage takes place have not yet been identified. Although the exact cleavage site was not determined, the general conclusion that exclusively the catalytic domain is translocated is convincing. Further support of the notion that the catalytic domain is sufficient to cause full cytotoxicity came from experiments showing that microinjection of the catalytic fragment of toxin B or application of chimeric toxin B-anthrax lethal toxin fusion protein results in the same cytotoxic features (Hofmann et al. 1997; Spyres et al. 2001, 2003). These data also show that the uptake process and the processing of toxin B is not essential for toxin B to exhibit enzyme activity. However, a further activation through the uptake process may be possible because toxin B concentration for microinjection was ten times higher than for extracellular application (Chaves-Olarte et al. 1997).

A pore leaky for monovalent ions is not large enough to pass an unfolded catalytic domain with a size of 63 kDa. Thus, it is likely that the catalytic domain can only pass the pore in an unfolded manner. This notion implies a correct folding at the cytoplasmic site after translocation. In the case of *C. botulinum* C2 and diphtheria toxin, the chaperone HSP90 has been identified to be essential for translocation, thereby allowing assisted refolding of the catalytic domains (Haug et al. 2003; Ratts et al. 2003). HSP90 is not involved in toxin B translocation but other chaperones than HSP90 have not been studied so far.

The current model how the *C. difficile* toxins enter their cells is the following (Fig. 2): The first step is the binding to an extracellular carbohydrate structure containing the terminal Gal-GlcNAc (in case of toxin A); the second step is the induction of endocytosis of the toxin-receptor complex followed by acidification of the endosomes; the third step is the pH-dependent refolding of the toxins and insertion into the endosomal membrane, thereby forming a pore (shown for toxin B); the fourth step includes the translocation of the catalytic domain through a toxin-formed pore (shown for toxin B) and a possibly assisted refolding in the cytoplasm.

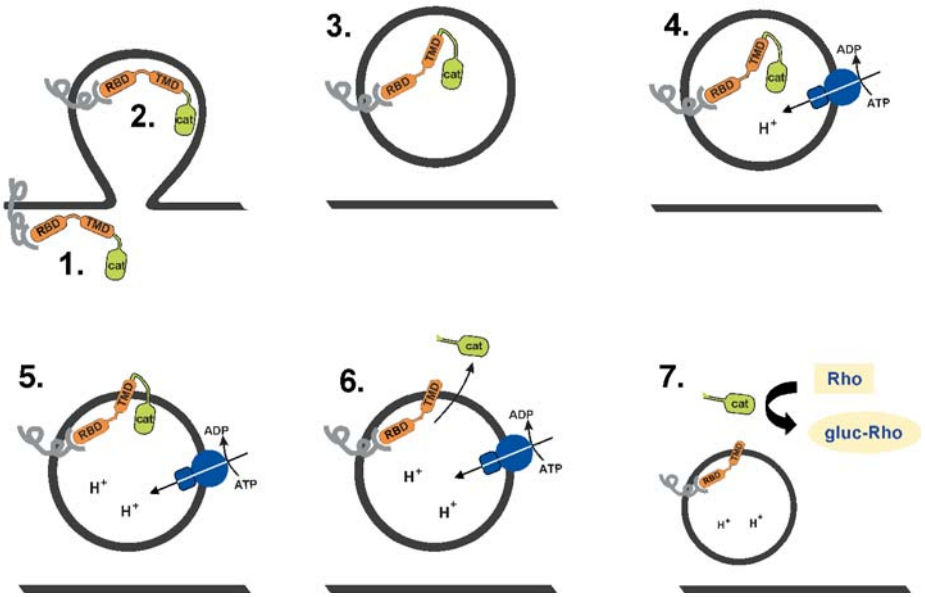

Fig. 2 Cell entry of *C.* toxin A/B. *1*Toxin A binds through its receptor-binding domain (*RBD*) to a membranous carbohydrate structure (the receptor of toxin B is unknown). *2* Toxin binding induces receptor-mediated endocytosis. *3* Endocytosed vesicle is processed to endosomes. *4* Vesicular-ATPase pumps protons into the endosomal lumen, thus decreasing the pH-value to about 5.5. Drop in pH induces refolding of the toxin, especially the hydrophobic transmembrane domain (*TMD*) which interacts with the endosomal membrane to form a pore (only shown for toxin B). Pore formation is blocked by inhibition of acidification. *6* The catalytic domain (*cat*) is translocated through the pore into the cytoplasm and it is released by proteolytic cleavage (only shown for toxin B). *7* The catalytic domain mono-glucosylates the Rho-GTPase targets in the cytoplasm.

