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Edited by K. Aktories and I. Just

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Rho-modifying C3-like ADP-ribosyltransferases

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Abstract C3-like exoenzymes comprise a family of seven bacterial ADP-ribosyltransferases, which selectively modify RhoA, B, and C at asparagine-41. Crystal structures of C3 exoenzymes are available, allowing novel insights into the structure-function relationships of these exoenzymes. Because ADP-ribosylation specifically inhibits the biological functions of the low-molecular mass GTPases, C3 exoenzymes are established pharmacological tools to study the cellular functions of Rho GTPases. Recent studies, however, indicate that the functional consequences of C3-induced ADP-ribosylation are more complex than previously suggested. In the present review the basic properties of C3 exoenzymes are briefly summarized and new findings are reviewed.

Introduction

Many bacterial ADP-ribosyltransferases are potent bacterial protein toxins and important virulence factors. After cellular uptake caused by highly efficient cell entry mechanisms, they modify eukaryotic target proteins with great specificity and often grossly affect biological functions of their targets. These properties of the toxins are the reason for their use as cell biological and pharmacological tools (Aktories 2000). Particularly successful pharmacological tools are ADP-ribosyltransferases of the C3 family, which modify Rho GTPases. In the 1990s, C3 exoenzymes turned out to be very valuable experimental keys to understand the wide array of diverse regulatory functions of Rho GTPases. In hundreds of papers, C3 exoenzymes have been widely employed as cell biological tools to elucidate the cellular functions of Rho GTPases. This holds true despite the fact that these ADP-ribosyltransferases are rather poorly taken up by eukaryotic targets cells and their roles as virulence factors are still not well defined. Here, we will briefly review the basic properties of these ADP-ribosyltransferases and will focus on novel findings on the functional consequences of C3-induced ADP-ribosylation and discuss recent reports on the structure and function of the enzymes.

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Recent reviews about C3 exoenzymes focused on different aspects of the transferases (Aktories et al. 1992; Aktories 1997a,b; Boquet et al. 1998; Just et al. 2001; Narumiya and Morii 1993; Wilde and Aktories 2001). The exciting follow-up of the initial major discoveries in the field of Rho GTPases, including the role of C3 in this process, was recently vividly communicated by Ridley and Hall (Ridley and Hall 2004).

Sources of C3-like ADP-ribosyltransferases

C3 exoenzymes are produced by different types of Gram-positive obligate and facultative pathogens. So far, seven C3-like isoforms have been described, which are produced by *Clostridium botulinum*, *Clostridium limosum, Bacillus cereus* and *Staphylococcus aureus.* C3 was first identified as a product of *Clostridium botulinum* types C and D (Aktories et al. 1987, 1988; Rubin et al. 1988). Later it was found that two isoforms are produced by these Clostridia, which are about 65% identical in their amino acid sequences (Nemoto et al. 1991). They have been termed C3bot1 and C3bot2. At least the gene for C3bot1 is located on the same phage, which also encodes *C. botulinum* neurotoxins type C (Popoff et al. 1990). C3lim is produced by *Clostridium limosum* (Just et al. 1992) and is about 63% identical with C3bot1. *Bacillus cereus* produces C3cer (Just et al. 1995a), which is about 30% identical with C3bot1. Three C3 isoforms have been described, which are produced by *Staphylococcus aureus* (C3stau1, 2, and 3). These exoenzymes are about 35% identical with C3bot1 and 66%–77% identical between each other. The C3stau exoenzymes are also termed EDINs (Epidermal differentiation inhibitor) (Inoue et al. 1991; Wilde et al. 2001b; Yamaguchi et al. 2001) (Fig. 1a, b).

Structure–function analysis of C3-like exoenzymes

C3-like ADP-ribosyltransferases are enzymes of about 25 kDa, which all share the same activity in the sense that they mono-ADP-ribosylate RhoA, B, and C at the same site at asparagine 41 (Aktories et al. 1989; Braun et al. 1989; Chardin et al. 1989; Just et al. 1992, 1995a; Quilliam et al. 1989; Sekine et al. 1989; Sugai et al. 1992; Wilde et al. 2001b). The bacterial exoenzymes possess no receptor-binding or translocation domain and, consist exclusively of the catalytic domain, which possess ADP-ribosyltransferase and like many other ADP-ribosyltransferases also NAD glycohydrolase activity (Fig. 1a). Most important for understanding of the structure-function-relationship of C3-like transferases were the analysis of the crystal structures of C3bot either bound or unbound to NAD (Han et al. 2001; Ménétrey et al. 2002). These studies showed that the exoenzymes are very similar in structure and folding and share almost all functionally pivotal residues despite the limited primary sequence homology (some are not more than ~30% identical in their amino acid sequences). These data also corroborated previous mutational analysis, which let to the identification of many functionally important residues and their possible role in the ADP-ribosylation reaction (Böhmer et al. 1996; Just et al. 1995a; Saito et al. 1995; Wilde et al. 2002b).

The active site of C3bot (and most likely of other C3-like ADP-ribosyltransferases) consists of a mixed α/β-fold with a β-sandwich core, consisting of a five-stranded mixed β-sheet perpendicularly packed against a three-stranded antiparallel β-sheet. Four consecutive α-helices surround the three stranded β-sheet. An additional α-helix flanks the five-

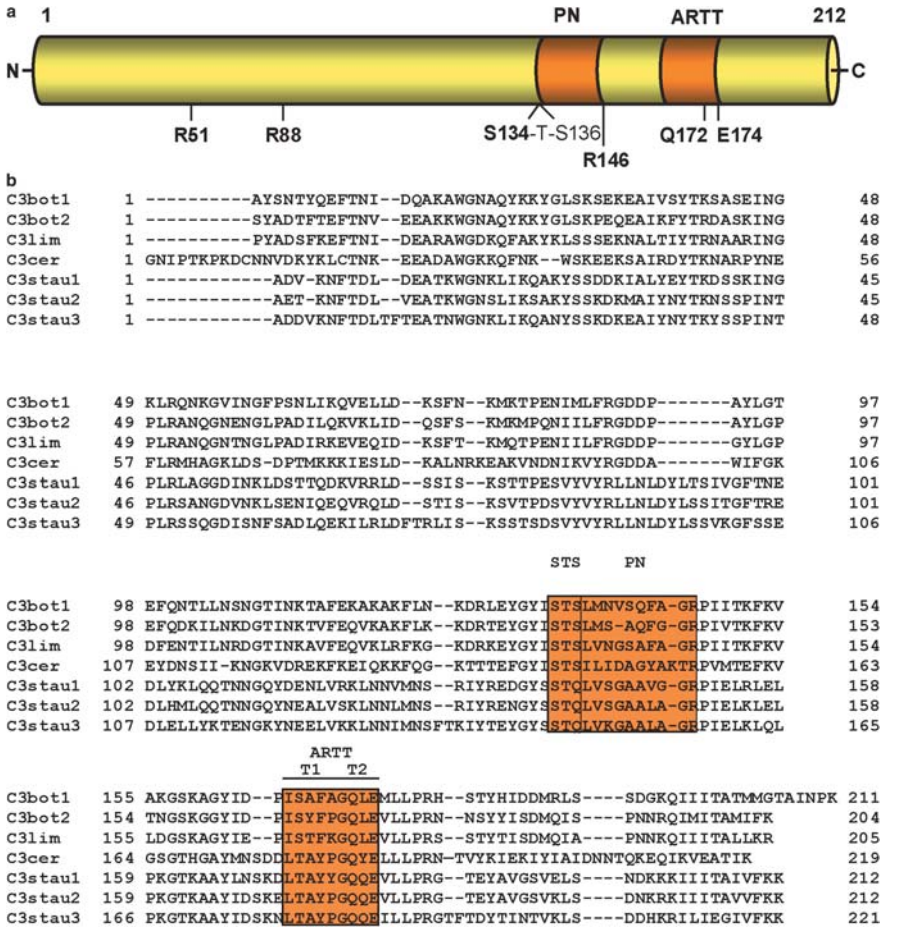
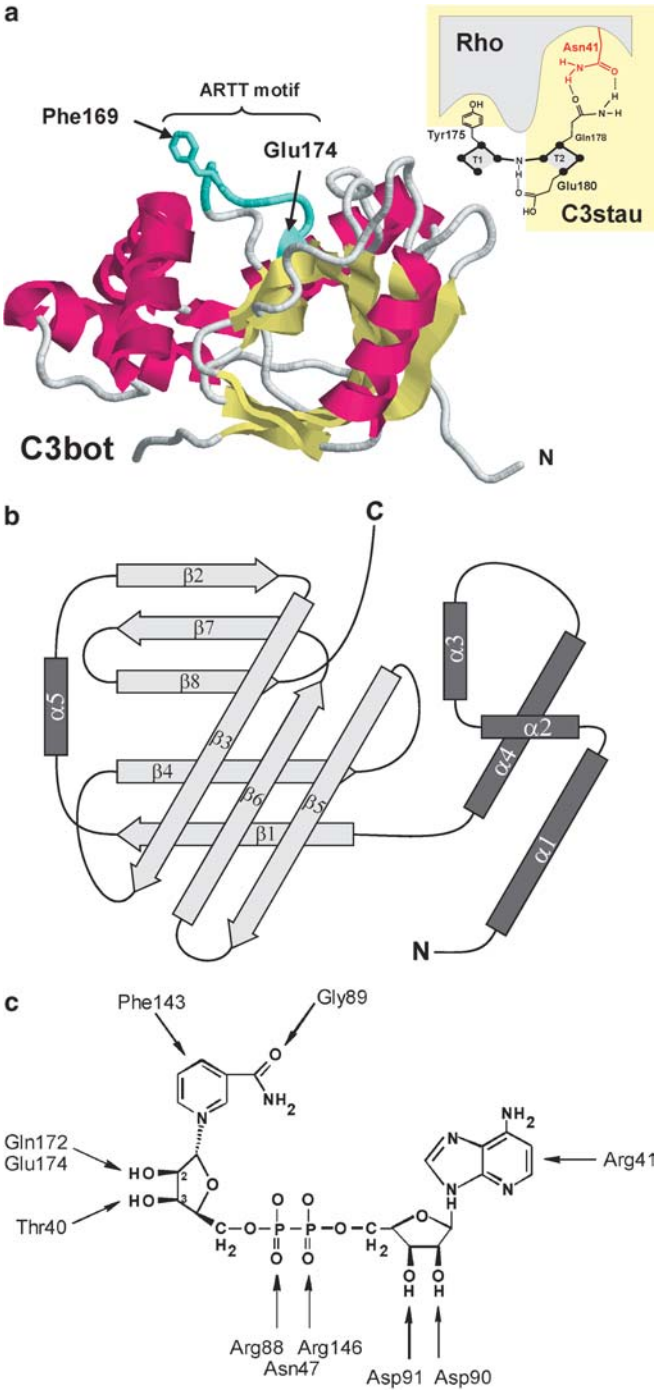


Fig. 1 Structure of C3 transferase. **a** Scheme of the primary sequence of C3bot showing the catalytic glutamate, residues of the ADP-ribosylation toxin-turn-turn (*ARTT)* loop, which is involved in protein substrate recognition, and PN-loop, which is involved in binding of phosphates of NAD. The STS-motif, which is conserved within the family of ADP-ribosyltransferases (C3stau isoforms possess an STQ motif), and several arginine residues involved in interaction with NAD are shown. **b** The sequences of the seven C3-like ADP-ribosyltransferases are given. *Clostridium botulinum* C3 transferase type I (C3bot1; Acc.Nr. P15879), *Clostridium botulinum* C3 transferase type II (C3bot2; Acc.Nr. Q00901), *Clostridium limosum* C3 transferase (C3lim; Acc.Nr. Q46134), *Bacillus cereus* C3 transferase (C3cer; Acc.Nr. AJ429241.1), *Staphylococcus aureus* C3 transferase A, B, and C (C3stau1; Acc.Nr P24121; C3stau2; Acc.Nr BAC22946, C3stau3; Acc.Nr;. NP_478345 ; also termed EDIN A,B,C).

stranded sheet (Han et al. 2001). After binding of NAD, a clasping movement ("Crab-claw" movement) of the transferase occurs which involves the structural elements α5, β2, β7 and β8, and α3 to enclose the substrate (Evans et al. 2003; Ménétrey et al. 2002) (Fig. 2). A novel structural motif, termed "ADP-ribosylating-toxin-turn-motif" (ARTT-motif) was proposed to be involved in the ADP-ribosylation reaction and suggested to be typical for all Rho-modifying C3-like transferases and also for the structurally related actin-modifying ADP-ribosyltransferases like *Clostridium botulinum* C2 toxin (Han and Tainer 2002). In C3bot, this motif consists of residues 167–170 (note that the counting is


a
Rho
Asn41
ARTT motif
Phe169
Glu174
Tyr175
Gln178
T1
T2
Glu180
C3stau
C3bot
N
b
C
β2
β7
β8
α5
α3
α2
β3
β4
α4
β6
β5
β1
α1
N
c
Phe143
Gly89
Gln172
Glu174
Thr40
Arg41
Arg88
Asn47
Arg146
Asp91
Asp90

without the signal sequence of 40 residues) for "turn 1" and residues 171–174 for "turn 2" (Figs. 1a, b, 2a). Turn 1 contains a conserved aromatic residue (C3bot^{Phe169}). The aromatic side chain points to the surface of the molecule and was suggested to recognize the substrate RhoA via hydrophobic patches around the acceptor amino acid residue Rho Asn41. The exchange of this critical residue to alanine or lysine in C3stau2 leads to a decreased binding of RhoA and abolishes the ADP-ribosyltransferase activity of these mutants (Wilde et al. 2002b). In the second turn, two residues (C3bot^{Gln172} and C3bot^{Glu174}) play important roles in enzyme activity. The side chain of C3bot^{Gln172} forms hydrogen bonds with the O2'-hydroxyl of the nicotinamide ribose (Fig. 1b), and is thought to be involved in the positioning of the ternary C3-NAD-Rho complex on turn 2. The side chain of C3bot^{Glu174} stabilizes the formation of an oxocarbenium transition state that arises during the enzymatic reaction (Han et al. 2001; Ménétrey et al. 2002; Oppenheimer 1994) (Fig. 2c). Exchange of either of these glutamine or glutamate residues to any other amino acids results in inhibition of the asparagine-modifying ADP-ribosyltransferase activity (Böhmer et al. 1996; Evans et al. 2003; Ménétrey et al. 2002; Saito et al. 1995; Wilde et al. 2002b).

Recently, it was reported that the ARTT-motif of C3bot undergoes conformational changes upon NAD-binding. While NAD is bound to C3bot, the complete motif is orientated into the inside of the protein and participates in NAD binding (Ménétrey et al. 2002). This form of NAD-binding was also observed in other ADP-ribosyltransferases (Bell and Eisenberg 1996; Choe et al. 1992; Han et al. 1999; Li et al. 1996). In C3stau2, the resting (NAD free) position of the ARTT loop is similar to the NAD bound state in C3bot. C3 exoenzymes produced by *S. aureus* are unique as compared to the other C3 transferases, because the loop before the ARTT loop possesses an additional two residues. These two residues are suggested to be responsible for positioning the ARTT loop of C3stau isoforms in a conformation identical not to that of the NAD-free C3bot1 structure but to that of the C3bot1-NAD-bound conformation. Therefore, conformational changes subsequent to NAD binding are minor in this region (Evans et al. 2003).

Deduced from the crystal structure of C3bot bound to NAD, a further structural element, termed phosphate-nicotinamide-loop (PN-loop), was suggested to be involved in NAD-binding (Fig. 1b). It covers residues C3bot 137–146 and is also located between strands β3–β4. At least one critical arginine within this loop is conserved in all C3-like ADP-ribosyltransferases. It was reported that this residue forms hydrogen bonds to the phosphate groups of NAD (Fig. 1b). Consequently, exchange to aspartate in C3bot or C3cer abolished both NAD glycohydrolase activity and ADP-ribosyltransferase activity of this mutant (Ménétrey et al. 2002; Wilde et al. 2003).

Enzyme activity and substrate specificity

Like typical ADP-ribosyltransferases, C3 exoenzymes split NAD into ADP-ribose and nicotinamide and transfer the ADP-ribose moiety onto Rho protein (Fig. 3). C3 modifies

Fig. 2 Structure of C3bot. **a** The crystal structure of C3bot shows the ADP-ribosylation toxin-turn-turn (*ARTT*) motif. This motif is suggested to be involved in the interaction with the protein substrate, e.g., RhoA (C3 was designed by Swiss-Pdb Viewer 3.7 (database code 1G24). The insert exhibits the putative interaction of C3stau with RhoA. Data are from Han et al. (Han et al. 2001). **b** Scheme of the folding of C3bot (see text). **c** Residues, which participate in the binding of NAD to C3bot. Note that the counting of residues is without the signal sequence.

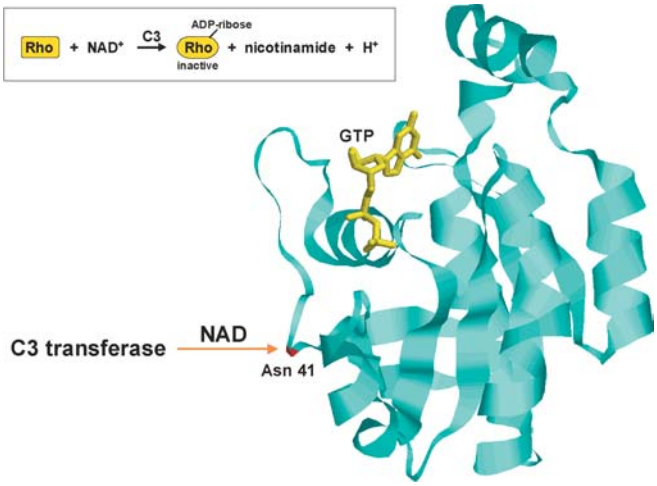


Fig. 3 ADP-Ribosylation of RhoA by C3. C3 transferases ADP-ribosylate RhoA at position Asn41. The acceptor amino acid is located in or very close to the so-called effector region (switch I-region) of RhoA. Rho structure was designed by Rasmol version 2.7.2 (database code 1FTN).

asparagine residue (Asn41) of the target protein (Sekine et al. 1989). This is unique for this family of ADP-ribosyltransferases. Many bacterial ADP-ribosyltransferases modify arginine residues, including cholera toxin, *Pseudomonas exoenzyme* S and T, and the actin modifying binary ADP-ribosyltransferases like *C. botulinum* C2 toxin. Cysteine is modified by pertussis toxin (Aktories 2000; Barbieri et al. 2002).

C3-like ADP-ribosyltransferases are characterized by their substrate specificity, because they modify preferentially Rho A, B, and C. Other Rho GTPases are poor substrates, including Rac and Cdc42. Recently, it was reported that the transferases C3stau1 and C3stau2 (EDIN A and EDIN B) from *S. aureus* ADP-ribosylate also RhoE and Rnd3. RhoE and Rnd3 are isoforms, identical except for a 15-residue N-terminal extension on Rnd3, that are antagonistic to RhoA (Guasch et al. 1998; Foster et al. 1996; Nobes et al. 1998; Riento et al. 2003). They bind GTP but lack GTPase activity. However, the kinetics of the modification of RhoE/Rnd3 is much more slower than that to modify RhoA (Wilde et al. 2001b).