Enzyme activity

The large clostridial cytotoxins are very potent agents that exhibit biological activity in the pico- to femtomolar range. The basis for this potent activity is their inherent enzyme activity (Just et al. 1995a, b). The toxins are transferases which catalyze the transfer of a glucose (in the case of α-toxin glcNAc) moiety to cellular targets, the Rho-GTPases. Toxins A and B mono-glucosylate Rho, Rac, Cdc42, RhoG, and TC10, all GTPases belonging to the Rho subfamily (Boquet and Lemichez 2003; Just et al. 1995a, b; I. Just, unpublished data). Lethal toxin (various isoforms) and the variant toxin B from *C. difficile* (strain B-1470) predominantly modify Rac from the Rho subfamily but also members from the Ras subfamily (Ras, Rap, Ral) (Chaves-Olarte et al. 1999, 2003; Genth et al. 1996; Hofmann et al. 1996; Just et al. 1996; Popoff et al. 1996; Schmidt et al. 1998). Differences in substrate specificity are evident and based on the isoform of the toxin (see Table 1). The glucose moiety is attached to the threonine residue residing in the effector region of the GTPases, i.e., Thr-37 in RhoA and Thr-35 in Rac, Cdc42, Ras. α-toxin from *C. novyi* recruits UDP-*N*-acetyl-glucosamine (UDPglcNAc) and transfers the glcNAc to the same threonine residue in Rho, Rac, and Cdc42 as toxins A and B do (Selzer et al. 1996) (Table 1).

The source of the transferred glucose is the ubiquitous nucleotide sugar UDP-glucose which is intracellularly present in micromolar range (Laughlin et al. 1988). UDP-glucose

Table 1 Large clostridial cytotoxins and their intracellular substrates

Toxin	Cosubstrate	Cellular targets		Producing microbe
		Rho subfamily	Ras subfamily	
Toxin A-10463	uDP-glucose	Rhu, Rau, Cuu42, RhuG,TC10	(Rap)	*C. difficile* strain VPI10463
Toxin A-C34	UDP-glucose	Rho, Rac, Cdc42	Rap	*C. difficile* strain C34
Toxin B-10463	UDP-glucose	Rho, Rac, Cdc42, RhoG, TC10	-	*C. difficile* strain VPI10463
Toxin B-1470	UDP-glucose	Rac	Ras, Ral, Rap	*C. difficile* strain 1470
Toxin B-C34	UDP-glucose	Rho, Rac, Cdc42	Ras, Ral, Rap	*C. difficile* strain C34
Lethal toxin CN6	UDP-glucose	(Rho), Rac, (Cdc42)	Ras, Ral, Rap	*C. sordellii* strain CN6
Lethal toxin VPI9048	UDP-glucose	Rac, (Cdc42)	Ras, Rap	*C. sordellii* strain VPI9048
Lethal toxin 6018	UDP-glucose	Rac, (Cdc42), RhoG.TC10	Ras, Ral, Rap	*C. sordellii* strain 6018
Lethal toxin IP82	UDP-glucose	Rac	Ras, Rap	*C. sordellii* strain IP82
Hemorrhagic toxin	UDP-glucose	Rho, Rac, Cdc42	-	*C. sordellii* strain VPI9048
α-Toxin	UDP-*N*-acetyl-glucosamine	Rho, Rac, Cdc42	-	*C. novyi* strain 19402

( ) Minor substrate, only partially modified

deficiency in a mutant cell line (Don cells) protects against cytotoxicity from *C. difficile* and *C. sordellii* toxins, proving the specificity of the cosubstrates (Chaves-Olarte et al. 1996).

The glucose is O-glycosidically bound to the acceptor amino acid. The configuration of the Rho-bound glucose was shown to be α-anomerical by applying crystal structure analysis and NMR spectroscopy of glucosylated Ras (Geyer et al. 2003; Vetter et al. 2000). Thus, the glucosylation reaction goes under retention of the configuration of the α-D-glucose at the C-1 position. As 1,5-gluconolacton inhibits Ras glucosylation by lethal toxin, it can be concluded that the reaction mechanism includes a glucosyl oxonium transition state (Geyer et al. 2003). Retention of configuration and the glucosyl transition state exclude a single S_N2 reaction mechanism but rather argues for a binucleophilic substitution (double replacement) or a stereospecific S_N1 reaction, where structural constraints of the catalytic pocket prevent formation of a racemate (Geyer et al. 2003; Vetter et al. 2000)

Besides the UDP-glucose, a divalent cation is required to form a ternary complex between the enzyme (toxin), UDP-glucose and the metal ion; the ion is involved in nucleotide sugar binding. After the glucose moiety is O-glycosidically attached to the target Rho-GTPase, the complex dissociates and the toxin, UDP, and glucosylated Rho-GTPase are released.

In addition to the transferase activity, large clostridial cytotoxins exhibit glycohydrolase activity in the absence of protein substrates to hydrolytically cleave the cosubstrate UDP-glucose, thereby releasing UDP and glucose (Busch et al. 2000; Chaves-Olarte et al. 1997; Ciesla and Bobak 1998; Just et al. 1995b). The glycohydrolase activity is lower than the transferase activity and it is unclear whether the hydrolase activity contributes at all to the biological activity of the LCTs.

Independently of the biological relevance of glycohydrolase activity, this activity can be used for studying the requirement of divalent cations for enzyme activity. Enzymes that

bind nucleotide diphosphate sugars often require the presence of divalent metal ions for optimal nucleotide binding. Such studies cannot be performed in the presence of Rho-GTPases because the GTPases essentially require Mg^{2+} ions which interfere with the defined metal ion conditions of the glycohydrolase assay. Thus, glycohydrolase is a useful model system to address this question. Removal of bound divalent metal ions by treatment of the toxins with EDTA or EGTA results in a complete inhibition of enzyme activity, which can be restored by addition of Mg^{2+} or Mn^{2+} (Ciesla and Bobak 1998; Just et al. 1996). However, divalent metal ions are not essential for correct folding or for cell entry of the toxins, because EDTA-treated cation-free lethal toxin is as cytotoxic as the nontreated one (H. Genth and I. Just, unpublished data). The following rating of the efficacy of divalent metal ions to stimulate glycohydrolase has been reported: $Mn^{2+} > Co^{2+} > Mg^{2+}$, $Zn^{2+} >> Ca^{2+}$, Cu^{2+} (H. Genth and I. Just, unpublished data). The k_D for Mn^{2+} was calculated as 11.6 μM (only one single metal binding site was found). The above-mentioned D-X-D motif is also involved in glycohydrolase activity as proven by site-directed mutagenesis of lethal toxin (H. Genth and I. Just, unpublished data). Divalent metal ions are necessary but not sufficient for glycohydrolase activity. In the absence of any monovalent cation no enzyme activity is detectable. Only K^+ costimulates but not Na^+, whereby Na^+ is not inhibitory (Ciesla and Bobak 1998; H. Genth and I. Just, unpublished data).

The cytotoxins (lethal toxin and toxins A/B) do not exhibit a restricted specificity for one exclusive divalent metal ion, but Mn^{2+} is the most efficacious stimulator. However, the in vivo concentration of Mn^{2+} in mammals is clearly below the k_D value of 11 μM and can therefore be excluded as physiological stimulator. Although Mg^{2+} is less efficacious than Mn^{2+}, its in vivo concentration is about 10 mM and thus, it is likely that Mg^{2+} is the probable physiological activator. Furthermore, under physiological in vivo conditions extracellular metal ion conditions are characterized by the presence of Na^+; inside the cell preferably K^+ is found. Since the cytotoxins modify intracellular substrates and the uptake process includes a translocation step through endosomal membranes—which probably strips off all noncovalently bound factors—the requirement for Mg^{2+} and coactivation by K^+ may reflect that the cytotoxins are optimally adapted to the metal ion condition of the intracellular milieu of the mammalian target cell.

The catalytic mechanism implies a specific recognition of the Rho/Ras substrate proteins by the toxins. Therefore, the toxins need—in addition to UDP-glucose binding site and the catalytic center—a substrate recognition site. Whereas toxin A and B exclusively modifies the Rho subfamily proteins, lethal toxin glucosylates Rac and Cdc42 from the Rho subfamily and Ras, Ral, and Rap from the Ras subfamily. Thus, it is unlikely that there is only one single substrate recognition site which is able to recognize variant combinations of the Rho and Ras GTPases. It is rather conceivable that the toxins possess different recognition sites. This notion makes it possible to explain the differences in the substrate specificity of the isoforms of lethal toxin and the variant toxin of *C. difficile* (Table 1). As toxin B and lethal toxin are highly homologous (90 %) but differ in their protein substrate specificity, chimeric toxins are helpful to study the site of substrate recognition. Testing the substrate specificity of various chimeras between the N-terminal part of toxin B and lethal toxin led to the restriction and separation of recognition sites for the Rho and Ras proteins. Amino acids 408–468 in toxin B determine the specificity for Rho, Rac, and Cdc42, whereas residues 364–408 in lethal toxin determine for Rac and Cdc42, but not for Rho recognition (Fig. 1). Interestingly, the substrate specificity of lethal toxin and toxin B is determined by different domains. The recognition of the Ras proteins is mediat-

ed from the region aa 408–516, which is adjacent to the Rho recognition site (Busch and Aktories 2000; Hofmann et al. 1998). It seems that the substrate recognition sites are modularly organized.