The targets of C3 exoenzymes are molecular switches

RhoA, B, and C, the main targets of C3 exoenzymes, belong to the Rho subfamily of low molecular mass GTP-binding proteins, which comprises more than twenty related GTP-binding proteins, including RhoA, B, C, Rac1, 2, 3, Cdc42, RhoD, Rnd1, Rnd2 (RhoN), RhoE/Rnd3, RhoF (Rif), RhoG, RhoH (TTF) and TC10, TCL, Chp, and Wrch (Jaffe and Hall 2002; Nagata et al. 1998; Ridley 2000; Wennerberg and Der 2004). Most of them, e.g., the prototypes RhoA, B, C, Rac, and Cdc42 cycle between an activated GTP-bound form and an inactive GDP-bound form (Fig. 4). The exchange is tightly controlled by regulating proteins: (a) guanine nucleotide exchange factors (GEFs; more than 60 have been identified) which activate Rho by promoting the exchange of GDP to GTP, (b) GTPase-

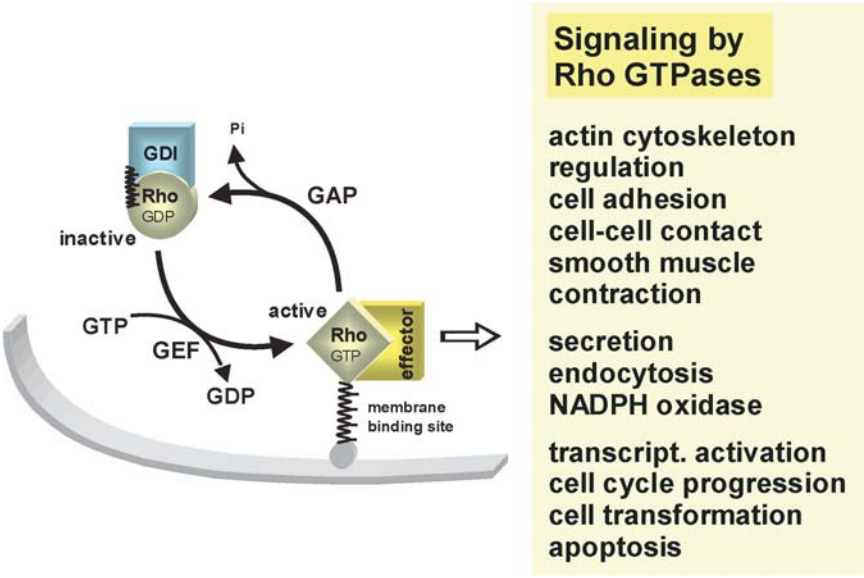


Fig. 4 RhoA GTPase cycle. Rho GTPases are inactive in the GDP bound form and activated by GDP/GTP exchange, which is facilitated by guanine nucleotide exchange factors (*GEFs*). In the active form RhoA interacts with a large array of effectors to induce various cellular effects indicated. The active form of RhoA is terminated by hydrolysis of the bound GTP, which is facilitated by GTPase-activating proteins (*GAPs*). The inactive form is extracted from the membrane by guanine nucleotide dissociation inhibitor (*GDI*), which keep the Rho GTPases in their inactive form in the cytosol.

activating proteins (GAPs; more than 70 have been identified), which inactivate Rho proteins by increasing their intrinsic rate of GTP-hydrolysis, and (c) guanine nucleotide dissociation inhibitors (GDIs; only three are known), which sequester the isoprenylated Rho proteins in the cytosol. The active GTP-bound form of Rho GTPases, which is mostly located at the cell membrane, interacts with multiple cellular effectors, including different protein kinases, lipid kinases, phospholipases and a still growing number of adaptor proteins, involved in a large array of distinct cellular functions, including regulation of the cytoskeleton (Burridge and Wennerberg 2004), cell and smooth muscle contraction, phagocytosis, polarity, activation of transcription, cell cycle progression, and cell transformation (Bishop and Hall 2000; Etienne-Manneville and Hall 2002; Jaffe and Hall 2002; Wennerberg and Der 2004).

Rho GTPases have been identified to be the preferred target of several other bacterial toxins and effectors. They can be activated by deamidation (*E. coli* cytotoxic necrotizing factors, CNF1, CNF2, and CNFy) (Flatau et al. 1997; Hoffmann et al. 2004; Schmidt et al. 1997) and by transglutamination (*Bordetella* dermonecrotizing toxin DNT) (Masuda et al. 2000) at Gln63/61 in Rho and Rac/Cdc42, respectively. Moreover, Rho GTPases are activated by *Salmonella* SopEs, which possess GEF activity and mimic the regulatory functions of endogenous activators (Hardt et al. 1998). An inactivation of Rho GTPases is caused by mono-O-glucosylation by the large clostridial cytotoxins, including toxins A and B of *Clostridium difficile* (Just et al. 1995b), lethal toxin from *Clostridium sordellii* (Just et al. 1996), and α-toxin from *Clostridium novyi* (Selzer et al. 1996). *Yersinia* YopE (von Pawel-Rammingen et al. 2000), *Salmonella* SptP (Fu and Galán 1999), or *Pseudomonas aeruginosa* ExoS (Goehring et al. 1999) inactivate Rho GTPases by mimicking en-

dogenous GAP activity. Moreover, it has been shown recently that *Yersinia* YopT acts as a protease, which cleaves Rho-GTPases at the C-terminal isoprenylated cysteine to inactivate the GTPase (Shao et al. 2002, 2003).

Functional consequences of the ADP-ribosylation of Rho

The ADP-ribosylation of RhoA (B, C) occurs at asparagine-41 (Sekine et al. 1989), which is part of or at least located in close vicinity to the switch-1 region (residues 28–40/41) of the GTPase (Fig. 3). The modification renders Rho biologically inactive (Paterson et al. 1990). The switch-1 region adopts different conformations depending on the nucleotide bound to the GTPase and is the molecular basis for the conduction of signals downstream. The inactivation of Rho by C3 exoenzyme-catalyzed ADP-ribosylation can be easily monitored by redistribution of actin filaments and depolymerization of stress fibers (Chardin et al. 1989; Paterson et al. 1990; Wiegers et al. 1991). ADP-ribosylation of Rho has only minor effects on the nucleotide binding, intrinsic, and GAP-stimulated GTP hydrolase activity. Also binding of ADP-ribosylated Rho with effector proteins, e.g., protein kinase N or Rho kinase (Sehr et al. 1998) and phospholipase D (Genth et al. 2003b) is possible (Fig. 5). Moreover, ADP-ribosylated RhoA is still able to activate its effectors (Genth et al. 2003a). However, this activation appears to depend on the fact that it is already in the active form before ADP-ribosylation. ADP-ribosylation appears to prevent the conformational changes occurring with activation of Rho proteins (Genth et al. 2003b). In line with this notion is the finding that activation of ADP-ribosylated Rho by GEFs (e.g., Lbc) is inhibited (Sehr et al. 1998) (Fig. 5b). Importantly, ADP-ribosylated RhoA seems to be trapped in the Rho/GDI-complex (Genth et al. 2003a). This was studied with a simple membrane filtration assay. The unmodified RhoA/GDI complex (mass ~45 kDa) is not able to pass a 30-kDa cut-off membrane filter. In the presence of phosphatidylinositol 4,5-bisphosphate (PIP2), the complex dissociates and releases RhoA (~20 kDa) and GDI (~24 kDa), which are able to pass the membrane filter. After ADP-ribosylation, however, PIP2 is not able to dissociate the RhoA/GDI-complex, indicating a tight interaction after modification of Asn41 by C3 (Fig. 5c). In line with the apparent increase in the affinity between modified RhoA and GDI, ADP-ribosylated RhoA is exclusively found in the cytosolic fraction of C3-treated cells. ADP-ribosylation reduces the binding of RhoA to membranes (Fujihara et al. 1997; Genth et al. 2003a). Taken together, inhibition of activation of ADP-ribosylated Rho by GEFs and sequestration of ADP-ribosylated RhoA in the GDI-complex are most likely the causes of C3-induced blockade of Rho-dependent signaling (Fig. 6).

Nonenzymatic actions of C3 exoenzymes

Recently, it was reported that C3-like exoenzymes interact directly with other small GTP-binding proteins not belonging to the Rho subfamily of GTPases. Wilde and coworkers showed that C3 exoenzymes from *C. botulinum* and *C. limosum* bind with high affinity (k_D ~10 nM, for *C. limosum*) to RalA (Wilde et al. 2002a) without modifying the GTPase by ADP-ribosylation. Ral is a member of the Ras subfamily of small GTPases and occurs in two isoforms, RalA and B, which share ~35% amino acid identity to RhoA. Ral has been implicated in several cellular processes, e.g., Ras-mediated cell transformation (Feig

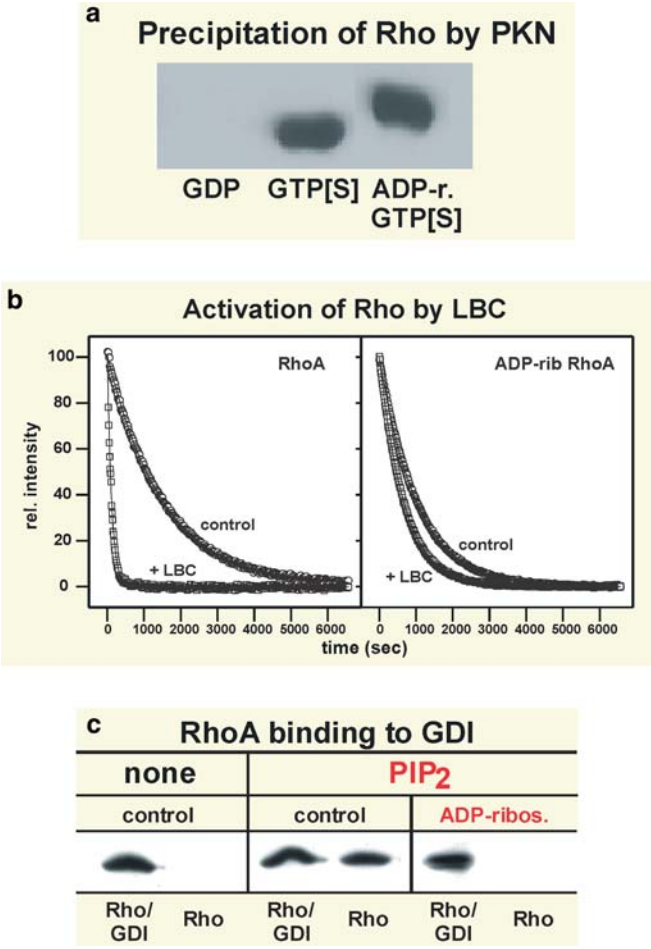


Fig. 5 Functional consequences of ADP-ribosylation of RhoA. **a** ADP-ribosylation of RhoA at Asn41 has no major effect on the interaction of the GTPase with the RhoA effector protein kinase N (*PKN*). Under control conditions, only the active GTP-form (here the GTPγS-form) but not the inactive GDP-form of RhoA is precipitated by the Rho-binding domain (*RBD*) of protein kinase N (*PKN*). In the experiment shown, this RBD-domain was coupled to Sepharose beads and used for precipitation. After ADP-ribosylation, which can be monitored by the shift of RhoA to an apparent higher molecular mass, RhoA is still able to interact with PKN (data from Sehr et al. 1998). **b** ADP-ribosylation decreases the rate of activation by the GEF protein LBC. The activation of RhoA was followed by the release of the fluorescently labeled mantGDP from RhoA to allow binding of GTP. Therefore, activation of RhoA causes decrease in fluorescence. Pretreatment of RhoA with C3 reduces the rate of RhoA activation (data from Sehr et al. 1998). **c** ADP-ribosylation increases the binding of RhoA to GDI. In the cytosol, Rho is in a complex with GDI. Therefore, only the complex is detected by gel or membrane filtration. Under control conditions phosphatidylinositol bisphosphate (*PIP2*) causes dissociation of this complex. Accordingly, Rho released from the complex is detected. After ADP-ribosylation PIP2 is not able to induce dissociation of the complex. Therefore, ADP-ribosylated Rho stays in the cytosol in a complex with GDI. (Data from Genth et al. 2003a).

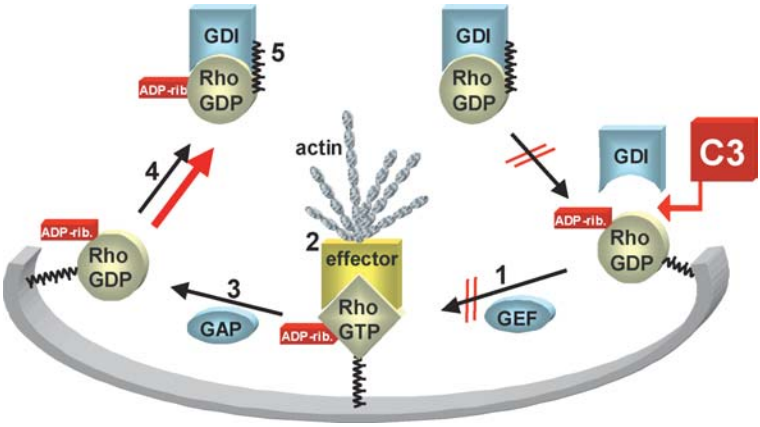


Fig. 6 Summary of the functional consequences of the ADP-ribosylation of RhoA by C3. RhoA is ADP-ribosylated by C3 in the GDI-free form. *1* ADP-Ribosylation inhibits the activation of RhoA by GEF. *2* ADP-ribosylated Rho is still able to interact at least with some effectors such as kinases. *3* The nucleotide binding and the GTP hydrolysis is almost not affected by ADP-ribosylation. *4* ADP-ribosylation decreases membrane-binding of RhoA. *5* Binding of ADP-ribosylated RhoA to GDI is increased. Therefore, ADP-ribosylated RhoA will remain in the inactive form in the cytosol.

et al. 1996; Urano et al. 1996), cytoskeleton rearrangement (Jullien-Flores et al. 1995; Ohta et al. 1999; Park and Weinberg 1995), and vesicle trafficking, e.g., by regulating the exocyst via binding to sec5 (Moskalenko et al. 2001). Ral acts on phospholipase D1 (PLD1) (Jiang et al. 1995; Luo et al. 1998) and it is suggested that both RalA and PLD1 modulate receptor endocytosis and vesicle transport. Binding of C3 to RalA inhibits its ADP-ribosyltransferase activity to modify RhoA (Fig. 7). Similarly, interaction of C3 with RalA reduces the ability of the GTPase to activate PLD1 in vitro, suggesting that the binding of the exoenzyme to Ral occurs in a region of the GTPase, which is important for the interaction with its effectors. Moreover, interaction of C3 with Ral prevents glucosylation of Ral by *Clostridium sordellii* lethal toxin in intact cells (Wilde et al. 2002a). Because glucosylation of Ral occurs in the functionally important switch-1 region, it is likely that interaction of C3 with this region also affects Ral functions in intact cells. Such a sequestration of Ral might be relevant at high concentration of C3, which can be achieved by microinjection or overexpression of C3 (see below). In contrast to the exoenzymes from *C. botulinum*, *C. limosum*, and *B. cereus*, the transferase C3stau2 from *S. aureus* is not capable of binding to RalA.

Recently another C3 effect, which is independent of the ADP-ribosyltransferase activity has been reported. It is well-known that Rho proteins regulate neurite outgrowth (see below). Several studies showed that C3 prevent neurite retraction induced by activated RhoA (see below). Surprisingly, Ahnert-Hilger and coworkers found that C3bot but not other C3 exoenzymes promote the axonal growth and branching independent of the enzyme activity (Ahnert-Hilger et al. 2004). Moreover, this effect depended on the extracellular application of the exoenzyme. Intracellularly expressed C3bot did not induce axon growth. They propose a novel neurotrophic function of C3bot independent of its transferase activity.

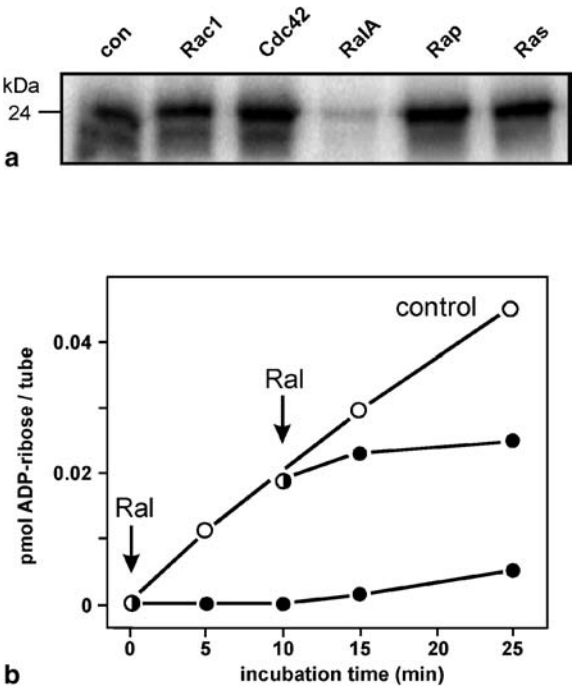


Fig. 7 Ral protein inhibits C3-induced ADP-ribosylation of RhoA. **a** RhoA was ADP-ribosylated by C3 with [^{32}P]NAD in the presence of various small GTPases (Rac, Cdc42, Ras, Rap, and Ral). Only Ral inhibited the ADP-ribosylation of RhoA. **b** Time course of ADP-ribosylation of RhoA in the presence and absence of Ral. Ral caused an immediate inhibition of the modification of RhoA. (Data from Wilde et al. 2002a).

Pathophysiological role of C3

Although much is known about the cellular functions of Rho GTPases, the roles of C3-like transferases in pathogenicity are not at all understood (Fig. 8). An action of C3 exoenzymes on the immune system of the eukaryotic target organism is most likely. Some of these effects have been already mentioned. Inhibition of immune cell functions including cytotoxicity of lymphocytes (Lang et al. 1992), adhesion (Nemoto et al. 1996), migration and invasion of lymphocytes (Stam et al. 1998; Verschueren et al. 1997), and leukocytes (Laudanna et al. 1996, 1997) by C3bot have been demonstrated. Rho GTPases have been proven to be important components of signal pathways used by antigen receptors, cytokine, and chemotaxins receptors to regulate the immune response (Heath and Holifield 1991; Henning and Cantrell 1998; Laudanna et al. 1996; Prepens et al. 1996; Price et al. 1995; Wojciak-Stothard et al. 1998). Moreover, Rho proteins participate in the barrier functions of epithelial cells (Nusrat et al. 1995; Vouret-Craviari et al. 1998) and in wound healing (Santos et al. 1997). However, considering the poor cell accessibility of C3 exoenzymes, an important question remains: how do these specific and potent agents get to their site of action? At least two possibilities are feasible. Recently, it was shown that pore-forming toxins appear to act as a delivery system for bacterial proteins. Madden and coworkers (Madden et al. 2001) reported that *Streptococcus pyogenes* uses streptolysin O, a cholesterol-dependent cytolysin, to translocate *S. pyogenes* NAD-glycohydrolase SPN into the target cells. This method of target-specific translocation appears to be comparable with the type-III secretion system frequently found in gram negative bacteria. Considering the fact that many of the bacteria, which synthesize C3 exoenzymes, also produce pore-forming agents, it is feasible that a similar mechanism is functional with these pathogens.