The diverse substrate specificity of the members of the large clostridial cytotoxins implies that the toxins recognize differing amino acids or domains of Rho and Ras GTPases. However, a common condition for all GTPases to be substrate is the nucleotide-dependent three dimensional structure. The GTPases serve only as substrates when bound to GDP but not when bound to GTP (Herrmann et al. 1998). In the GTP-bound state the side-chain of threonine-35 in Ras/ Rac and threonine-37 in Rho points to the core of the GTPase by coordination of the Mg^{2+} ion. Thus, the hydroxyl group of the threonine is not accessible. Only in the GDP-bound state is the hydroxyl group located at the surface of the GTPase molecule and accessible for glucosylation.

In addition to the nucleotide-bound state, little is known about other conditions determining whether a GTPase is substrate or not. Only the amino acid Thr-25 in Rac was reported to be important for lethal toxin-catalyzed glucosylation. The exchange of Thr-25-Lys mutation in Rac mimicking the situation in Cdc42 resulted in strongly diminished glucosylation. Accordingly, exchange of Lys-25 to Thr in Cdc42 made Cdc42 a substrate (Müller et al. 1999).

Functional consequences of glucosylation

The cellular functions of the low molecular mass GTPases of the Rho and Ras subfamily are governed by their ability to cycle between the inactive and the active state, a process which is regulated by various regulatory proteins (Fig. 3).

The common features of the Ras-like small GTP-binding proteins, also called small GTPases, are their molecular mass (18–28 kDa), their C-terminal polyisoprenylation, and their property to bind to and hydrolyze guanine nucleotides. They are molecular relays which transmit signals when bound to GTP and stop doing so when bound to GDP. Based on their sequence and functional homology, the superfamily of Ras-like proteins is subdivided into the subfamilies of Ras, Rho, Rab, Arf, and Ran. The Rho subfamily comprises the RhoA-related (RhoA, B, and C), Rac1-related (Rac1, 2, 3, and RhoG), Cdc42-related (Cdc42, G25 K, TC10, TCL, Chp/Wrch-2, and Wrch-1), Rnd subfamily (Rnd1/2 and Rnd3/RhoE), RhoD, Rif, and RhoH/TTF (Wennerberg and Der 2004). The best-characterized GTPases are Rho, Rac, and Cdc42. Rho/Rac/Cdc42-dependent signal pathways are stimulated by receptor-tyrosine kinases (PDGF for Rac) and by G-protein-coupled receptors (LPA for Rho, bradykinin for Cdc42). Stimulation of the Rho signalling starts with the guanine nucleotide exchange factors (GEF), which catalyze the exchange of nucleotides, resulting in binding of GTP to Rho (Fig. 3). Binding of GTP induces changes in the conformation of the effector region (covering residues 30–42) which allows interaction of Rho with its so-called effector proteins. Effectors are often serine/threonine kinases which are activated by binding of Rho (e.g., ROKα/Rho kinase) to phosphorylate downstream targets. In addition to kinases, Rho effectors also comprise multi-domain proteins without enzymatic activity (rhotekin, rhophilin, WASP) which may serve as nucleus for multi-protein complexes to connect different signalling pathways. Thus, the effector proteins amplify and execute the Rho signals. The downstream signalling is terminated by the GTPase-activating protein (GAP), which strongly enhances the inherent GTP-hydrolyzing activity