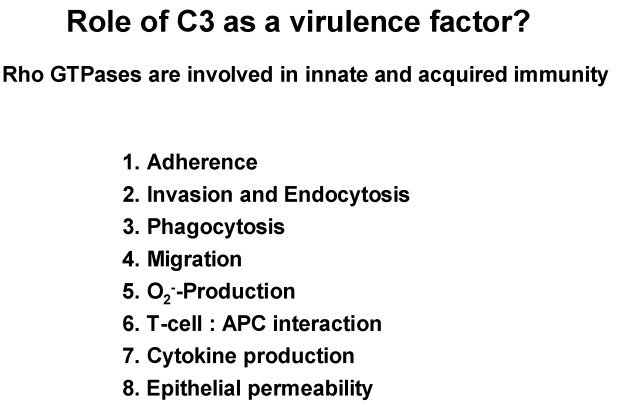


Fig. 8 The role of C3 as a virulence factor is not clear. However, Rho GTPases (including other members of the family) have been shown to be involved in several processes, which are important for innate and acquired immunity, including adhesion invasion and endocytosis of immune cells, migration, superoxide anion production (at least true for Rac), interaction of T-cells with antigen-presenting cells (*APC*), cytokine production, and epithelial permeability (see also Table 1).

The other possibility is based on recent findings that more pathogens than previously suggested are capable of invading host cells. This also applies to *Staphylococci* (Lowy 2000; Mempel et al. 2002). Moreover, it was suggested that the pathogens enter the cytosol of target cells (Bayles et al. 1998). This implies that the bacterium is able to release the Rho-ADP-ribosylating enzyme directly into the cytosol, where its protein target is localized (Wilde et al. 2001a; G.S. Chhatwal et al., unpublished data).

C3-like exoenzymes as pharmacological tools

Because C3-like ADP-ribosyltransferases are highly specific for Rho GTPases, they are established pharmacological and cell biological tools to study the physiological functions of Rho GTPases. On the other hand, C3-like ADP-ribosyltransferases lack a specific receptor binding and translocation domain and, therefore, their cellular uptake is rather poor. Due to this fact, the toxins have to be applied in high concentrations and/or for long incubation periods. Quite often the toxins were introduced into target cells by microinjection (Paterson et al. 1990; Watanabe et al. 1997). Another approach to overcome this problem is the use of C3-toxin chimeras. Aullo et al. (Aullo et al. 1993) fused C3bot to diphtheria toxin. DC3B, a fusion protein of C3 and the binding and translocation domain of diphtheria toxin, has a high affinity for the DT receptor, but apparently enters the target cell by a mechanism different from the typical pathway of diphtheria toxin. Because the action of this fusion toxin is limited to cells with receptors for diphtheria toxin, other chimeras were constructed. Very efficient is a fusion toxin, which is based on the binary C2 toxin from *Clostridium botulinum.* C2 toxin consists of the actin-ADP-ribosylating enzyme component C2I and the binding and translocation component C2II, which are both separated proteins (Aktories et al. 1986; Barth et al. 2002; Ohishi et al. 1980). After proteolytic activation of C2II, the activated C2II monomers oligomerize to heptamers (Barth et al. 2000) and upon binding of C2I to C2II, both components are internalized by receptor-mediated endocytosis. The N-terminal part (C2IN) of C2I, which alone is sufficient for the interaction with the binding component C2II, was fused to full-length ADP-ribosyltransferases C3lim or C3stau, respectively (Barth et al. 1998; Wilde et al. 2001b). This chimeric toxin increases the potency of C3 several hundred-fold (Meyer et al. 2000; Valderrama et al. 2000; Vischer et al. 2000; Wahl et al. 2000). Because the binding component of C2 toxin

appears to bind to complex and/or hybrid carbohydrates present on all vertebrate cells (Eckhardt et al. 2000), all these cells are sensitive towards the fusion toxin. Also the adaptor domain of the enzyme component and the binding component of iota toxin, which are similar to C2 toxin, have been effectively used for delivery of C3-like toxins into cells (Marvaud et al. 2002).

Recently, it was reported that C3bot could be transported into cells by adding short peptides to the C-terminal end of the exoenzyme. For this purpose short sequence of the human immunodeficiency virus transcription activator Tat was used (Park et al. 2003; Sauzeau et al. 2001). The transport of C3bot into cells can also be accomplished by fusing the third helix of the *Antennapedia* homeodomain protein from *Drosophila* to C3bot. In addition, short proline-rich peptides and highly basic arginine-rich peptides were C-terminally fused to C3 exoenzyme to facilitate the uptake of the transferase (Winton et al. 2002).

Another method to use C3-specific inhibition of Rho GTPases is the intracellular expression of the gene (Hilal-Dandan et al. 2004). Transgenes based on the thymocyte-specific lck promoter have been used for expression of C3 in thymus. Transgenic mice showed maturational, proliferative, and cell survival defects during T-cell development (Henning et al. 1997). Recently, a transgenic mouse model expressing C3 exoenzyme in a lens-specific manner was utilized (Maddala et al. 2004). Under transcriptional control of the lens-specific alphaA-crystallin promoter mice, expressing the C3 exoenzyme transgene, exhibited selective ocular defects, including cataract and microphthalmia (Rao et al. 2002).

In the following paragraph, cell biological effects, which are observed with the "C3 tool," are briefly summarized. Quite early studies showed that treatment of Vero cells with C3bot induces morphological changes characterized by rounding up of the cells with concomitant destruction of stress fibers (Chardin et al. 1989). The same findings were obtained with many other cell types and with different C3-like ADP-ribosyltransferases. Many of the classical studies on the functions of Rho GTPases performed in the laboratory of Alan Halls depended on the usage of C3 (Paterson et al. 1990; Ridley et al. 1992; Ridley and Hall 1992).

After C3 treatment, actin-staining by rhodamine-phalloidin usually reveals loss of stress fibers; treated cells remain in contact via small extensions. After removal of toxin from the medium, cells are still viable and the phenotype reverses after a few hours to days by neosynthesis of Rho (Barth et al. 1999). The reversal appears to be especially fast with the C3–C2I fusion toxin, which appears to be degraded rapidly (Barth et al. 1999). In many studies, C3 was shown to prevent the formation of stress fibers and focal adhesions induced by growth factor (Hall 1994; Mackay et al. 1997; Ridley and Hall 1992) or by integrins (Barry et al. 1997). In contrast, processes that are mediated by Rac or Cdc42, like lamellipodia and microspike formation in fibroblasts, are not affected by C3 (Kozma et al. 1995; Nobes and Hall 1995; Ridley and Hall 1992). Although C3bot induces rounding up in adherent cells, the toxins cause cell spreading in monocytes (Aepfelbacher et al. 1996) and in T cells (Borroto et al. 2000).

C3bot was frequently used as a tool to study the role of Rho in cell motility, migration and cell invasion (see Table 1). The exoenzyme was successfully applied in studies on the regulatory function of Rho GTPases in neurite outgrowth, branching, and neuroregeneration. Similarly the role of Rho GTPases in the control of phospholipase D and in phospholipid metabolism was studied with C3. The role of Rho GTPases in transcriptional activa-

Table 1 List of studies with C3 exoenzymes applied as cell biological and pharmacological tools

Rho in regulation of the actin cytoskeleton	Barry et al. 1997; Kozma et al. 1995; Nobes and Hall 1995; Ridley et al. 1992; Ridley and Hall 1992; Wiegers et al. 1991; Wilde et al. 2001b
Rho in cell adhesion, migration, chemotaxis and invasion	Adachi et al. 2001; Anderson et al. 2000; Kusama et al. 2001; Nguyen et al. 2002; Saurin et al. 2002; Strey et al. 2002; Takaishi et al. 1994; Worthylake et al. 2001; Yoshioka et al. 1998
Rho in cell cycle progression, cell division and cytokinesis	Eda et al. 2001; Fiorentini et al. 1998; Kato et al. 2001; Liberto et al. 2002; O'Connell et al. 1999; Olson et al. 1995; Takaishi et al. 1995
Rho and neurite growth	Dergham et al. 2002; Grunwald and Klein 2002; Jalink et al. 1994; Lehmann et al. 1999; Neumann et al. 2002; Niederost et al. 2002; Nishiki et al. 1990; Tigyi et al. 1996; Wahl et al. 2000; Yuan et al. 2003
Rho in endocytic and exocytic processes	Caron and Hall 1998; Doussau et al. 2000; Lamaze et al. 1996; Park et al. 2003; Schmalzing et al. 1995; Vögler et al. 1999
Rho, phospholipase D and phospholipid metabolism	Balboa and Insel 1995; Chong et al. 1994; Kanumilli et al. 2002; Kuribara et al. 1995; Meacci et al. 1999; Ren et al. 1996; Schmidt et al. 1996; Schmidt et al. 1999; Weernink et al. 2000; Xie et al. 2002
Rho and transcription and differentiation	Alberts et al. 1998; Chen et al. 2002; Dreikhausen et al. 2001; Hill et al. 1995; Lu et al. 2001; Mack et al. 2001; Sahai et al. 1998; Sotiropoulos et al. 1999; Su et al. 2001; Takemoto et al. 2002
Rho in signal transduction by heptahelical receptors	Buhl et al. 1995; Fromm et al. 1997; Gohla et al. 2000; Klages et al. 1999; Le Page et al. 2003; Mao et al. 1998a; 1998b; Nguyen et al. 2002; Ohmori et al. 2001; Sagi et al. 2001; Sah et al. 1996, 2000; Xie et al. 2002; Yamakawa et al. 2000
Rho and apoptosis	Bobak 1999; Dubreuil et al. 2003; Fiorentini et al. 1998; Hippenstiel et al. 2002; Mills et al. 1998; Reuveny et al. 2004

tion was another important topic, which was frequently addressed with C3 as a tool. Moreover, C3 was successfully employed in delineation of the role of Rho in the signaling of various heptahelical receptors to the actin cytoskeleton, phospholipases, and the nucleus via heterotrimeric G proteins. Especially important was C3 in studies on the functions of $G\alpha_{12/13.}$ Finally, C3 was studied in cell division and apoptosis (for references see Table 1).

Conclusion

Our information about C3 ADP-ribosyltransferases, their structures and mode of actions has increased enormously in recent years. We do understand a lot about the functional consequences of the ADP-ribosylation of Rho GTPases, when C3 is applied as a tool. However, additional potentially important functions and properties of C3 have been described recently, which are not clearly defined or not really understood at present, including the high affinity interaction with Ral and the action as a neurotrophic factor. Moreover, many open questions remain concerning the pathogenic role of C3 exoenzymes. With respect to further progress in the structure function analysis, it would be of major importance to solve the crystal structure of C3 in the complex with its Rho substrate. Hopefully, we will get this information in the near future.

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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

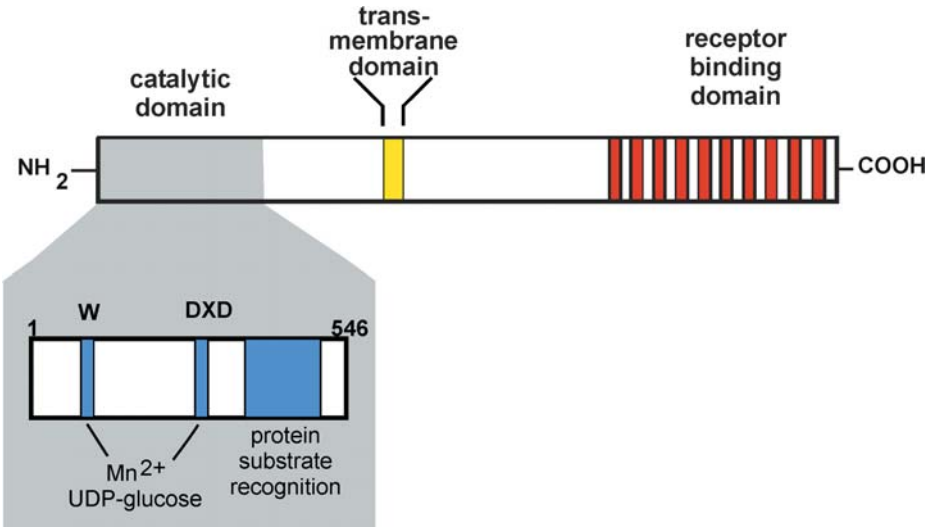

Fig. 1 Structure of the large clostridial toxins illustrated for toxin B. Toxin B is constructed of three functional domains. The receptor-binding domain is composed of repetitive oligopeptide elements commonly accepted as motif for binding to sugar structures. The possible multivalent binding to cell 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.

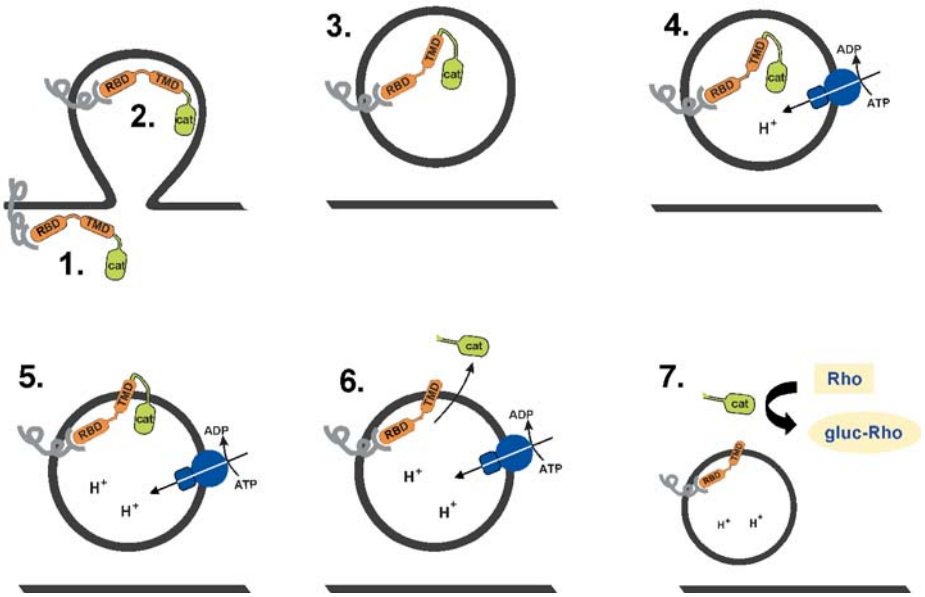

Fig. 2 Cell entry of *C.* toxin A/B. *1*Toxin A binds through its receptor-binding domain (*RBD*) to a membranous carbohydrate structure (the receptor of toxin B is unknown). *2* Toxin binding induces receptor-mediated endocytosis. *3* Endocytosed vesicle is processed to endosomes. *4* Vesicular-ATPase 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

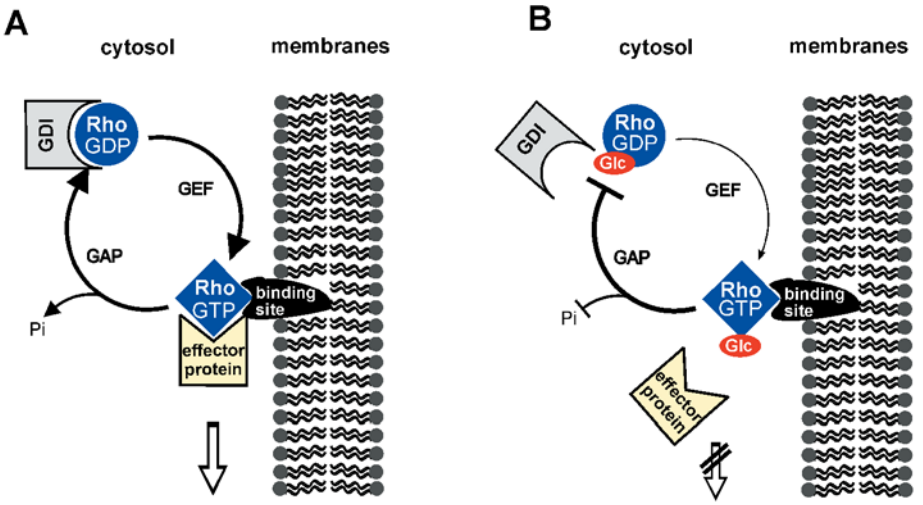

Fig. 3 Molecular mode of action of toxin A/B. **A** The Rho-GTPases are molecular switches which are regulated by guanine nucleotide binding. Nonsignalling, inactive Rho is complexed with the guanine nucleotide dissociation inhibitor GDI to be localized to the cytosol. Signal input induces an activation 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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CNF and DNT

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Abstract The actin cytoskeleton of mammalian cells is involved in many processes that affect the growth and colonization of bacteria, such as migration of immune cells, phagocytosis by macrophages, secretion of cytokines, maintenance of epithelial barrier functions and others. With respect to these functions, it is not surprising that many bacterial protein toxins, which are important virulence factors and causative agents of human and/or animal diseases, target the actin cytoskeleton of the host. Some toxins target actin directly, such as the C2 toxin produced by *Clostridium botulinum.* Moreover, bacterial toxins target the cytoskeleton indirectly by modifying actin regulators such as the low-molecular-mass guanosine triphosphate (GTP)-binding proteins of the Rho family. Remarkably, toxins affect these GTPases in a bidirectional manner. Some toxins inhibit and some activate the GTPases. Here we review the Rho-activating toxins CNF1 and CNF2 (cytotoxic necrotizing factors) from *Escherichia coli*, the *Yersinia* CNF_Y and the dermonecrotic toxin (DNT) from *Bordetella* species. We describe and compare their uptake into mammalian cells, mode of action, structure–function relationship, substrate specificity and role in diseases.

Rho GTPases

Rho guanosine triphosphatases (GTPases) belong to the Ras superfamily of low-molecular-mass GTPases and act as molecular switches in various signalling pathways. Rho GTPases are ubiquitously expressed. The family consists of more than 15 members, which share more than 50% sequence identity (for review see Takai et al. 2001). Like all members of GTP-binding proteins, they are active in the GTP-bound form and inactive when guanosine diphosphate (GDP)-bound. Activation occurs by nucleotide exchange catalysed by guanine nucleotide exchange factors (GEFs) and is inhibited by binding of guanine nucleotide dissociation inhibitors (GDIs). Inactivation occurs by hydrolysis of the bound

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GTP. The intrinsic GTPase activity is catalysed by GTPase-activating proteins (GAPs) (Symons and Settleman 2000; Van Aelst and D'Souza-Schorey 1997).

Low-molecular-mass GTPases of the Rho family are known as master regulators of the actin cytoskeleton. Best studied are the morphological changes induced by RhoA, Rac1 and Cdc42. Activation of RhoA leads to the formation of actin stress fibres, bundles of actin filaments associated with myosin and other proteins. Cdc42 is known to induce the formation of filopodia, small actin protrusions at the leading edge of migrating cells, which appear to act as sensory elements more than to be driving forces for migrating cells.

The third well-characterized member of the Rho family, Rac, is involved in the formation of lamellipodia and membrane ruffles. These actin-containing structures are necessary for phagocytosis and cell movement. Besides their actin-regulating functions, Rho GTPases are involved in the control of transcriptional activation, cell transformation, apoptosis, cell polarization and others. Many Rho GTPase effectors involved in these processes have been identified, including protein kinases, lipid kinases, phospholipase D, scaffolding proteins and numerous adaptor proteins (for review see Takai et al. 2001; Etienne-Manneville and Hall 2002; Burridge and Wennerberg 2004).

As mentioned above, Rho GTPases are the eukaryotic targets of a variety of bacterial protein toxins that either inhibit or activate the Rho proteins.

Rho-inactivating toxins

One of the first toxins, identified as an inactivator of RhoA, was the C3 toxin from *Clostridium botulinum.* This toxin is an adenosine diphosphate (ADP)-ribosyltransferase that covalently modifies the small GTPases RhoA, B and C at Asn41 (Sekine et al. 1989). Other C3-like toxins, which share 30–70% amino acid sequence identity, are produced by *C. limosum* and *Staphylococcus aureus.* These toxins also ADP-ribosylate Rho GTPases, thereby inactivating them. The large clostridial cytotoxins modify GTPases by glucosylation (Just et al. 1995, 2000; Busch and Aktories 2000). Members of this toxin family are *C. difficile* toxins A and B, the lethal and the hemorrhagic toxins from *C. sordellii* and the alpha-toxin from *C. novyi*. These toxins modify a highly conserved threonine residue (Thr37 in RhoA) in the switch I region of the GTPases, which is involved in Mg^{2+}, nucleotide and effector binding. Thus, modification of this threonine causes inactivation of the GTPases (Sehr et al. 1998; Genth et al. 1999; Herrmann et al. 1998). The *Yersinia pseudotuberculosis* outer protein T (YopT) inactivates Rho GTPases by cleaving off the isoprene moiety of the GTPase thereby preventing membrane localization (Shao et al. 2002).

Recent studies indicate that Rho proteins are not exclusively covalently modified by bacterial toxins. Some bacterial effectors like the *Salmonella* SopEs and SptP modulate the activity of Rho GTPases by acting as regulatory proteins with GAP (SptP) or GEF (SopEs) functions (Galan and Fu 2000; Stender et al. 2000).

Rho-activating toxins

Besides the Rho-inactivating toxins, bacteria have evolved mechanisms for constitutive activation of Rho GTPases. Interestingly, not only inactivation, but also permanent activation of the GTPases leads to inhibition of immune responses of the host (Hofmann et

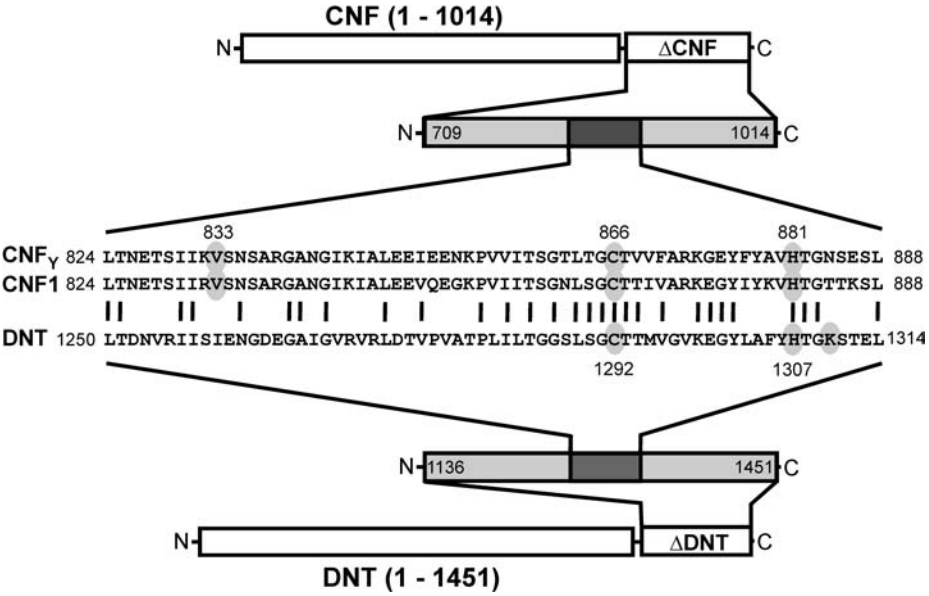

Fig. 1 Homology between CNF1, CNF_Y and DNT: CNF, CNF_Y and DNT consist of 1014 or 1451 (DNT) amino acids. The toxins share homology within their catalytic domains (ΔCNF and ΔCNF_Y, aa 709–1014 and ΔDNT, aa 1136–1451) which are located at the C termini of the proteins. Highest sequence similarity between DNT and the CNFs is observed in a stretch of 64 amino acid residues, which covers residue 1250–1314 of DNT, showing ~45% sequence identity (DNT and CNF1), whereas the amino acid sequence of the whole catalytic domain of DNT is only ~13% identical with the sequence of the catalytic domain of CNF1. Catalytically essential residues are marked with a *grey circle*

al. 2000; Rippere-Lampe et al. 2001a) and, for example, to opening of tight junctions (Gerhard et al. 1998).

More than 20 years ago, a toxin from *Escherichia coli* obtained from enteritis-affected children was isolated by Caprioli and co-workers. Because of its necrotizing effects on rabbit skin, they called the toxin cytotoxic necrotizing factor (CNF) (Caprioli et al. 1983). CNF1 and the homologue CNF2, which was identified later [90% sequence identity, (Oswald et al. 1994)], are produced by various pathogenic *E. coli* strains isolated from animals [e.g. piglets and calves (de Rycke et al. 1987)] and man (de Rycke et al. 1990). In contrast to CNF1, which is chromosomally encoded, CNF2 is located on a transmissible plasmid (Oswald and de Rycke 1990).

More recently, the gene for a CNF of *Yersinia pseudotuberculosis* (CNF_Y) with 65% nucleotide sequence identity was identified, and it is not located on the so-called virulence plasmid encoding the *Yersinia* outer proteins but is on the genome of the bacteria (Lockman et al. 2002).

All three CNFs have molecular masses of about 115 kDa. They are single-chain AB toxins with an N-terminal cell-binding domain [amino acids (aa) 53–190] and a C-terminal catalytic domain (aa 720–1014, Fig. 1) (Lemichez et al. 1997). The central part contains two hydrophobic helices (aa 350–372 and aa 387–412), which appear to be involved in membrane translocation (Pei et al. 2001) (see below).

DNT, which is produced by *Bordetella pertussis, B. parapertussis* and *B. bronchiseptica*, received its name from dermonecrotic effects caused by its intradermal injection

(Horiguchi et al. 1989). DNT is a large, 160-kDa, heat-labile protein that shares significant sequence similarity to the *E. coli* toxins. The homology with CNF1 and CNF2 is restricted to the catalytic domains at the C termini of the toxins, suggesting that both toxins share a similar molecular mechanism (see below).

Mechanism of uptake of CNFs

CNFs are cytotoxic for a wide variety of cells, including 3T3 fibroblasts, Chinese hamster ovary cells, Vero cells, T cells, epithelial cells, HeLa cells and cell lines of neuronal origin. This implies that a commonly expressed receptor is responsible for the uptake of CNF1. Recently, the receptor for the entry of the toxin into cells was suggested to be the 37-kDa laminin receptor precursor, which is ubiquitously expressed and interacts with the N-terminal part of the toxin in the yeast two-hybrid screen (Chung et al. 2003). Moreover, CNF-mediated RhoA activation and the uptake of CNF1-producing bacteria were inhibited by the addition of laminin receptor precursor (Chung et al. 2003). For the uptake of CNF, clathrin-dependent and independent endocytosis is crucial as has been shown with the use of filipin, which blocks clathrin-independent endocytosis (Torgersen et al. 2001). In both experiments, the cells were sensitive to CNF1. Following endocytosis and acidification of the endosomal compartment, CNF1 crosses the endosomal membrane to get into the cytosol (Contamin et al. 2000). For this translocation step, the translocation domain containing two hydrophobic helices connected by a loop of 5 amino acids seems to be necessary. It was suggested that the low pH of the endosome causes unfolding of CNF1 and leads to protonation of acidic residues and facilitates insertion into the membrane. According to this model, mutation of specific acidic residues in the translocation domain inhibits translocation of the toxin (Pei et al. 2001). These acidic residues are identical in CNF_Y, indicating that translocation of the *Yersinia* toxin occurs similar to that shown for the *E. coli* CNF1. It is not yet clear whether the CNF holotoxin or a fragment is translocated into the cytosol.

Uptake of DNT

The *Bordetella* DNT is a large, 160-kDa, heat-labile single-chain toxin that is composed—similar to CNFs—of a receptor-binding domain at the N-terminus and a C-terminal catalytic domain. Like CNF, it is endocytosed and released from the endosomal compartment after acidification. It was found that aa 1–531 of DNT blocked the intoxication of cells by full-length DNT, suggesting that this fragment retains the cell-binding domain of DNT (Kashimoto et al. 1999). The very N-terminal aa 1–54 of DNT are sufficient for binding to the cellular receptor. The iodinated DNT fragment (aa 1–54) binds to target cells. Moreover, the fragment competitively inhibits DNT uptake (Matsuzawa et al. 2002). More recently, a furin cleavage site within this binding domain was identified. Proteolytic processing of DNT by furin seems to be necessary for translocation of the toxin across cellular membranes. After cleavage, the translocation seems to be independent of the DNT receptor (Matsuzawa et al. 2003).

Mode of action of Rho-activating toxins

CNFs have been shown to induce the enlargement, spreading and multinucleation of culture cells. The toxins lead to the formation of filopodia, membrane ruffles and a dense network of actin stress fibres, indicating an activation of Rho proteins (compare Fig. 3) (Fiorentini et al. 1997). Moreover, CNFs change the migration behaviour of RhoA in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Oswald and de Rycke 1990; Oswald et al. 1994), a finding, which quite early suggested that a covalent modification of RhoA is catalysed by the toxins. Indeed, CNF1 deamidates glutamine 63 of RhoA (Schmidt et al. 1997; Flatau et al. 1997) or glutamine 61 of Rac and Cdc42 (Lerm et al. 1999a). Glutamine 63/61 is essential for the GTP hydrolysis catalysed by the GTPase. Exchange to any other amino acid blocks the GTP hydrolysing activity. Thus, GTP hydrolysis is blocked after treatment of Rho with CNF1/2 or CNF_Y also in the presence of a GAP and RhoA is held constitutively active (Hoffmann et al. 2004). CNF is highly specific for Rho GTPases. The minimal Rho sequence allowing deamidation or transglutamination (see below) by CNF1 is a peptide covering mainly the switch II region (Asp59–Asp78) of RhoA (Lerm et al. 1999a; Flatau et al. 2000). The high specificity of CNF1 can be explained by the localization of the catalytic core of CNF1 in a deep cleft, as was deduced from the crystal structure of the catalytic domain [see below (Buetow et al. 2001)]. In contrast to CNF1 and CNF2, CNF_Y has even higher substrate specificity. It has been shown to selectively activate RhoA, and only moderately activate other Rho GTPases like Rac and Cdc42 in HeLa cells and Rat hippocampal cells, whereas no difference could be detected in vitro (Hoffmann et al. 2004). The reason for the RhoA specificity of CNF_Y in living cells might be due to a different uptake mechanism or different localization within the cell and remains to be analysed.

Transglutaminases are mammalian enzymes that catalyse the deamidation of glutamine residues within protein chains (Lorand and Graham 2003). In the presence of primary amines, however, these enzymes covalently link the amine to glutamine, thereby polyaminating the amino acid. Moreover, transglutaminases are protein crosslinking enzymes that connect glutamine residues with lysines of a different protein. CNFs ability to crosslink Rho GTPases or to polyaminate them has been analysed. Besides CNFs deamidating ac-

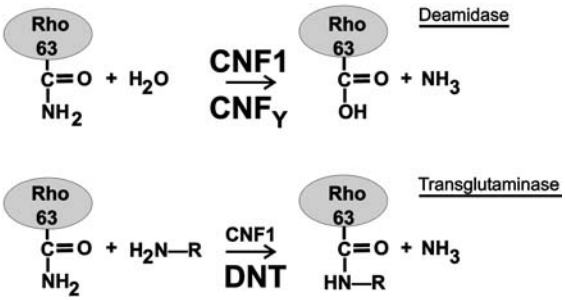

Fig. 2 Molecular mechanism of Rho activation by CNF1, CNF_Y and DNT: Glutamine 63 of RhoA (Gln 61 of Rac and Cdc42) is essential for the hydrolysis of bound GTP. CNF1, CNF_Y and DNT deamidate this glutamine residue, creating glutamic acid, and the GTP hydrolysing activity of the GTPase is blocked. In the presence of primary amines, CNF1 and DNT can transglutaminate Rho at the same residue, thereby also blocking hydrolysis of GTP. Polyamination is the preferred activity of DNT. During deamidation as well as during transglutamination ammonia is released

tivity, they have been shown to catalyse the transglutamination of the same glutamine residue of Rho. This modification also leads to permanent activation of the GTPases (Schmidt et al. 1998). In contrast, no crosslinking activity has been found.

A similar mechanism of Rho modification as catalysed by CNF1 was reported for the CNF1-related DNT from *Bordetella* species (Horiguchi et al. 1997; Kashimoto et al. 1999; Schmidt et al. 1999b; Horiguchi 2001).

It was demonstrated that DNT causes two different covalent modifications of Rho resulting in either slightly slower or slightly faster migration of the GTPase in SDS-PAGE. The faster migrating RhoA represents the transglutaminated GTPase, whereas the slower form is the deamidated GTPase (Schmidt et al. 1999b). DNT causes the polyamination of glutamine 63/61 of Rho GTPases in the presence of primary amines and can also catalyse the deamidation with water. Both modifications of Rho occur at position Gln63. No crosslinking activity has been found for DNT. However, it appears that DNT prefers transglutamination, whereas CNF is primarily a deamidase (Fig. 2) (Schmidt et al. 1999b). Recently, putrescine, spermidine and spermine have been identified as in vivo substrates for the transglutamination (Masuda et al. 2000; Schmidt et al. 2001). At least in vitro, lysine is a preferred substrate for transglutamination by DNT (Schmidt et al. 2001). Similar to CNF, the DNT targets are not only Rho but also Cdc42 and Rac.

Although CNF and DNT modify the same set of Rho GTPases, they differ in their respective requirements for substrate recognition. A peptide covering the switch II region of RhoA (Asp59–Asp78) is sufficient for deamidation by CNF1 even though the reaction is about 110 times slower. Mutations of amino acids Arg68 and Leu72 within this region of RhoA block its modification by CNF, indicating the involvement of these residues in substrate recognition (Lerm et al. 1999a). However, a shorter peptide (aa 59–69) has been identified as the minimal RhoA sequence required for deamidation (Flatau et al. 2000). In contrast to CNF1, no deamidation or transglutamination of these peptides by DNT is detected, supporting the notion that other regions besides switch II are essentially required. A good candidate is the switch I region, since prior glucosylation of Thr37 by toxin B prevents modification by DNT. Modification by DNT is dependent on the type of nucleotide bound to RhoA, and only GDP-bound RhoA is substrate (Masuda et al. 2000; Schmidt et al. 1999a), while CNF1 equally accepts the GTP- and the GDP-bound form. Taken together, these findings show that structural requirements for DNT substrate recognition are more stringent than for CNF1 and that additional elements in RhoA are needed.

Cell biological effects

As mentioned above, cultured mammalian cells treated with CNFs are characterized by dramatic changes of actin-containing structures, including stress fibres, lamellipodia and filopodia (Fig. 3). The formation of lamellipodia and membrane ruffles is required for phagocytosis and endocytosis. Accordingly, activation of Rho GTPases by CNF induces phagocytic behaviour and macropinocytosis in mammalian cells (e.g. human epithelial cells), which are non-professional phagocytes (Falzano et al. 1993; Fiorentini et al. 2001). One of the most striking effects of CNF1 is the formation of multinucleated cells (Oswald et al. 1989). It is not yet clear whether activation of all Rho GTPases is needed for this effect or which GTPases are involved. The effect might be caused by blocking cell division without changes in nuclear cycling, or by increasing in the rate of nuclear cycles within

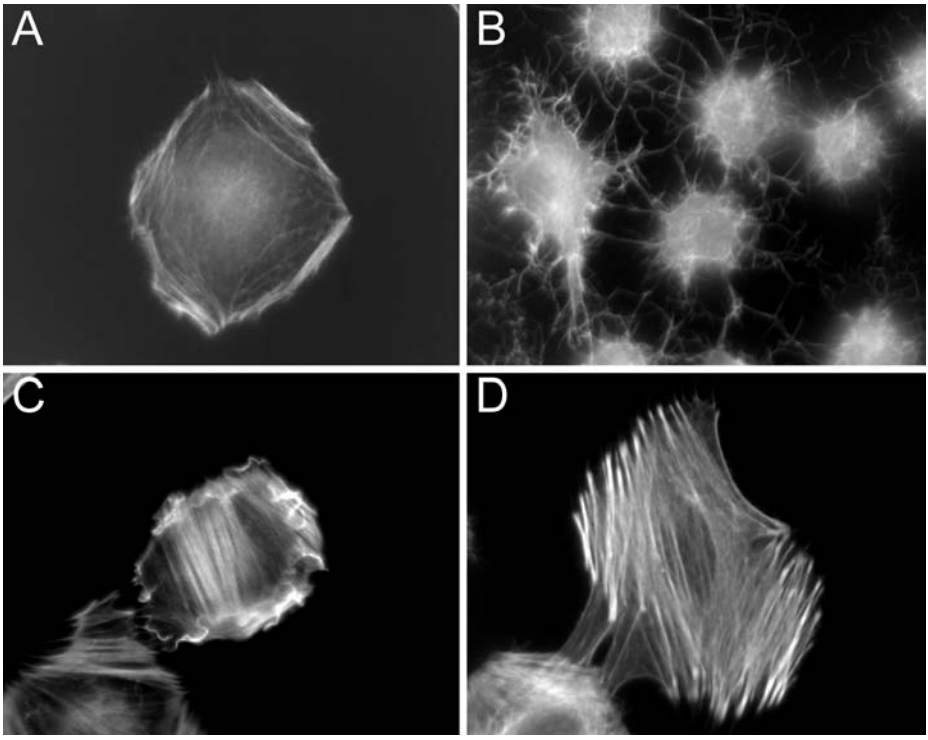

Fig. 3A–D Changes of the actin cytoskeleton of HeLa cells induced by ToxB, CNF1 and CNF_Y, respectively. HeLa cells (untreated; **A**) have been treated with *Clostridium difficile* toxin B (ToxB; **B**), *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1; **C**) or *Yersinia pseudotuberculosis* cytotoxic necrotizing factor 1 (CNF_Y; **D**) for 2 h at 37°C, fixed and stained with rhodamine-phalloidine (F-actin staining)

one cell division cycle. It has been shown that CNF2 uncouples S-phase from mitosis (Denko et al. 1997). Thus, it affects the synthesis of cytoplasmic material without a complete mitosis before starting another S-phase. Quite early it was found that CNF causes phosphorylation of paxillin and Fak kinase, which are known to also be involved in nuclear signalling. This pathway is Rho dependent but does not involve the classical Map kinase pathway (Lacerda et al. 1997).