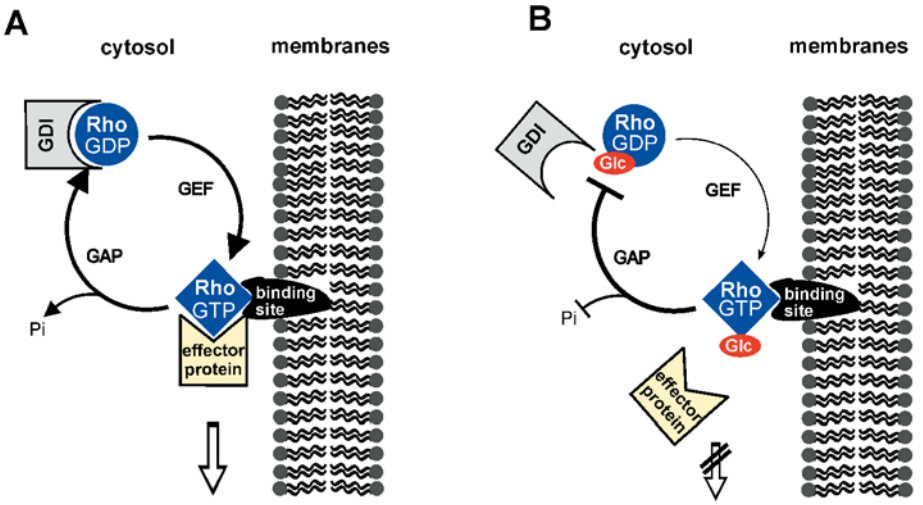

Fig. 3 Molecular mode of action of toxin A/B. **A** The Rho-GTPases are molecular switches which are regulated by guanine nucleotide binding. Nonsignalling, inactive Rho is complexed with the guanine nucleotide dissociation inhibitor GDI to be localized to the cytosol. Signal input induces an activation cascade resulting in translocation to the plasmamembranes and nucleotide exchange catalyzed by GEF (guanine nucleotide exchange factor harboring a Dbl domain). GTP binding causes a conformational change of especially the effector region allowing Rho to interact with effector proteins. The effector proteins comprise Thr/Ser-kinases, lipid kinases, lipases, or scaffold proteins which execute and amplify Rho signals. Rho signalling is terminated by an additional regulatory protein called GTPase-activating protein GAP which increases GTP-hydrolysis, resulting in inactive GDP-bound Rho which is delivered to the cytosol again. **B** Toxin-catalyzed mono-glucosylation alters the properties of Rho-GTPases. Glucosylation promotes entrapment of Rho-GTPases at membranous binding sites. The glucose moiety stabilizes the effector region so that Rho-GTPases are incapable of interacting with their effector proteins, thereby completely blocking downstream signalling. Furthermore, the glucosylation inhibits binding to GDI, thereby preventing extraction of inactive Rho from the membranes. Entrapment at restricted membranous binding sites is thought to be the basis for the dominant negative mode of action of glucosylated Rho.

of Rho, resulting in inactive GDP-bound Rho. In addition to the cycling between the inactive GDP-bound and active GTP-bound state, Rho concomitantly cycles between membranes (active Rho) and cytosol (inactive Rho). Inactive Rho is localized to the cytosol by binding to the guanine nucleotide dissociation inhibitor (GDI), which traps the inactive GDP-bound form in a high-affinity complex. GDI, however, is not only a mere negative regulator but is also involved in the correct subcellular addressing of active Rho to the membranes. In this respect, GDI cooperates with the ERM proteins (ezrin, radixin, moesin). After termination of the signalling job of Rho by GAP, inactive Rho is extracted by GDI from the membranes and relocalized to the cytosolic pool of inactive Rho (Fig. 3).

The Rho-GTPases are in general the master regulators of the actin cytoskeleton. The isoforms, however, regulate different aspects: Cdc42 is involved in the formation of filopodia (microspikes); Rac governs the formation of lamellipodia (ruffles), whereas Rho is responsible for stress fibers. In addition, they are involved in many actin-dependent processes (such as cell motility, cell adhesion, cell-cell contact), as well as in membrane trafficking (endo- and exocytosis, phagocytosis). However, Rho functions are also beyond the regulation of the cytoskeleton: Transcriptional activity is governed via the JNK, p38 and NFκB pathway; the cell cycle is regulated (G1-S phase transition) and finally, the forma-

tion of reactive oxygen species is stimulated through activation of the NADP oxidase of neutrophils (for reviews on the Rho proteins see Bishop and Hall 2000; Etienne-Manneville and Hall 2002; Hall 1998; Ridley 2001; Takai et al. 2001; Van Aelst and D'Souza-Schorey 1997; Wettschureck and Offermanns 2002; Zohn et al. 1998).

A glucose moiety attached to the conserved threonine residue causes various alterations of Rho functions (Fig. 3) (Just et al. 1995a): (1) Rho activation by exchange factors (GEFs) is reduced, (2) intrinsic GTPase is reduced but GAP-stimulated GTPase is completely inhibited, (3) coupling to the effector proteins is completely blocked (Herrmann et al. 1998; Sehr et al. 1998). Inhibition of effector coupling is based on the effect of the glucose moiety to stabilize the inactive conformation of the effector region, although GTP can be bound (Vetter et al. 2000). In addition to the GTPase cycling, also the cytosol-membrane cycling of Rho-GTPases is altered. Surprisingly, glucosylation renders Rho properties so that GDP-bound glucosylated Rho is bound to the membranes and cannot be complexed anymore to GDI. Glucosylation blocks the cytosol-membrane cycling of Rho proteins, thereby leading to an entrapment at the membranes, which is the basis for the dominant negative effect of glucosylated Rho (Genth et al. 1999). Thus, glucosylation redundantly switches off Rho signalling to completely block all Rho-dependent signal pathways. The entrapment at the membranes, however, indicates rather a gain of function with respect to a negative regulator than a mere inactivation of the GTPases.

The actin depolymerizing activity of the cytotoxins can be fully explained by the inactivation of Rho-GTPases. RhoA is the upstream activator of the serine/threonine kinase ROCK (Rho-kinase) which, in cooperation with the scaffold protein mDia, regulates actin-myosin assembly and contractility as well as actin polymerization, resulting in alteration of stress fiber formation and cell motility. Rac1 acts through PAK/WAVE/PIP_2, whereas Cdc42 acts through WASP. Their inactivation causes actin depolymerization in filopodia and lamellipodia as well as deinhibition of F-actin stabilization, resulting in inhibition of cell motility and phagocytosis.