Rac is involved in the activation of c-Jun kinase. Therefore, CNF1 causes activation of this kinase. Surprisingly, c-Jun kinase activity is only transiently increased after CNF treatment of cells, although the GTPases are constitutively activated (Lerm et al. 1999b). Recently, the reason for the transient activation was identified. It was shown that Rac, which induces c-Jun kinase activation, is ubiqitinated and markedly degraded by a proteasome-dependent pathway in CNF1-treated HEK293 cells (Lerm et al. 2002; Doye et al. 2002). Thus, it appears that the targeted cell is able to block the persistent activation of deamidated Rac by rapid degradation. This effect is functionally comparable with the tight regulation of GTPases by *Salmonella* strains producing a GAP as well as a GEF for Rho GTPases (Kubori and Galan 2003). Thereby the bacteria are able to cause activation of Rho GTPases directly followed by their inactivation. An important functional role of the degradation of Rac in the infection process is proposed, whereby elimination of the Rac GTPase increases the mobility of CNF-affected target cells and facilitates invasion of host

cells and crossing of the epithelial barrier by the CNF-producing *E. coli* (Doye et al. 2002). A further function of limited activation of Rho GTPases by deamidation and subsequent degradation could be the restriction of an inflammatory response of the host cells, since activated Rho GTPases lead to upregulation of inflammatory mediators in human umbilical vein endothelial cells (HUVEC) cells, mostly triggered by Rac and Cdc42 (Munro et al. 2004).

The DNT-induced morphological changes have not been precisely analysed so far. DNT modifies the same target amino acid in the same set of Rho GTPases leading to their constitutive activation. This suggests that both toxins induce comparable morphological changes in mammalian cells. In fact, DNT induces similar but not identical morphological changes (enlargement of cells, multinucleation, actin polymerization) as CNF. For example, DNT did not induce membrane ruffling in HeLa cells. Such differences could be explained by a possible sterical hindrance of interactions with some effectors due to transglutamination of Rac. In line with this, it was observed recently that DNT-modified Rac is activated but not degraded in mammalian cells (Pop et al. 2004). Although RhoA, Rac and Cdc42 are activated by CNF1, the set of Rho-GTPases that are degraded seem to be cell-type specific. In HUVEC cells CNF-activated RhoA, Rac and Cdc42 are degraded (Munro et al. 2004), whereas in HEK293 cells degradation is restricted to Rac and even isotype-specific Rac1 (Pop et al. 2004). Therefore, the requirements for degradation of CNF-activated Rac have been analysed in detail, recently. Interaction of this protein with Rac depends on the integrity of the effector domain (Doye et al. 2002). Moreover, the presence of a putative destruction box, correct localization of the GTPase and/or specific protein interactions at the C-terminus seem to be essential for degradation. The polybasic region and amino acids 90, 107, 147 and 151 define isotype-specific degradation of Rac in HEK293 cells. Although these residues have not been identified as crucial for any effector interaction, they are located on the same side of the Rac1 molecule and may be involved in binding of as-yet-unidentified proteins that specify Rac1 function (Pop et al. 2004).

Structure–function relationship

Recently, the crystal structure of the C-terminal fragment harbouring the catalytic domain of CNF1 was solved (Buetow et al. 2001). The structure provided new insight into the catalytic mechanism and how restriction to the specific substrate is achieved. It revealed a protein fold previously unknown that is likely to be unique to the family of deamidating/transglutaminating toxins. The centre of the catalytic domain is formed by a β-sandwich consisting of two mixed β-sheets (Fig. 4). It is surrounded by α-helices and extensive loop regions that line the entrance to a deep and narrow pocket, with the catalytic residues situated at the base of the pocket. This may explain the tight substrate specificity of CNF (see above). CNF possesses a catalytic triad reminiscent of that found in cysteine proteases and other members of the catalytic triad superfamily, e.g. eukaryotic transglutaminases.

The amino acids crucial for catalytic activity, Cys866 and His881, were first identified by mutation studies (Schmidt et al. 1998). These residues are conserved in all members of the CNF/DNT family. Mutation of Cys866 and His881 to serine and alanine, respectively, abolishes enzyme activity of CNF1. The crystal structure definitely defined the role of these residues and, moreover, identified the third amino acid residue involved in catalysis as Val833. Most catalytic triad enzymes have an asparagine (e.g. papain) or an aspartate

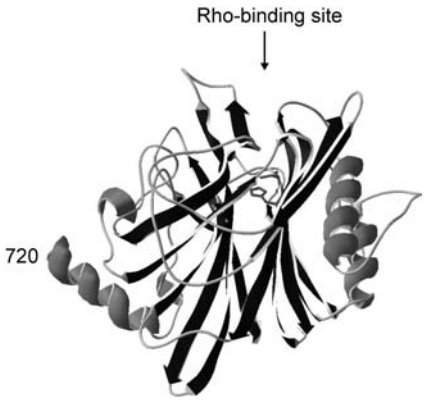

Fig. 4 Structure of the CNF1 catalytic domain. Ribbon representation of CNF1 aa 720–1014. The catalytic residues Cys866 and His881 are situated in a pocket—formed by two extensive β-sheets—that is surrounded by α-helices. The entrance to the pocket (indicated by an *arrow*) is lined by *flexible loops*. (The figure was created using Swiss-PDBViewer 3.7)

(e.g. blood coagulation factor XIII) that takes the role of orienting the imidazole ring of the histidine during catalysis. However, in CNF1 it is the main chain carbonyl of Val833 that instead interacts with the histidine (Buetow et al. 2001). Although CNF1 does not share the overall structure of other catalytic triad enzymes, the positions of key atoms at the active site superimpose impressively well with those of factor XIII and the amidotransferase GMP synthetase (Buetow et al. 2001). Additional residues that affect RhoA deamidation are Asn835 and Ser864 that are located in close proximity to the catalytic residues, but their mutation does not completely abolish enzymatic activity (Buetow et al. 2001).

It has been hypothesized that the flexible loops lining the entrance to the catalytic pocket could rearrange during RhoA binding. In their recent publication, Buetow and Ghosh have deleted five of these nine loops to further investigate their role in substrate recognition and binding of RhoA. Deletion of loop 8 (aa 964–970) and loop 9 (aa 996–1003) nearly or completely abolished the deamidation activity of the C-terminal fragment of CNF1 (aa 720–1014), while the complete fragment possesses full deamidation activity towards RhoA (Buetow and Ghosh 2003). This hints at an important role of loops 8 and 9 in substrate recognition and/or binding. In contrast, deletion of loop 2 (aa 790–795) resulted in a five- to sevenfold increase in deamidation of RhoA. Therefore the authors suggest that loop 2 might have to rearrange during RhoA binding (Buetow and Ghosh 2003).

The crystal structure of DNT has not been obtained thus far. However, some information has been gained from biochemical and other data. CNF1 and DNT can both act as a deamidase and a transglutaminase. CNF1 mainly catalyses the deamidation reaction while DNT preferentially catalyses transglutamination (Schmidt et al. 1999b). Buetow and colleagues (Buetow et al. 2001) suggest that a possible contribution to that preference might be the negatively charged active site pocket of DNT that is predicted by modelling the structure of DNT. The negative charges within the DNT molecule could attract positively charged amines to the active site. CNF1, in contrast, does not expose such charges (Buetow et al. 2001).

CNFs as virulence factors

Several studies have analysed the role of CNFs as virulence factors for *E. coli*-caused diseases (Table 1). Using CNF1-deficient *E. coli* strains, it was shown that the colonization and tissue damage of the urinary tract of mice was less pronounced when no CNF was produced than with the isogenic wild-type strains (Rippere-Lampe et al. 2001b). The same was found for tissue damage of rat prostates (Rippere-Lampe et al. 2001a). In contrast, no difference in lung and serosal inflammation of infected piglets has been observed when CNF1-producing and -deficient strains have been compared (Fournout et al. 2000). In order to study the influence of the toxin on the epithelial barrier function, its effect on the resistance of epithelial monolayers has been analysed in vitro. CNF1 increases the intestinal permeability in human colon carcinoma cells (Caco-2) (Gerhard et al. 1998). Similarly, a marked reduction in tight junctions gate function, accompanied by displacement of the tight junction proteins occludin and zonula occludens-1 (ZO-1) was observed in intestinal T84 cells (Hopkins et al. 2003). Recently, it was shown that CNF1-activated T lymphocytes are cytotoxic against intestinal epithelial cells, probably by an enhanced cytokine release. Loss of epithelial cells could also reduce the barrier function of the intestinal epithelium (Brest et al. 2003). Moreover, the involvement of the toxin in the traversal of the blood–brain barrier in a meningitis animal model has been studied. Only bacteria producing CNF were able to cross the blood–brain barrier (Khan et al. 2002). Taken together, several findings support the notion that CNFs are major virulence factors in various infection models.

Without doubt, effects of CNFs on immune cells are of major importance for their role as virulence factors. The actin as well as the microtubule cytoskeleton is known to play an important role in the activity of cell-mediated immune responses. Thus, constitutive activation of regulators of the actin cytoskeleton (Rho GTPases) should influence immune responses. For example, it was reported recently that CNF1 increases the cytotoxicity of natural killer (NK) cells by Rac-mediated effector cell binding and NK cell recruitment (Malorni et al. 2003). Moreover, it has been shown that CNF-producing bacteria are able to resist killing by human neutrophils (Rippere-Lampe et al. 2001b). CNF activates T lym-

Table 1 Involvement of CNFs in bacterial virulence. The deamidating and transglutaminating toxins CNFs and DNT, which activate Rho GTPases, have been shown to be major virulence factors in various infection models (*NK* natural killer, *PMN* polymorphonuclear)

Tissue damage	Urinary tract of mice	Rippere-Lampe et al. 2001b
	Rat prostates	Rippere-Lampe et al. 2001a
Barrier functions	Decrease in resistance of Caco2 cells	Gerhard et al. 1998
	Displacement of tight junction proteins in T84 cells	Hopkins et al. 2003
	Increased cytotoxicity of T lymphocytes against epithelial cells	Brest et al. 2003
	Crossing the blood–brain barrier	Khan et al. 2002
Immune cells	Increased cytotoxicity of NK cells	Malorni et al. 2003
	Activation of cytokine release of T lymphocytes	Brest et al. 2003
	Increase in oxidative burst and decrease in phagocytosis of PMN leucocytes	Hofmann et al. 2000 Capo et al. 1998
	Induction of phagocytic activity of non-professional phagocytes	Falzano et al. 1993
Development of cancer	Increased expression of Cox-2	Thomas et al. 2001
	Possible tumorigenic potential	Lax and Thomas 2002

phocytes to release several cytokines that are known to augment inflammatory processes (Brest et al. 2003) and the toxin increases the oxidative burst of human polymorphonuclear leucocytes (Hofmann et al. 2000). By changing the cytoskeletal organization, CNF1 was shown to decrease the phagocytosis of polymorphonuclear leucocytes and monocytes probably by preventing the co-distribution of CR3 with F-actin (Hofmann et al. 2000; Capo et al. 1998). In contrast, CNF induces the phagocytic activity of non-professional phagocytes (Falzano et al. 1993).

Doye and coworkers recently showed that degradation of Rac, which is induced by CNF (Lerm et al. 2002), increases the motility of target cells and increases the dynamics of cellular junctions (Doye et al. 2002). Moreover, they showed that CNF increases the internalization of bacteria by 804G-rat bladder carcinoma cells after degradation of Rac, indicating that down-regulation as well as activation of Rho GTPases might be essential for CNF action. Taken together, CNFs largely interfere with the innate and with the acquired immunity by switching Rho GTPases on and off (degradation).

Rho GTPases are increasingly recognized as essential factors in the development of cancer and metastasis. This fact has initiated a discussion about whether activation of Rho proteins by CNFs might be involved in tumourigenesis. It has been shown that CNF1 increases the expression of the cyclooxygenase-2 (Cox-2) gene in fibroblasts (Thomas et al. 2001). Interestingly, an increased expression of Cox-2 is also observed in some types of tumours e.g. colon carcinoma. Lipid mediators produced by the enzyme are suggested to be responsible for tumour progression (Oshima et al. 1996). Thus, it is feasible that chronic infections with CNF-producing *E. coli* might carry a tumourigenic potential important for the pathogenesis of some types of cancer (Lax and Thomas 2002).

DNT as virulence factors

The role of DNT in the pathogenesis of respiratory diseases in swine has been studied. Turbinate atrophy and pneumonia of pigs developed only after infection with a DNT-producing *Bordetella bronchiseptica* strain and not with an isogenic DNT-deficient strain (Brockmeier et al. 2002). DNT has vasoconstrictive and splenoatrophic activities (Cowell et al. 1979; Nakai et al. 1985) and induces mucosal damage in swine nasal tissue as well as degenerative changes of the periosteum after injection into subcutaneous tissues in neonatal rats (Horiguchi et al. 1995). Moreover, DNT has been shown to be involved in turbinate atrophy in mice. In contrast, DNT does not play a role in the lethal mouse model, since DNT-negative strains are as virulent as wild-type *Bordetella* strains (Magyar et al. 2000).

Conclusion

The deamidating and transglutaminating toxins CNFs and DNT, which activate Rho GTPases, have been shown to be major virulence factors in various infection models. Rho GTPases are crucial switches in signalling pathways responsible for cell transformation and metastasis. Thus CNFs may also play a potential role in pathogenesis of certain types of cancer. CNF-activated Rac is degraded, but it is still not clear whether degradation of Rac is also a physiological mechanism to down-regulate Rac signalling. Degradation of

RhoA has recently been shown to play a role in cell polarity and directed migration of cells (Wang et al. 2003).

Moreover, because the toxins constitutively activate Rho GTPases, they can be used as pharmacological tools to study Rho function.

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Modulation of Rho GTPases by type III secretion system translocated effectors of *Yersinia*

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Abstract Pathogenic species of the bacterial genus *Yersinia* subdue the immune system to proliferate and spread within the host organism. For this purpose yersiniae employ a type III secretion apparatus which governs injection of six effector proteins (*Yersinia* outer proteins; Yops) into host cells. Yops control various regulatory and signalling proteins in a unique and highly specific manner. YopE, YopT, and YpkA/YopO modulate the activity of Rho GTP-binding proteins, whereas YopH dephosphorylates phospho-tyrosine residues in focal adhesion proteins. Furthermore, YopP/YopJ and YopM affect cell survival/apoptosis and cell proliferation, respectively. In this review the focus will be on the biochemistry and cellular effects of YopT, YopE, YopO/YpkA, and YopH.

Introduction

There are three human pathogenic *Yersinia* species: (1) *Y. pestis* is the causative agent of plague, (2) *Y. pseudotuberculosis*, and (3) *Y. enterocolitica* cause acute or chronic gastroenteric infections. In contrast to other enteropathogenic bacteria such as salmonellae and shigellae, which can replicate intracellularly, yersiniae proliferate extracellularly. The latter also have a preference for lymphatic tissues. To resist the attack of the immune system, pathogenic *Yersinia* spp. are endowed with a 70-kb virulence plasmid (called pYV) which carries genes encoding (1) a protein type III secretion system (TTSS), (2) a set of six effector proteins (*Yersinia* outer proteins, Yops), (3) regulators for gene expression and Yop-secretion/translocation, and (4) a *Yersinia* adhesin (YadA), which among other functions can mediate *Yersinia* attachment to host cells (Cornelis et al. 1998; Hoiczyk et al. 2000). *Y. enterocolitica* and *Y. pseudotuberculosis* also express a chromosomally encoded outer membrane protein—Invasin—that binds with high affinity to $\beta 1$ integrin receptors (Isberg and Leong 1990). In the initial phase of an oral infection invasin triggers internalization of yersiniae into M-cells of the intestinal mucosa and thereby allows transversal of

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the intestinal mucosa. Details about type III secretion, the pYV plasmid, as well as the structure and function of the *Yersinia* adhesins have been reviewed elsewhere (Cornelis et al. 1998; El Tahir and Skurnik 2001; Hueck 1998; Isberg et al. 2000).

Upon contact with target cells, yersiniae employ their TTSS to translocate the six effector proteins named YopH, YopO/YpkA, YopP/YopJ, YopE, YopM, and YopT into the cell. Yops interfere with a variety of cell functions including cytoskeletal regulation, cytokine production, and control of apoptosis to suppress the immune response to infection (Aepfelbacher and Heesemann 2001; Bliska 2000; Cornelis and Wolf Watz 1997; Juris et al. 2002). Controlling the actin cytoskeleton seems to be the major function of at least four Yops, namely YopT, YopE, YopO/YpkA, and YopH. To do so YopT, YopE, and YopO/YpkA modulate the function of Rho GTPases whereas YopH dephosphorylates tyrosine residues of focal adhesion proteins. Noteworthy, *Y. pseudotuberculosis* also produces a homolog of the *E. coli* exotoxin cytotoxic-necrotizing-factor (CNF), which was named CNF-Y (Hoffmann et al. 2004; Lockman et al. 2002). CNF-Y specifically activates RhoA by deamidation of Gln-63, in contrast to CNF from *E. coli* which modifies RhoA, Rac, and Cdc42 (Hoffmann et al. 2004).

Rho GTP-binding proteins constitute ideal targets for pathogens because they are master regulators of the actin cytoskeleton, control cell cycle, intracellular vesicle transport, and gene transcription (Symons and Settleman 2000; van Aelst and D'Souza-Schorey 1997). Most Rho-family proteins are molecular switches which are "on" when bound to GTP and "off" when bound to GDP. In the GTP-bound state Rho proteins stimulate a variety of effector proteins, which include protein kinases, lipid kinases, and multidomain scaffolds (Bishop and Hall 2000). The GDP/GTP cycling of Rho GTPases is regulated by (1) their intrinsic GTPase activity; (2) GTPase activating proteins (GAPs), which greatly increase the intrinsic GTPase activity; and (3) guanine nucleotide exchange factors (GEFs), which promote the exchange of GTP for bound GDP. Furthermore, by binding to guanine nucleotide dissociation inhibitors (GDIs) the hydrophobic isoprenoid moiety at the C-terminus of Rho GTPases is neutralized and this is involved in cytosol/membrane cycling. GDIs also "freeze" Rho GTPases in their actual guanine nucleotide bound state (GDP or GTP) and block their interaction with regulators and effectors (Fig. 1; Olofsson 1999).

An emerging theme is that Yops modulate and exploit the signal transduction pathways which are stimulated initially in host cells after infection with yersiniae (Ruckdeschel et al. 2001; Ruckdeschel 2002). Rho GTPases can get activated through different *Yersinia* factors. Invasin-triggered uptake has been shown to involve Rac, either individually or in cooperation with Rho and Cdc42Hs, dependent on the cell type (Alrutz et al. 2001; McGee et al. 2001; Wiedemann et al. 2001). With the help of cofactors such as phosphatidylinositolphosphates Cdc42Hs and Rac activate N-WASp and WAVE, respectively, which greatly enhance the actin polymerizing activity of Arp2/3 complex (Higgs and Pollard, 1999). Assembly of an actin-rich phagocytic cup by these mechanisms then mediates bacterial uptake. Besides by surface adhesins, Rho and Rac could be activated by lipolysaccharide (LPS) of yersiniae acting via Toll-like receptors (Arbibe et al. 2000). In this review we will focus on the biochemistry and cell biology of the Rho GTPase modulating Yops YopT, YopO/YpkA, YopE, and the focal adhesion regulator YopH. Important features of YopM and YopP will also be mentioned; a more detailed description of their function has been published elsewhere (Juris et al. 2002; Ruckdeschel 2002). Figure 1 de-

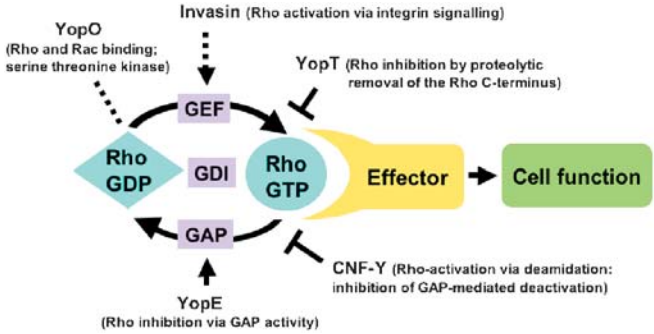

Fig. 1 Interference of *Yersinia* virulence factors with the Rho activation/deactivation cycle. The major biochemical mechanisms by which the respective factors modulate Rho GTPases are in *brackets*. *CNF-Y*, cytotoxic necrotizing factor of yersiniae; *GAP*, GTPase activating protein; *GEF*, guanine nucleotide exchange factor; *GDI*, guanine nucleotide dissociation inhibitor; *RhoGDP*, inactive, GDP-bound Rho GTPase; *RhoGTP*, active, GTP-bound Rho GTPase; *Invasin*, *Yersinia* adhesin binding to β1-integrins.

picts a scheme of the multiple "mechanisms" whereby *Yersinia* virulence factors interfere with the Rho activation/deactivation cycle.

YopT: a cysteine protease removing the isoprenoid group of Rho GTPases

YopT is a 35-kDa protein (322 amino acids) that belongs to the CA clan of cysteine proteases. It is expressed by *Y. enterocolitica*, *Y. pestis*, and only some *Y. pseudotuberculosis* strains (Iriarte and Cornelis 1998; Shao et al. 2002; Zumbihl et al. 1999). In addition to the three *Yersinia yopT* sequences, 16 homologous open reading frames derived from animal pathogens such as *Haemophilus ducreyi*, *E. coli* O157:H7, or *Pasteurella multocida,* plant pathogens such as *Pseudomonas syringae pv. phaseolicola* and entomopathogenic *Photorhabdus luminescens* have been extracted from databases. Despite great sequence diversity, all YopT family members possess conserved C/H/D amino acid residues which appear to be essential for protein activity. The cDNA-inferred YopT family proteins can be divided into two groups. The first group, which includes YopT, contains proteins of 30–40 kDa; the second group contains proteins of >300 kDa which harbor additional functional domains (Shao et al. 2002).

Cellular overexpression and in vitro studies showed that YopT proteolytically removes the geranylgeranyl isoprenoid moiety of RhoA, Rac1, and Cdc42 and this activity is dependent on the invariant C/H/D residues C139, H258, and D274. YopT cleaves just before the C-terminal cysteine to which the geranylgeranyl group is attached via a thioether bond (Shao et al. 2002, 2003). Catalytically inactive YopTC139S can still bind to RhoA and thus can be used for pull-down experiments (Aepfelbacher et al. 2003; Shao et al. 2002; Sorg et al. 2003). Although YopT requires an isoprenoid group for binding and activity, it does not distinguish between geranylgeranylated or farnesylated RhoA. YopT also seems to act equally well on GDP- and GTP-bound RhoA. In the test tube and in cells overexpressing the reaction components, YopT works best on RhoA, is less active on Rac and Cdc42, and does not cleave H-Ras. When the 5 basic amino acid residues (3 x lysine and 2 x arginine) at the C-terminus of RhoA were mutated to glutamine residues, the resulting

RhoA mutant became insensitive to YopT cleavage. Furthermore, an intracellularly expressed GFP fusion construct containing the last 13 amino acids of RhoA (including the 5 basic residues), but not a construct containing the last 4 amino acids of RhoA, was cleaved by YopT, although both GFP constructs became isoprenylated (Shao et al. 2002, 2003). Together these data suggest that YopT recognizes an isoprenoid group in combination with a stretch of basic amino acid at the C-terminus of Rho GTPases. The structural requirements of YopT for binding and cleavage of RhoA were also tested. Whereas deletion of 8 amino acids from the C-terminus abrogated YopT activity, deletion of 74 amino acids from the N-terminus had no effect. When more than 100 amino acids were deleted from the N-terminus of YopT its activity was abolished. In comparison, binding to RhoA was not greatly affected by deleting 14 amino acids from the C-terminus, whereas the N-terminal 74 amino acid deletion mutant displayed considerably reduced RhoA binding. These findings suggest that catalytic activity is mainly located at the C-terminus, whereas Rho GTPase binding also involves the very N-terminal amino acids of YopT (Sorg et al. 2003).

Considering that yersiniae inject only minute amounts of Yops into target cells and intracellular Yop activity is temporally and spatially controlled, cellular infection models provided specific informations as to the physiological functions of Yops. In fact, destruction of actin stress fibers in *Y. enterocolitica*-infected cells was the first activity pointing to the function of YopT (Iriarte and Cornelis 1998). The basis for this YopT effect was subsequently shown to be modification and inactivation of the Rho GTPase RhoA (Zumbihl et al. 1999). Thereafter, the biochemical activity of YopT was identified (Shao et al. 2002, 2003).

RhoA modification by removal of the isoprenoid group has a variety of consequences in *Yersinia*-infected cells. RhoA is released from the plasma membrane and from its cytoplasmic binding partner guanine nucleotide dissociation inhbitor-1 (GDI-1) and accumulates as a monomeric protein in the cytoplasm. Notably, neither Rac1 nor Cdc42 are removed from cell membranes or GDI-1, suggesting that YopT does not work on these proteins in infected cells (Aepfelbacher et al. 2003; Zumbihl et al. 1999). As part of a systematic approach, the role of YopT in preventing opsonized and unopsonized phagocytosis of *Yersinia enterocolitica* by human neutrophils and mouse macrophages was investigated also. Mutant bacteria lacking YopT were phagocytosed in significantly higher amounts than wild-type bacteria both under opsonizing and nonopsonizing conditions. *Yersinia* mutants translocating only YopT were not resistant to phagocytosis by neutrophils or macrophages (Grosdent et al. 2002). However, in primary macrophages YopT overexpressing mutants disrupted actin rich phagocytic cups induced by *Yersinia* invasin as well as podosomal adhesion structures required for chemotaxis (Aepfelbacher et al. 2003). Hence, YopT alone and in combination with partner Yops can disrupt immune cell function thereby promoting *Yersinia* infection. At least in some cell types RhoA seems to be the major target of YopT, whereas Rac and Cdc42 are not affected. However, at present it cannot be excluded that YopT has additional substrates that may be within the large RhoGTPase family or even unrelated to it.

In infected cells YopT is located in membranes, whereas the majority of its target protein RhoA is complexed to GDI in the cytosol. Furthermore YopT can modify RhoA complexed to GDI-1 in vitro only when additional factors such as the membrane lipid phosphatidylinositolbisphosphate (PIP2) are present or when RhoA is artificially loaded with GTP-γS (Aepfelbacher et al. 2003). Thus, YopT likely requires additional signalling molecules (such as PIP2 production and GEFs) to modify RhoA in cells. Taken all these data

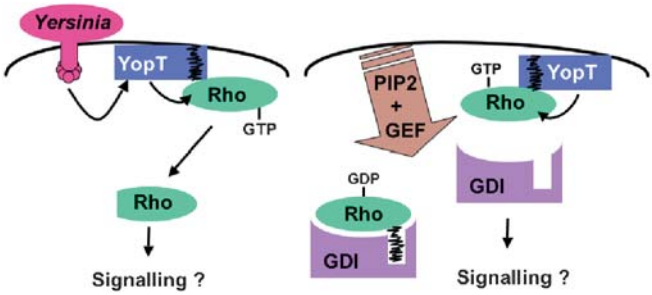

Fig. 2 How YopT may function after translocation into cells. The effects of YopT in cells infected with yersiniae is depicted taking into account temporal and spatial considerations (*left*). Upon injection by the *Yersinia* TTS YopT locates to the plasma membrane where it binds RhoA via its isoprenoid membrane anchor. Through proteolytic removal of the isoprenoid group RhoA is released into the cytosol (*right*). Through action of PIP2 and/or guanine nucleotide exchange factors (*GEFs*) RhoA is released from its cytosolic complex with GDI and then cleaved by YopT. It is unclear what signalling activities the cleaved RhoA and the free GDI in the cytosol have. For details see the section entitled "YopT: A cysteine protease removing the isoprenoid group of Rho GTPases" and reference Aepfelbacher et al. 2003.

together, a model of YopT function within *Yersinia*-infected cells is proposed which includes temporal and spatial considerations: YopT translocated into target cells by the *Yersinia* TTS binds to the plasma membrane where it "meets" RhoA and cleaves off its isoprenoid membrane anchor. The truncated RhoA is released from the membrane and trapped in the cytosol. By a mechanism likely involving membrane lipids and exchange factors, the remaining isoprenylated RhoA is released from GDI and then translocates either directly to YopT or to cell membranes where it is cleaved by YopT. This cycle proceeds until all of the RhoA is modified (Fig. 2).

Within the last years much has been learned about the biochemistry and cell biology of YopT. An exact understanding of the structural basis of YopT cleavage of Rho GTPases will require crystallographic data. The crystal structure of the remote YopT-homolog AvrPphB has been solved and revealed similarities with papain-like cysteine proteases. Consistent with the differing substrate specificities of YopT and AvrPphB, the residues corresponding to the substrate binding sites are highly divergent among the YopT family proteins (Zhu et al. 2004).

YopE: GAP activity for rapid and specific downregulation of Rho GTPases

YopE is a 25-kDa protein (219 amino acids) which, like the homologous domains within exoenzyme S from *Pseudomonas aeruginosa* and SptP from *Salmonella* Typhimurium, works as GAP for Rho-family proteins (Andor et al. 2001; Black and Bliska 2000; Fu and Galan 1999; Goehring et al. 1999; Pawel-Rammingen et al. 2000). The Rho GAP domain of YopE ranges from amino acids 96 to 219. Residues 54–75 have been shown to be both necessary and sufficient for targeting of YopE to an unidentified perinuclear compartment (Krall et al. 2004). The bacterial RhoGAPs, like all mammalian Rho GAPs, possess an arginine finger motif known to be essential for activity (Scheffzek et al. 1998; Wurtele et al. 2001). Mutation of the arginine finger in YopE (R144A) abolished most of the YopE activities in cells and mouse infection models (Aili et al. 2003; Pawel-Rammingen et al.

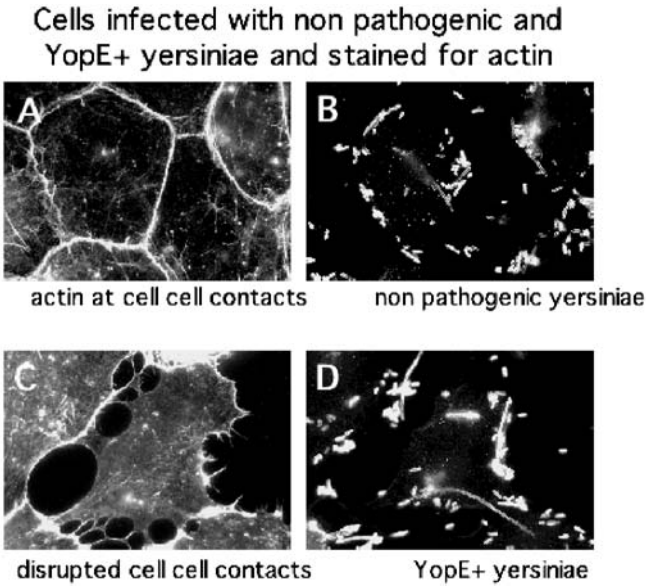

Fig. 3 Disruption of cell–cell contacts by yersiniae expressing YopE. Confluent human endothelial cells were infected with nonpathogenic yersiniae or yersiniae expressing YopE (*YopE+*). Thereafter the actin cytoskeleton of the cells (**A**, **C**) and the bacteria (**B**, **D**) were stained and recorded in separate channels. **A** In confluent cells infected with the nonpathogenic yersiniae, actin is exclusively located at cell-cell contacts. **B** Bacteria attached to the cell in **A** are shown. **C** *Yersinia* YopE+ disrupts actin at cell-cell contacts and changes cell shape. **D** Bacteria attached to the cell in **C** are shown.

2000; Black and Bliska 2000; Andor et al. 2001). Five different triple alanine substitution mutants within amino acids 166–208 abrogated in vitro GAP activity but not cellular cytotoxicity of YopE. It was therefore suggested that YopE may have additional targets/functions within cells which are dependent on the arginine 144 residue but not on GAP activity (Aili et al. 2003). In vitro YopE and ExoS work on Rho, Rac, and Cdc42 whereas SptP acts on Rac and Cdc42 (Black and Bliska 2000; Fu and Galan 1999; Goehring et al. 1999; Pawel-Rammingen et al. 2000). Introduction of YopE into transformed cell lines by *Yersinia* infection or microinjection was found to disrupt actin filaments (Black and Bliska, 2000; Pawel-Rammingen et al. 2000; Rosqvist et al. 1991). In primary endothelial cells, a *Y. enterocolitica* strain that translocates YopE but none of the other effector Yops did not affect direct Rho activation by thrombin, direct Rac activation by sphingosine-1-phosphate, or direct Cdc42 activation by bradykinin, as tested by normal formation of actin stress fibers, membrane ruffles, or filopodia respectively. However, a basal Rac activity required for maintaining cell-cell contacts, as well as Rac activation by Cdc42 resulting in ruffling, was blocked by YopE (Fig. 3; Andor et al. 2001). This finding demonstrates that YopE can modulate Rho GTPase-dependent signal pathways with a remarkable specificity in primary target cells of yersiniae. It also confirms earlier findings showing that the substrate specificity of GAPs may differ in vitro and inside cells (Moon and Zheng 2003; Ridley et al. 1993). The molecular basis for the intracellular specificity of YopE is not known, but YopE may act in a compartmentalized fashion, i.e., may not get access to the membrane compartments in which Cdc42 and Rho are localized. The specific perinuclear membrane localization of YopE supports this notion (Krall et al. 2004). It has not been

tested, however, to what extent the compartments containing the RhoGTPases and YopE overlap.

YopE contributes to the antiphagocytic activity of *Y. enterocolitica* and *Y. pseudotuberculosis* in cooperation with fellow Yops. It blocks phagocytosis of opsonized or unopsonized yersiniae by macrophages, neutrophils, and epithelial cells (Grosdent et al. 2002; Fällman et al. 1995; Ruckdeschel et al. 1996). Furthermore, *Y. pseudotuberculosis* mutants deleted in YopE are clearly attenuated in general virulence/lethality (Rosqvist et al. 1998). Interestingly, in a mouse infection model a YopE-deficient *Y. pseudotuberculosis* strain showed only minor defects in colonization of and persistence in intestinal and lymphoid tissues. However, a YopH/E double mutant was essentially avirulent whereas a YopH mutant wasn't. Furthermore, the YopE-deficient *Y. pseudotuberculosis* strain was outcompeted by *Y. pseudotuberculosis* wild type in coinfection models (Logsdon and Mecsas 2003).

Together these findings suggest that YopE has both individual and redundant functions and synergizes with other Yops during the complex infection cycle of yersiniae. Likely there are additional, hitherto unknown functions of YopE. First, YopE without in vitro GAP activity still has cytotoxic effects (Aili et al 2003). Second, specific intracellular membrane localization of YopE may indicate effects on vesicle transport (Krall et al 2004). Third, although the GAP function individually shuts down activity of Rho GTP-binding proteins, the coordinated activities of GEFs and GAPs can promote some cell functions by enhancing the on-off cycling of RhoGTPases (Symons and Settleman 2000). In addition, some GAPs also work as effector proteins by binding preferentially to the GTP-bound proteins (Moon and Zheng 2003). Thus, introduction of bacterial GAPs into cells may in cooperation with other cellular factors also result in a gain of function.

YopO/YpkA: a serine threonine kinase activated by G-actin and binding to Rho GTPases

YopO from *Y. enterocolitica* (called YpkA in *Y. pseudotuberculosis*) is a 80-kDa (729 amino acids) multidomain protein for which different activities have been demonstrated: (a) serine/threonine kinase activity causing autophosphorylation and phosphorylation of artificial basic substrates, (b) actin binding which results in kinase activation, and (c) binding to the Rho GTPases RhoA and Rac. The protein organization of YopO reflects these distinct activities. An N-terminal secretion/translocation region (aa 1–77) is followed by the predicted catalytic domain (aa 150–400). Mutation of a critical lysine residue in YopO (K269A) or aspartic acid residue in YpkA (D270A), located within the putative catalytic domain, abolished kinase activity (Cornelis et al. 1998; Dukuzumuremyi et al. 2000; Galyov et al. 1993; Juris et al. 2000). The C-terminal half of YopO contains four regions (within aa 436–710) with homology to Rho binding domains (RBDs). In yeast two hybrid assays a construct (residues 442–732) consisting of the RBDs of YpkA was able to bind to RhoA, whereas deletion of residues 543–640 (which include three of the RBDs) from YpkA abolished RhoA binding. The very C-terminal stretch of 21 amino acids (aa 709–729) displays some homology to the actin bundling protein coronin (Dukuzumuremyi et al. 2000; Juris et al. 2000). Removal of these 21 C-terminal amino acids abolished actin binding and actin induced autophosphorylation (Juris et al. 2000). The protein organization of YopO is depicted in Fig. 4.