The primary target tissue of the *C. difficile* toxins is the colonic epithelium. Human colon carcinoma (T84) cells are polarized in culture and form tight junctions and therefore they are an established model for the colonic epithelial barrier. Toxins A and B disrupt the barrier function by opening the tight junctions (Gerhard et al. 1998; Hecht et al. 1988, 1992; Johal et al. 2004; Moore et al. 1990; Triadafilopoulos et al. 1987, 1989). This effect is not merely caused by the breakdown of actin filaments, but by inactivation of the Rho function to regulate the tight junction complex (Chen et al. 2002; Nusrat et al. 1995). These barrier-disrupting effects of toxins A and B are supposed to increase the colonic permeability, the basis of the watery diarrhea, which is a typical feature of the *Clostridium difficile*-induced, antibiotic-associated diarrhea.

Toxins A and B from the reference strain 10463 cause morphological changes at fibroblasts which are characterized by cell rounding and intermediate formation of "neurite-like" protrusions, the intoxicated cells remaining attached to the substratum. In contrast, variant toxin B (from strains 1470 and 8864) and lethal toxin from *C. sordellii* induce rounding with formation of some filopodia-like structures, however, accompanied by massive cell detachment (Chaves-Olarte et al. 2003). The variant toxin B is related to the lethal toxin which glucosylates Rac1 and Ras-GTPases but not RhoA. R-Ras but not Rho is involved in the control of cell adhesiveness through modulation of the integrin avidity (Zhang et al. 1996). Thus, differences in substrate specificity are responsible for different cytotoxic effects (Chaves-Olarte et al. 2003).

Toxins A and B have been reported to induce apoptosis (Brito et al. 2002; Calderón et al. 1998; Fiorentini et al. 1998; Mahida et al. 1996, 1998; Qa'Dan et al. 2002). Apoptosis is induced by many signals, particularly by detachment of cells from their extracellular matrix (Ruoslahti 1997). The cytotoxins which are known to induce detachment through their actin filament-disrupting properties may induce apoptosis in the same way as EDTA or neutrophil elastase do (Shibata et al. 1996). Lethal toxin from *C. sordellii* was also reported to induce apoptosis in the myeloid cell line HL-60 through inactivation of Rac. Apoptosis was detected after 16 h of intoxication involving caspases 9 and 3 as well as the cytochrom C release from mitochondria. Furthermore, it was shown that lethal toxin partially colocalized with mitochondria (Petit et al. 2003). Independently of detachment of cells, inactivation of especially Rac and Cdc42 is a satisfactory explanation for toxin-induced apoptosis. As Rac and Cdc42 function as prosurvival signalling proteins, they functionally shut off further apoptotic processes (Coleman and Olson 2002; Gomez et al. 1998).

Glucosyltransferase-independent properties of toxins A and B

Recently, findings have been reported raising the question whether there are additional nonenzymatic or non-Rho-dependent biological effects of toxins A and B. The mitochondrial targeting by toxin A is now the focus of research on the pathogenesis of *Clostridium difficile*-associated diarrhea (He et al. 2002). The release of interleukin-8 (IL-8) from colonocytes was reported to be based on damage of mitochondria by toxin A. Toxin-induced damage resulted in a decreased ATP production and formation of reactive oxygen ROS, which induced a sequelae of reactions involving activation of NFkB and eventually release of the proinflammatory cytokine IL-8. Since this sequelae takes place in a time frame of 30 min in which no glucosylation of Rho-GTPases were detected, it was concluded that the mitochondrial effects of toxin A are Rho-independent, particularly because isolated mitochondria are also directly affected by toxin A (He et al. 2000, 2002). However, a contrary finding was reported showing that mitochondrial damage as initiation of apoptosis started after 18–24 h of intoxication and was clearly enzyme-dependent. Since enzymatically inactive toxin A, generated by covalent modification of toxin A, had no effect on apoptosis, it was concluded that this toxin effect is mediated by a Rho-dependent mechanism (Brito et al. 2002)

A further non-Rho-dependent effect was reported by Warny et al. showing that activation of the MAP-kinases ERK, p38-kinase, and JNK started 1–2 min after toxin A application to the monocytic THP-1 cell line (Warny et al. 2000). This early onset of kinase cascades is clearly before cell entry of toxin A and the glucosylation of the Rho-GTPases. Especially p38-kinase activation results in monocyte activation and IL-8 production, thus explaining how toxin A may cause the inflammatory process of the colitis. The authors suggest that the interaction of the toxin with the toxin-receptor may trigger the MAP kinase cascade (Warny et al. 2000).