Purified actin was found to bind to and activate YopO, resulting in autophosphorylation and phosphorylation of artificial substrates (Juris et al. 2000). There may be additional YopO/YpkA activators, because in fetal calf serum the highest YpkA stimulatory activity

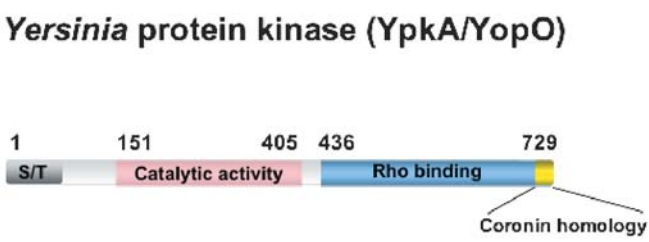

Fig. 4 Domain organization of YpkA/YopO. This figure is based on and summarizes the work of Barz et al. 2000; Cornelis et al. 1998; Dukuzumuremyi et al. 2000; Galyov et al. 1993; and Juris et al. 2000. *S/T*, secretion and translocation domain required by the type III secretion system; *Rho binding*, a region containing four amino acid stretches with homology to Rho binding domains; *coronin homology*, a stretch of 20 amino acids showing homology to the actin bundling protein coronin from *D. discoideum*. The amino acid *numbers* correspond to the sequence of the YopO protein.

was found between 100- to 300-kDa proteins where no actin was present (Dukuzumuremyi et al. 2000).

By yeast two hybrid assays and in immunoprecipitation experiments YpkA was shown to associate with Rho and Rac but not Cdc42Hs (Barz et al. 2000; Dukuzumuremyi et al. 2000). Rho and Rac binding were independent of the nucleotide bound state (GDP or GTP) of the GTPases, although in one report the GDP-bound form of RhoA bound three times more efficiently to YpkA than the GTP-bound form (Dukuzumuremyi et al. 2000). Moreover, YpkA did not alter guanine nucleotide exchange factor-stimulated GDP-release on RhoA in vitro. Although the in vitro data provide no basis for this so far, YpkA was able to reduce the level of GTP-bound RhoA in *Y. pseudotuberculosis*-infected HeLa cells (Dukuzumuremyi et al. 2000). In experiments aimed to relate the phosphorylating and Rho/Rac binding activities of YpkA, it was observed that (a) neither the GDP- nor the GTP-bound forms of Rho or Rac affect autophosphorylation, (b) autophosphorylated YpkA does not show altered binding to the GDP- or GTP-bound forms of Rho or Rac, and (c) in cells the kinase dead D270A mutant of YpkA reduces the levels of GTP-bound Rho as effectively as wild-type YpkA (Barz et al. 2000; Dukuzumuremyi et al. 2000).

HeLa cells infected with a *Y. pseudotuberculosis* mutant overexpressing YpkA responded by cell contraction and formation of pronounced retraction fibers whereby focal adhesions remained intact. In these experiments YpkA was localized at the plasma membrane, a property that was later shown to reside in the N-terminal half of the protein (Dukuzumuremyi et al. 2000; Hakansson et al. 1996). Divergent results where reported concerning the effects of kinase inactive YpkA/YopO mutants on the actin cytoskeleton. In one study, actin filament disruption by YpkAD270A was normal (Dukuzumuremyi et al. 2000), whereas in another study YopOK269A showed considerably attenuated actin filament destroying ability (Juris et al. 2000). In cooperation with YopT and YopE, YpkA contributes to the antiphagocytic activity of yersiniae towards neutrophils and macrophages (Grosdent et al 2002). It also synergizes with other Yops to confer upon yersiniae the ability to colonize and persist in different tissues (Logsdon and Mecsas 2003).

Important information concerning the biology and molecular functions of YopO/YpkA are missing to date. Its physiological substrates in cells are unknown and which of its different biochemical activities are responsible for the cellular and in vivo effects is not clear. Whether these activities work independently or synergistically with each other is worth investigation as well.

YopH: a tyrosine phosphatase working on focal adhesion proteins

YopH is a highly active phosphotyrosine phosphatase of 50 kDa (468 amino acids) with multiple substrates, many of which are components of focal adhesions (Cornelis 2002; Juris et al. 2002). The catalytic domain of YopH is situated in the C-terminal half of the protein (from amino acids 206–408). Mutation of a cysteine residue implicated in phosphate hydrolysis abolishes catalytic activity and leads to a substrate trapping protein (YopHC403S or YopHC403A) which localizes to focal adhesion complexes (Black and Bliska 1997; Persson et al. 1997). The N-terminal 129 amino acids of YopH both harbor the secretion/translocation domain and are crucial for substrate recognition (Black et al. 1998). Four single amino acid mutations (Q11R, V31G, A33D, and N34D) in YopH were identified that interfered with binding to tyrosine-phosphorylated $p130^{Cas}$ but not with YopH translocation into cells. One of these mutants (V31G) was also unable to localize to focal adhesions and dephosphorylate target proteins (Montagna et al. 2001). In addition, deletion of residues 223–226 blocks YopH targeting to focal adhesions and at the same time impairs antiphagocytic activity of *Yersinia* towards macrophages and mouse virulence (Persson et al. 1999).

YopH dephosphorylates $p130^{Cas}$, focal adhesion kinase (Fak), paxillin, Fyn-binding protein (FyB), and the scaffolding protein SKAP-HOM in different cell types such as neutrophils, macrophages, and epithelial cells (Black and Bliska 1997; Persson et al. 1997; Hamid et al. 1999). These YopH substrates regulate the interaction between the actin cytoskeleton and extracellular matrix-binding integrins in focal adhesions/complexes or similar structures (Brakebusch and Fässler 2003; Giancotti 2000). Cytoskeletal uptake structures formed upon interaction of *Yersinia* YadA or invasin with cellular integrins are thought to resemble focal adhesions, and their disruption by YopH may thus explain its antiphagocytic activity.

Previous studies using *Y. enterocolitica* or *Y. pseudotuberculosis* mutants suggested that YopH is responsible for up to 50% of the antiphagocytic activity of yersiniae towards neutrophils and J774 macrophages (Fällman et al. 1995; Ruckdeschel et al. 1996). Further studies showed that translocation of wild-type YopH but not of the catalytically inactive YopHC403S into HeLa cells or J774 macrophages prevented invasin/β1 integrin-mediated uptake of yersiniae (Persson et al. 1997, 1999). YopH has a variety of additional activities in *Yersinia*-infected cells, including suppression of the oxidative burst in macrophages, reduction of Ca^{++} signalling in neutrophils, inhibition of T- and B-lymphocyte activation, and blockage of monocyte chemoattractant protein 1 production by macrophages (Cornelis 2002; Alonso et al. 2004). Whether these functions are due to dephosphorylation of the known YopH substrates or involve unknown substrates is not clear at the moment. Nonetheless, YopH is among the most effective Yops with regard to mouse virulence and antiphagocytic activity.

YopM and YopP/YopJ: effectors modulating cell growth and survival

YopM is an approximately 40-kDa protein the exact size of which depends on the *Yersinia* strain from which it is isolated (Cornelis 2002). Size variation is due to varying numbers (13–20) of a 19-residue leucine-rich-repeat motif (LRR). LRRs which have been implicated in protein-protein interactions make up most of the YopM protein. YopM coimmuno-

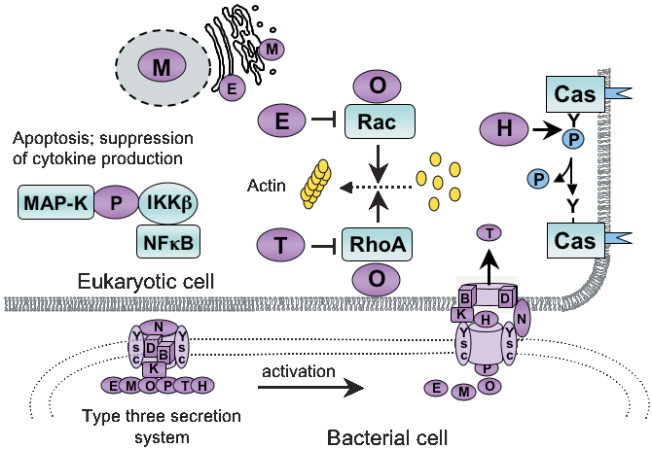

Fig. 5 *Yersinia* type III secretion system mediated injection of Yops into cells and Yop functions. Upon contact of yersiniae with target cells the type III secretion system is activated and a translocation needle is formed that spans the two bacterial membranes and the membrane of the eukaryotic host cell. Through this needle the effector Yops (*single letters in purple circles*) are translocated into the cell where they locate to distinct compartments such as plasma membrane (*YopO*) or Golgi apparatus (*YopE* and *YopM*). The proteins targeted by the Yops are depicted within *blue objects*. *MAP-K*, MAP kinase; *NFκB*, nuclear factor κB; *IKKβ*, inhibitory κB kinase *β*; *Ysc*, yop secretion proteins; *Cas*, p130 Crk-associated substrate; *LcrV*, V-antigen; *P*, phosphate group. For more detailed information, see specific sections of this article.

precipitates from cell lysates with two kinases, protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1). RSK1 is directly activated by YopM and this is required for stimulation of PRK2 (McDonald et al. 2003). The known cellular targets of YopM go well with its ability to affect the expression of genes involved in cell growth and cell cycle (Sauvonnet et al. 2002). It is also noteworthy that YopM is transported to the nucleus in a membrane-vesicle mediated pathway (Skrzypek et al. 1998). It is unclear, however, how this transport event relates to the role of YopM in cell growth or its association with the targets. Although YopM has no obvious antiphagocytic function, it is clearly required for mouse virulence of yersiniae (Leung et al. 1990).

YopP/YopJ (*Y. pseudotuberculosis*) has limited homology to the ubiquitin-like protease Ulp1 of yeast, which cleaves a small ubiquitin related modifier (SUMO1). YopP modulates SUMO activity and concentration in cells overexpressing these reaction components. Moreover, mutation of a critical cysteine or histadine residue within the putative catalytic region prevented some YopP/YopJ functions (Orth et al 1999). YopP/YopJ also associates with members of the MAPK kinase (MKK) superfamily, which represent upstream MAPK activators. Furthermore, it binds and inhibits the IκB kinase-*β* (IKK-*β*), which is the major NF-κB-activating kinase (Orth et al. 1999). Most likely through these properties YopP/YopJ disrupts NF-κB and MAPK signaling pathways, which leads to blockage of the release of TNF*α* by macrophages and of IL-8 by epithelial cells (Ruckdeschel 2002).

Interestingly, by downregulation of NF-κB signaling YopP/YopJ triggers apoptosis in infected macrophages (Ruckdeschel 2002). By enhancing the synthesis of antiapoptotic proteins, NF-κB is thought to protect cells that encounter bacteria or LPS (Karin and Lin 2002). Thus it has been proposed that LPS-responsive signaling by *Yersinia* infection co-

operates with the NF-κB-inhibitory action of YopP/YopJ to mediate apoptosis (Ruckdeschel et al. 2001).

Together, these findings emphasize the variety of strategies pathogenic *Yersinia* spp. have developed to undermine host defense mechanisms. Through the activity of different Yops, yersiniae disrupt the physiological sequence of immune functions that work on distinct cellular and molecular levels (Fig. 5). Initial effects are characterized by blockage of phagocytosis, oxidative burst, and cytokine production, and subsequent responses involve an effect on cell growth/proliferation and/or the induction of an intrinsic macrophage cell death program.

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Pseudomonas aeruginosa ExoS and ExoT

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Abstract ExoS and ExoT are bi-functional type-III cytotoxins of *Pseudomonas aeruginosa* that share 76% primary amino acid homology and contain N-terminal RhoGAP domains and C-terminal ADP-ribosylation domains. The Rho GAP activities of ExoS and ExoT appear to be biochemically and biologically identical, targeting Rho, Rac, and Cdc42. Expression of the RhoGAP domain in mammalian cells results in the disruption of the actin cytoskeleton and interference of phagocytosis. Expression of the ADP-ribosyltransferase domain of ExoS elicits a cytotoxic phenotype in cultured cells, while expression of ExoT appears to interfere with host cell phagocytic activity. Recent studies showed that ExoS and ExoT ADP-ribosylate different substrates. While ExoS has poly-substrate specificity and can ADP-ribosylate numerous host proteins, ExoT ADP-ribosylates a more restricted subset of host proteins including the Crk proteins. Protein modeling predicts that electrostatic interactions contribute to the substrate specificity of the ADP-ribosyltransferase domains of ExoS and ExoT.

Abbreviations *GAP:* GTPase activating protein · *ADP-r:* Adenosine diphosphate ribose · *MLD:* Membrane localization domain · *FAS:* Factor activating exoenzyme S · *Crk:* CT-10 regulator of kinase · *SBTI:* Soybean trypsin inhibitor · *GEF:* Guanine nucleotide exchange factor

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen and the most common Gram-negative bacterium associated with nosocomial infections. *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 12% of hospital-acquired urinary tract infections, 8% of surgical wound infections, and 10% of bloodstream infections (Van

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Delden and Iglewski 1998). Immunocompromised individuals, such as patients with cystic fibrosis, neutropenic cancer, burn wound, and bone marrow transplantation, are susceptible to infection by this pathogen and the prevalence of multi-drug resistant strains limits antibiotic therapy. *P. aeruginosa* pathogenesis depends on cell associated and secreted virulence factors. Cell-associated virulence factors include flagellum, pilus, adhesins, alginate, and lipopolysaccharide. Secreted virulence factors include: hemolysins, lipases, proteases, Exotoxin A and the type-III cytotoxins; ExoS, ExoT, ExoU, and ExoY.

The type-III secretion system delivers cytotoxins directly from the bacterium into a mammalian cell through a contact-dependent mechanism (Blocker et al. 2001). The type-III secretion apparatus is composed of 20–30 proteins that are conserved among Gram-negative bacteria. The genes encoding the *Pseudomonas* type-III secretion system are clustered in the 55 min region of the *P. aeruginosa* PA01 chromosome and are designated *psc*, *pcr*, *exs*, and *pop*. *psc* and *pcr* encode proteins of the type-III secretion apparatus and proteins involved in the regulation of expression of the system. In addition, PopB and PopD are responsible for the translocation of the type-III cytotoxins into mammalian cells (Frank 1997).

Pseudomonas aeruginosa type-III cytotoxins inhibit host innate immunity. ExoU is a phopholipase (Sato et al. 2003) and is correlated with acute cytotoxicity in epithelial cells and macrophages, and contributes to injury in models systems (Finck-Barbancon et al. 1997; Vallis et al. 1999). ExoY is an adenylate cyclase, which elevates the intracellular cAMP levels in cultured mammalian cells and causes actin cytoskeleton reorganiza tion (Yahr et al. 1998). ExoS and ExoT are similar, yet distinct, possessing N-terminal RhoGAP and C-terminal ADP-ribosyltransferase domains. The RhoGAP domains of ExoS and ExoT stimulate the reorganization of actin cytoskeleton through inactivation of Rho GTPases, while the ADP-ribosyltransferase domains ADP-ribosylate distinct host proteins.

This review will discuss the structural and biochemical properties of ExoS and ExoT and their relative roles in *P. aeruginosa* pathogenesis.

History of *Pseudomonas aeruginosa* exoenzyme S

Exoenzyme S was first described as a secreted ADP-ribosyltransferase that was isolated from *P. aeruginosa* strain 388, possessing properties distinct from Exotoxin A (Iglewski et al. 1978). Exoenzyme S did not ADP-ribosylate elongation factor 2, as did Exotoxin A and diphtheria toxin, but ADP-ribosylated several proteins present in crude extracts of wheat germ or rabbit reticulocytes. In addition, the ADP-ribosyltransferase activity of exoenzyme S was not neutralized by α-Exotoxin A antibody and relative to Exotoxin A was heat stable. Together, these properties indicated that exoenzyme S was different from the previously described Exotoxin A.

From the culture supernatant fluid of *P. aeruginosa* strain 388, two proteins with molecular weight 53-kDa and 49-kDa (termed the 53- and 49-kDa forms of exoenzyme S) copurified with the ADP-ribosyltransferase activity of exoenzyme S (Kulich et al. 1993). The 53- and 49-kDa forms of exoenzyme S had similar N-terminal amino acid sequences (Coburn 1992), immunoreactivity (Nicas and Iglewski 1984), and peptides upon proteolytic cleavage (Kulich et al. 1994). Upon fractionation, the purified 49-kDa form of exoenzyme S possessed ADP-ribosylation activity, while the 53-kDa form lacked ADP-ribosylation activity. α-49-kDa-protein IgG inhibited the ADP-ribosyltransferase activity of purified exoenzyme S in a dose-dependent manner. This suggested that the 53-kDa form of

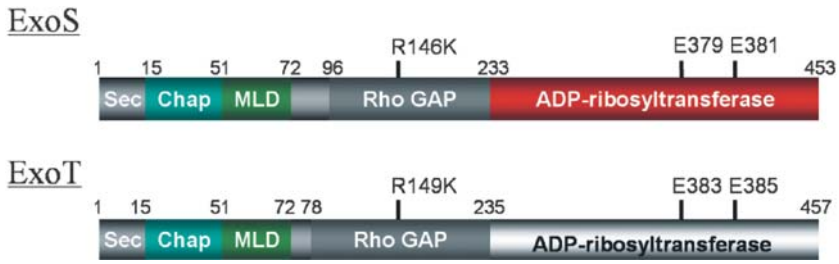

Fig. 1 Functional domains of *Pseudomonas aeruginosa* ExoS and ExoT. ExoS and ExoT are similar yet distinct. They share 76% amino acid identity and similar functional domains with N-terminal RhoGAP activity and C-terminal ADP-ribosyltransferase activity. The N terminus of ExoS and ExoT are similar, containing secretion signals (*Sec*), chaperon binding domains (*Chap*), membrane localization domains (*MLD*) and RhoGAP domains (*RhoGAP*). The RhoGAP domains of ExoS and ExoT stimulate reorganization of the actin cytoskeleton through inactivating Rho, Rac and Cdc42. R146 of ExoS and R149 of ExoT are catalytic residues of the RhoGAP activity. ExoS and ExoT have distinct C-terminal ADP-ribosylation domains

exoenzyme S was an inactive precursor of the 49-kDa form of exoenzyme S. Subsequent studies showed that the 53- and 49-kDa forms of exoenzyme S are encoded by distinct genes, termed *exoT* and *exoS*, respectively (Yahr et al. 1996).