The group of Ballard studied toxin B-induced apoptosis by comparing the effects of the catalytic domain of toxin B covering amino acids 1–556 with the holotoxin B (Qa'Dan et al. 2002). Whereas holotoxin B induced apoptosis of intoxicated cells via activation of caspase 3, the catalytic domain—delivered as chimeric anthrax toxin or expressed in the cells—did so via a caspase-independent mechanism. The authors concluded that inactivation of Rho-GTPases was involved in a caspase-dependent way but that the holotoxin had

the property to do so also via a non-Rho and noncaspase-dependent mechanism. However, this finding is hard to reconcile with the finding that only the proteolytically cleaved catalytic domain of toxin B is delivered to the cytoplasm, whereas the remainder cleavage product and the nontranslocated holotoxin B remain in endosomes (Pfeifer et al. 2003).

Also the increase in paracellular permeability was reported to be Rho-independent. Through an unknown mechanism, toxin A stimulated the protein kinases PKCα and β, which regulate sorting and assembly of the tight junction protein ZO-1. After toxin A application, ZO-1 time-dependently translocated from the tight junctions accompanied by a decrease in the transepithelial resistance TER (Chen et al. 2002). PKC activation preceded the glucosylation of Rho and, furthermore, the specific PKC inhibitor "myristoylated PKCα/β peptide" blocked toxin A-mediated glucosylation, indicating Rho-independent effects of toxin A. However, the applied PKC inhibitor in fact does not block glucosylation reaction of Rho but rather inhibits cell entry of toxin A (I. Just et al., unpublished data). Only a small pool of Rho-GTPases, i.e., the membrane-bound, has to be glucosylated to achieve the full cytotoxic effect although the majority of Rho is still unmodified and therefore substrate for subsequent C3-ADP-ribosylation (see the section entitled "The large clostridial cytotoxins as tools in cell biology"). Thus, assessing Rho glucosylation by C3-catalyzed [^{32}P]ADP-ribosylation of whole cell lysates does not reflect the exact time course of inactivation of functional Rho in cells by toxin A/B, but rather do so with a delay of hours (Just et al. 1997). In this way the reported effects of toxin A to activate PKC by a non-Rho-dependent mechanism seem to be questionable.

The large clostridial cytotoxins as tools in cell biology

Prerequisites for LCTs to serve as biological tool are: (1) cell accessibility, (2) target specificity, (3) well-known mode of action, and (4) no secondary effects. All cell lines studied so far are sensitive to the LCTs although there are great differences in sensitivity. For example, CHO cells are highly sensitive to toxin B acting at femtomolar range, whereas HEp2 cells are quite insensitive; most fibroblasts and epithelial cells are insensitive to lethal toxin, but RBL (mast) cells are highly sensitive (see also "Cellular uptake"). Thus, the LCTs have access to cultured cell lines, primary cell culture, or isolated cells from organs. The basis for cell accessibility is the property of the LCTs to cause their own cell entry by receptor-mediated endocytosis, so that techniques such as cell permeabilization, electroporation, or transfection are not needed. Treatment of cells with LCTs usually results in more than 90% of intoxicated cells, which is an essential prerequisite for biochemical studies of the cell lysates.

The cellular targets of LCTs are the Rho-GTPases and in case of lethal toxin Rho and Ras GTPases (Table 1). Rho, Rac, and Cdc42 are the canonical substrates of toxins A and B, but the Rho subfamily comprises more than these three members. Recently, is was reported that in addition to the canonical substrates also RhoG and TC10 are glucosylated and that in case of toxin A even Rap, a member of the Ras subfamily, is modified. Furthermore, it is conceivable that additional substrates may exist which have been overlooked, because they are only expressed in faint amounts (thus escaping the identification through [^{14}C]glucosylation) or solely present in some cell types. Unless the structural features of the substrate recognition by the toxins are not well understood, additional cellular substrates cannot be definitely ruled out.

The Rho-GTPases are the master regulators of the actin cytoskeleton, but they are also involved in cytoskeleton-independent processes such as gene expression, cell cycle progression, apoptosis, and cell transformation. To distinguish between cytoskeletal and non-cytoskeletal effects, application of agents or toxins which directly interfere with the actin cytoskeleton can be used. Two of such agents/toxins are available, latrunculin B and the binary C2 toxin from *C. botulinum*. The C2 toxin consists of the enzyme component C2I and the binding component C2II, which is only active after trypsin treatment. C2II translocates C2I through receptor-mediated endocytosis into the cytoplasm, where C2I mono-ADP-ribosylates G-actin. ADP-ribosylated actin is incapable of polymerization and eventually leads to complete depolymerization of the actin filaments (Aktories et al. 1997a, b; Barbieri et al. 2002).

The morphological changes (cell rounding and formation of arborized morphology) induced by the LCTs are an excellent read out to check cell sensitivity and toxin activity. However, in nonadherent cells, changes in cell-shape are hard to detect. The visualization of the submembranous actin cytoskeleton is applicable but gives no strong evidence for intracellular toxin activity. The only reliable method is to perform a differential glucosylation. The rationale for this experiment is the fact that toxin-catalyzed glucosylation in intact cells prevents a second [^{14}C]glucosylation of the lysates. A decrease in radioactive labeling of the Rho-GTPases in lysates compared to nontreated cells indicates previous glucosylation in the intact cell and proves active toxin. A less expensive alternative with the advantage of stronger signal in autoradiography/phosphorimaging is the usage of the C3-catalyzed [^{32}P]ADP-ribosylation which, however, only proves modification of Rho but not of Rac or Cdc42. The differential glucosylation and ADP-ribosylation, respectively, is also applicable to estimate the amount of inactivated (glucosylated) Rho-GTPases. This approach is generally applied but implies a pitfall: Surprisingly, cell rounding precedes intracellular glucosylation when C3-catalyzed [^{32}P]ADP-ribosylation is performed with whole cell lysates. However, when only membranes—reflecting functional membranous RhoA—are used, a clear temporal correlation between intracellular glucosylation and cell rounding exists (Just et al. 1997). Thus, intracellular glucosylation of a small fraction of cellular Rho is sufficient to cause effects even if subsequent C3-ADP-ribosylation indicates little or no modification of Rho.

The mode of action of LCTs is well-characterized, i.e., glucosylation of the pivotal threonine in the effector region of Rho and Ras GTPases resulting in functional inactivation. Whether the toxins possess additional nonenzymatic effects is under debate. One enzyme-independent effect has been reported—a cytochrom C-release from mitochondria induced by toxin A (He et al. 2000, 2002). An additional uncertainty is whether LCTs are able to induce secondary effects which are not directly associated with the Rho/Ras GTPases such as mere cytoskeleton-based effects that can easily be checked, as discussed above. Thus, the LCT can only be used for the initial orienting studies. Mandatorily, the findings have to be checked by other methods, such as expression of dominant negative GTPases or application of siRNA.

Usually, toxin B is used as tool because it is more active than toxin A. Toxin activity is the only reliable parameter to guarantee reproducible results in cell biology. Toxin activity is meant as titer of cytotoxicity but not as enzyme activity. The latter is less reliable, whereas mere protein concentration is absolutely unreliable. (for reviews see Bobak 1999; Boquet 2002; Just and Boquet 2000; Just et al. 1997; Schiavo and van der Goot 2001).

Involvement of *Clostridium difficile* toxins A and B in disease

Toxins A and B are the major pathogenicity factors of the antibiotic-associated diarrhea and the pseudomembranous colitis. Based on animal studies, toxin A has been assigned as the true enterotoxin which is biologically active and secondarily allows access of toxin B to subepithelial tissue. However, in human disease it seems that both toxins are comparable or that even toxin B is of more relevance (Savidge et al. 2003). Human colonic epithelial cells are about equisensitive to both toxins. In about 5%–7% of cases of *Clostridium difficile*–associated diarrhea variant strains are involved which lack functional toxin A, but possess a variant toxin B (Barbut et al. 2002; Johnson et al. 2003; Kuijper et al. 2001; Rupnik et al. 2003b). Variant toxin B resembles lethal toxin from *C. sordelli* with respect to substrate specificity but harbors the identical receptor-binding domain as toxin B-10463. The variant toxin B is detected in human disease but is not able to induce diarrhea and PMC in animals. These findings support the notion that the animal model, in which only toxin A possesses a biological activity, does not correctly reflect the conditions of the human disease.

The symptoms of CDAD are characterized by secretory diarrhea and inflammatory processes of colonic mucosa. Thus, mucosal barrier function is decreased, immune cells of the mucosa are activated triggering inflammation and, finally, the enteric nervous system is altered. Mast cells and macrophages are activated to release cytokines, especially IL-1, IL-6, IL-8, TNF, and IFN-γ (Pothoulakis et al. 1998; Pothoulakis and LaMont 2001; Thelestam and Chaves-Olarte 2000). Release of chemoattractants causes invasion of neutrophils which are characteristic for PMC. Furthermore, the enteric nervous system (ENS) is activated by the toxins through an unknown sequel of steps, causing secretory diarrhea and amplifying inflammation (Farrell and LaMont 2000; Jones and Blikslager 2002; Mantyh, McVey et al. 2000; Neunlist et al. 2003; Pothoulakis et al. 1998). However, the cellular and molecular mechanisms through which the toxins induce inflammatory processes are less clear and cannot be fully explained so far by their intracellular transferase activity to inactivate small GTPases (for reviews on the clinical aspects see Bartlett 2002; Farrell and LaMont 2000; Poxton et al. 2001; Stoddart and Wilcox 2002).

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