Functional domains of ExoS and ExoT

ExoS and ExoT share 76% amino acid identity (Fig. 1) and are bi-functional type-III cytotoxins with RhoGAP domains, which inactivate Cdc42, Rac and Rho, and C-terminal ADP-ribosyltransferase domains, which ADP-ribosylate unique cellular host proteins. The N termini of ExoS and ExoT also comprise a signal sequence required for secretion through the type-III apparatus, a chaperone binding region, and a membrane localization domain (MLD). The MLD, a hydrophobic region that is enriched with leucines, localizes ExoS and ExoT to intracellular membranes after translocation into host cells through the type-III secretion system.

Membrane localization domain

The MLD was first determined to be N-terminal to the GAP domain (Pederson et al. 2000), which resided within residues 96–219 of ExoS. The MLD was not required for expression of ExoS RhoGAP in cultured cells. Subsequent studies mapped the MLD to residues 51–72 of ExoS, and observed that these residues were necessary and sufficient for membrane localization within mammalian cells (Pederson et al. 2002). A deletion protein of ExoS that lacked the MLD (ExoS(ΔMLD) could be secreted through the type-III secretion system of ExoS and translocated into mammalian cells. This indicated that the MLD was not required for secretion through the type-III apparatus or translocation into mammalian cells. Although ExoS(ΔMLD) ADP-ribosylated Ras in vitro, type-III delivered ExoS(ΔMLD) did not ADP-ribosylate Ras, a membrane-associated target protein of ExoS (Riese and Barbieri 2002). This indicated that the subcellular localization of ExoS contributed to the targeting of Ras and other host proteins. Type III-delivered ExoS(ΔMLD)

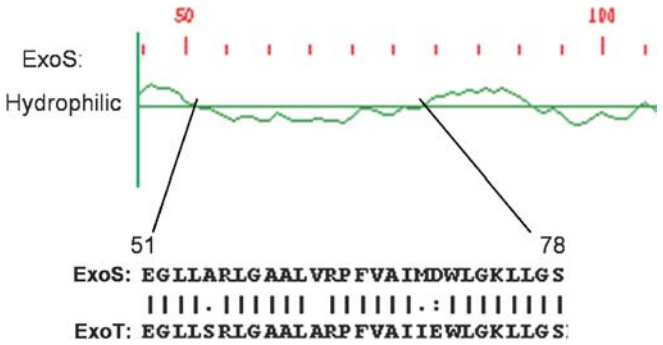

Fig. 2 Membrane localization domains of *Pseudomonas aeruginosa ExoS and ExoT. Upper panel:* the hydrophilic plot of N-terminal ExoS (residues *50–100*). *Lower panel:* the sequence alignment of the membrane localization domains of ExoS (*51–78*) and ExoT (*51–78*)

and ExoS showed similar capacities to elicit a cytotoxic response in CHO cells, which uncoupled the ADP-ribosylation of Ras from the cytotoxic action of ExoS (Riese and Barbieri 2002). Subsequently, the N terminus of *Yersinia* YopE was also shown to possess an intracellular membrane localization domain and that the MLD of YopE colocalized with the MLD of ExoS within mammalian cells and that the MLD of YopE functionally complemented the ExoS MLD for intracellular targeting in mammalian cells (Krall et al. 2004).

While the membrane localization domain of ExoT has not been experimentally mapped, primary amino acid sequences between the MLD of ExoS and the analogous region of ExoT are highly homologous (Fig. 2), indicating that ExoT possesses a similar targeting sequence to the ExoS MLD. This is supported by the determination that the type-III-delivered ExoT has a similar intracellular fractionation pattern with ExoS and that the RhoGAP domains of ExoS and ExoT have the same eukaryotic targets, Rho, Rac, and Cdc42 (Kazmierczak and Engel 2002; Krall et al. 2002). While the ADP-ribosylation domains of ExoS and ExoT have different in vivo target specificity, switching the ADP-ribosylation domains of ExoS and ExoT switched the in vivo substrate specificity of ExoS and ExoT. This suggests that the MLDs of ExoS and ExoT target these two toxins to a common intracellular trafficking pathway, where the intrinsic biochemical properties of the toxins become the only determinant for their different target specificity (Sun and Barbieri 2004).

RhoGAP domains

When ExoS was expressed in *Yersinia*, a mutated form of ExoS with deficient ADP-ribosyltransferase activity retained partial ability to disrupt the actin cytoskeleton in host cells and was resistant to phagocytosis, indicating ExoS could act through two different mechanisms on host cells (Frithz-Lindsten et al. 1997). Subsequently, the intracellular expression of the N-terminal 234 amino acids of ExoS (ExoS91–234) was observed to elicit the rounding of Chinese Hamster Ovary (CHO) cells and to disrupt the actin cytoskeleton. Cytoskeleton rearrangement elicited by ExoS(1–234) was reversed by the addition of cytotoxic necrotizing factor 1 (CNF 1), a toxin that constitutively activated Rho proteins

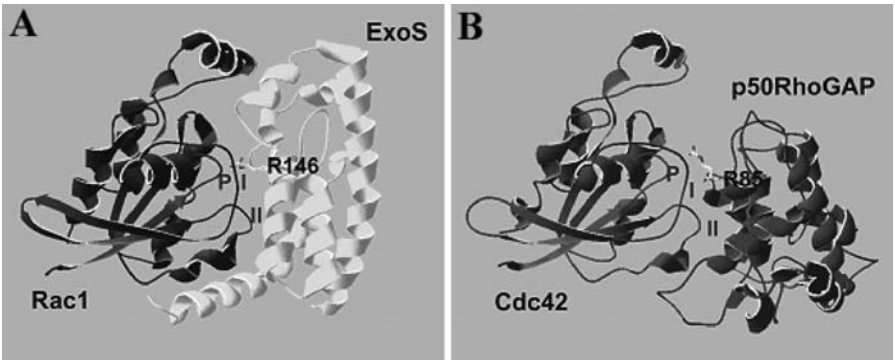

Fig. 3A, B Structures of bacterial GAP (*ExoS*) and eukaryotic GAP (*p50rhoGAP*). The atomic coordinates of Cdc42-p50rhoGAP complex (*1AM4*) and Rac1-ExoS RhoGAP complex (*1HE1*) were downloaded from PDB, and the structures were displayed in Swiss-Pdb-viewer v3.7. **A** Structure of Rac1-ExoS RhoGAP complex. Switch I (*I*), Switch II (*II*), and P-loop (*P*) in Rac1 are shown. The catalytic residue R146 in ExoS is shown. **B** Structure of Cdc42-p50RhoGAP complex. Switch I (*I*), Switch II (*II*), and P-loop (*P*) in Cdc42 are shown. The catalytic residue R85 in p50RhoGAP is shown

through deamidation of Gln61/63, which implicated a role for Rho GTPases in the disruption of the actin cytoskeleton by ExoS (Pederson et al. 1999). ExoS(1–234) was then shown to be a Rho GTPase Activating Protein (RhoGAP) for Rho, Rac, and Cdc42 and that Arg146 was a catalytic residue required for the expression of RhoGAP activity (Goehring et al. 1999). The 3-D crystal structure of the Rac-ExoSGAP complex showed that although ExoSGAP and the mammalian RhoGAPs did not share structural homology, like the eukaryotic RhoGAPs, ExoS RhoGAP stabilized the transition state of GTPase reaction, indicating ExoS RhoGAP was a functional mimic of eukaryotic GAPs (Wurtele, Renault et al. 2001; Wurtele, Wolf et al. 2001) (Fig. 3).

Similar to ExoS, the N terminus of ExoT is a Rho GAP for Rho, Rac, and Cdc42 in vitro and in vivo (Krall et al. 2000; Kazmierczak and Engel 2002), and that Arg149 (analogous to Arg146 on ExoS) is required for the Rho GAP activity of ExoT (Garrity-Ryan et al. 2000; Geiser et al. 2001). The function of ExoT Rho GAP activity has been proposed to be the inhibition of phagocytosis of bacterium by polarized epithelial cells and macrophage-like cells (Garrity-Ryan et al. 2000).

ADP-ribosyltransferase domains

ExoS was first described as an ADP-ribosyltransferase activity that was distinct from Exotoxin A, catalyzing the transfer of ADP-ribose from NAD^+ to a number of unknown eukaryotic proteins, but not EF-2 (Iglewski et al. 1978). The intermediate filament protein vimentin was first identified as a cellular target of ExoS (Coburn, Dillon et al. 1989). Ras and several Ras related proteins, including Rab3, Rab4, Ral, Rap1A, and Rap2, were subsequently identified as targets of ExoS (Coburn, Wyatt et al. 1989; Coburn and Gill 1991). The ADP-ribosylation of Arg41 by ExoS disrupted Ras signaling by inhibiting Guanine Nucleotide Exchange Factor (GEF) catalyzed nucleotide exchange, which uncoupled Ras signal transduction (Ganesan et al. 1998, 1999). ExoS also ADP-ribosylated Rap at Arg41, which also inhibited the ability of Rap GEF, C3G, to stimulate nucleotide exchange (Riese

et al. 2001). Type-III-delivered ExoS was found to be auto-ADP-ribosylated, which may regulate the toxin's catalytic potential (Riese et al. 2002). ExoS ADP-ribosyltransferase activity was dependent on a mammalian 14–3-3 protein termed *F*actor *A*ctivating Exo*S* (FAS) (Coburn et al. 1991; Fu et al. 1993). While FAS directly bound ExoS and Raf, activation of the ADP-ribosylation activity of ExoS by FAS appeared complex (Zhang et al. 1997, 1999; Petosa et al. 1998; Henriksson et al. 2000). The ADP-ribosyltransferase activity of ExoS was mapped to C-terminal residues 234–453 (Knight et al. 1995), where Glu 381 was determined to be a catalytic residue in the ADP-ribosylation reaction. The E381D mutation of ExoS reduced ADP-ribosyltransferase and NAD glycohydrolase activities. In contrast, the E379D mutation did not affect NAD glycohydrolase active, but inhibited ADP-ribosyltransferase activity, indicating that Glu379 contributed to the transfer of ADP-ribose to the target proteins (Liu et al. 1996; Radke et al. 1999). This identified ExoS as a bi-glutamic acid transferase.

While the C terminus of ExoT also has a FAS-dependent ADP-ribosyltransferase activity, it only possesses ~0.1% of ADP-ribosyltransferase activity relative to ExoS when soybean trypsin inhibitor (SBTI) is used as an artificial substrate. Interestingly, ExoT only possessed ~2% NAD glycohydrolase activity of ExoS (Liu et al. 1997). Intracellular expression of ExoS in the ADP-ribosylation domain was cytotoxic to culture cells, but ExoT was not cytotoxic to cultured cells (Note: the term "cytotoxicity" in the present review is defined as the ability of toxins to cause cell death). This implied that ExoT was a defective ADP-ribosyltransferase. However, recent studies observed that ExoT elicited reorganization of the actin cytoskeleton without interfering with Ras signal transduction, and the mutated form of ExoT (ExoTR149A) that was deficient in Rho GAP activity caused a morphological change of infected HeLa cells, suggesting that ExoT may ADP-ribosylate unique host proteins, distinct from Ras (Sundin et al. 2001). The observation that bacterial strains carrying point mutations at Arg149 of ExoT were internalized to an intermediate extent, compared to strains with a deletion of ExoT versus strains expressing wild-type ExoT, also suggested that the Rho GAP activity of ExoT accounted for only part of the anti-internalization activity (Garrity-Ryan et al. 2000). This implied that the ADP-ribosyltransferase domain of ExoT was active. Subsequent studies showed that ExoT ADP-ribosylated host proteins that were distinct to ExoS, Crk-I and Crk-II (CT10-regulator of kinase) both in vitro and in vivo. ExoT ADP-ribosylated Crk at a rate similar to the rate of ADP-ribosylation of SBTI by ExoS (Sun and Barbieri 2003). Crk proteins are SH2-SH3 domain-containing adaptor proteins that play an essential role in integrin-mediated phagocytosis and focal adhesion. Therefore, the ADP-ribosylation of the Crk proteins linked the antiphagocytic activity of ExoT to a mechanism distinct from the modulation of Rho GTPases by the RhoGAP domain. This leads to a hypothesis that ExoT ADP-ribosylates Crk proteins to block Rap1- and Rac1-mediated focal adhesion and phagocytosis.

Current models predict that the ADP-ribosylation of Crk by ExoT could either block interaction with upstream focal adhesion proteins or block interaction with downstream factor DOCK180, which would inhibit Rac-1-mediated phagocytosis (Fig. 4). Alternatively, ExoT ADP-ribosylation of Crk proteins may not block a physical interaction of Crk with its binding partners, but may result in nonfunctional signal complexes. Future studies will identify the sites of ADP-ribosylation on Crk proteins and investigate the mechanism of ExoT-mediated antiphagocytosis through ADP-ribosylation of Crk proteins.

Crk-mediated phagocytosis and focal adhesion appears to be a common pathway that is modulated by bacteria to either prevent uptake of bacteria by professional phagocytes or facilitate bacterial invasion into mammalian cells. *Yersinia* YopH, a type-III secreted

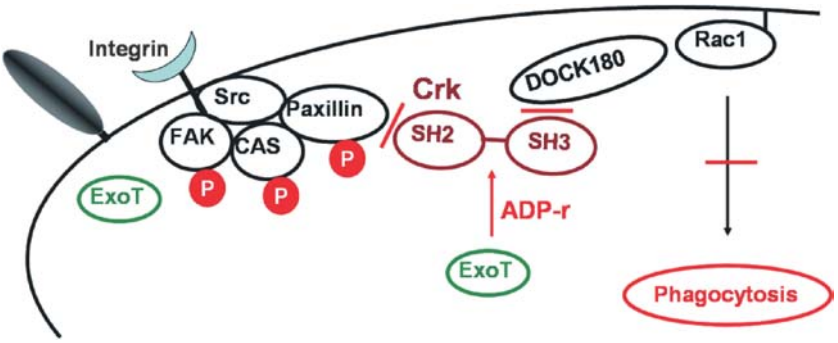

Fig. 4 . ADP-ribosylation of Crk proteins by ExoT inhibits phagocytosis. Once integrin receptors are activated by extracellular stimuli, Src kinase phosphorylates FAK (focal adhesion kinase), Cas (Crk-associated substrate) and Paxillin, which subsequently form a focal adhesion complex on the membrane. The phosphorylated focal adhesion complex recruits Crk proteins to the membrane through interaction with SH2 domain of Crk. The SH3 domain of Crk proteins brings the downstream factor DOCK180 that is the guanine nucleotide exchange factor for Rac1. Activated Rac1 stimulates downstream signal transduction leading to phagocytosis. The type-III delivered ExoT ADP-ribosylates Crk proteins. The ADP-ribosylation of Crk proteins either blocks the upstream interaction between focal adhesion complex and SH2 domain of Crk or blocks the downstream interaction between SH3 domain of Crk with DOCK180 to Rac-mediated phagocytosis

phosphatase, dephosphorylates focal adhesion complex proteins, which downregulate Crk-mediated phagocytosis (Persson et al. 1997; Black et al. 2000). *Shigella flexneri*, an opportunistic intracellular bacterial pathogen, activates Crk-mediated pathways to facilitate invasion. The Abl family of tyrosine kinases (Abl and Arg) is required during *Shigella* internalization. Abl and Arg were reported to be activated during a *Shigella* infection, accumulated at the site of bacterial entry, and were required for efficient bacterial uptake, as internalization was blocked upon deletion of these kinases or treatment with a specific inhibitor. The Abl kinases targeted Crk proteins during *Shigella* uptake, and a phosphorylation-deficient Crk mutant significantly inhibited bacterial uptake. Thus, *Shigella* appears to facilitate bacterial invasion through the modulation of a signal pathway that links Abl kinase phosphorylation of Crk to Rac-mediated phagocytosis (Burton et al. 2003).

Substrate recognition by the ADP-ribosyltransferases of ExoS and ExoT

While sharing 76% amino acid identity, ExoS and ExoT ADP-ribosylate different host proteins (Fig. 5). How ExoS and ExoT recognize different proteins is an intriguing question. Current structural and biochemical studies of bacterial ADP-ribosylating toxins provide insight into the mechanisms of NAD binding, cleavage, and ADP-ribose transfer; however, the mechanism by which bacterial ADP-ribosylating toxins recognize protein substrates is poorly defined. This is partially due to the lack of the structures of enzyme-protein substrate complexes. Recently, the 3-D structures of the ADP-ribosylation domains of ExoS and ExoT were generated by SWISS-MODEL, using VIP2, Iota, and C3 exoenzyme as templates (Fig. 6). Regions B (active site loop), C (ARTT motif), and E (PN loop) on ExoS were necessary and sufficient to recognize ExoS targets, while regions B, C and E on ExoT were necessary, but not sufficient, to recognize ExoT targets, the Crk

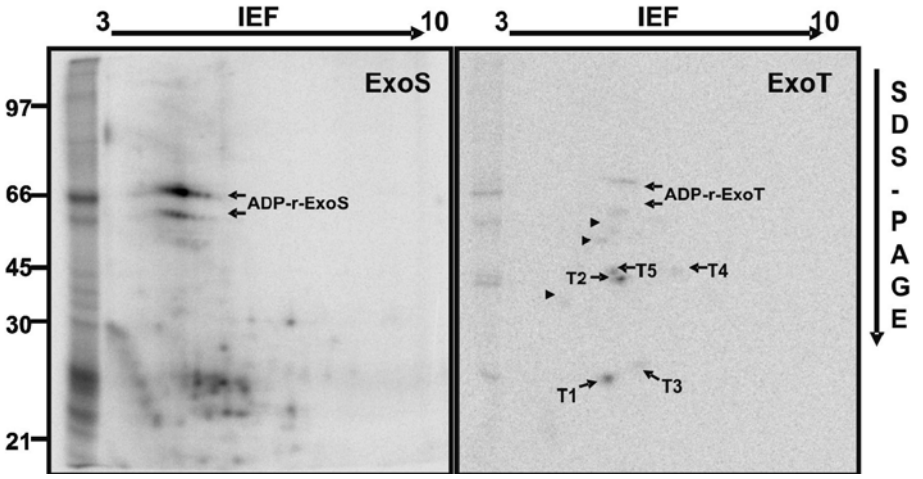

Fig. 5 Type-III delivered ExoS and ExoT ADP-ribosylated unique proteins in CHO cells. Postnuclear supernatant from CHO cells infected with *P. aeruginosa* PA103 Δ*exo*U,*exo*T::Tc expressing ExoS-HA (*ExoS*) or ExoT-HA (*ExoT*) were applied to 2-D SDS-PAGE, (pH 3–10), followed by auto-radiography. Auto-ADP-ribosylated ExoS and ExoT, and proteins that were ADP-ribosylated by ExoT are labeled with indicated *arrows*. Arrows indicate the migration of five radiolabeled spots. Spot T1, T2 and T3 correspond with Crk-I, Crk-II, and PGK-1. (Reproduction from J. Biol. Chem., Vol. 278, 32794–32800, *Pseudomonas aeruginosa* ExoT ADP-ribosylates CT10 Regulator of Kinase (Crk) Proteins. (Jianjun Sun and Joseph T. Barbieri)

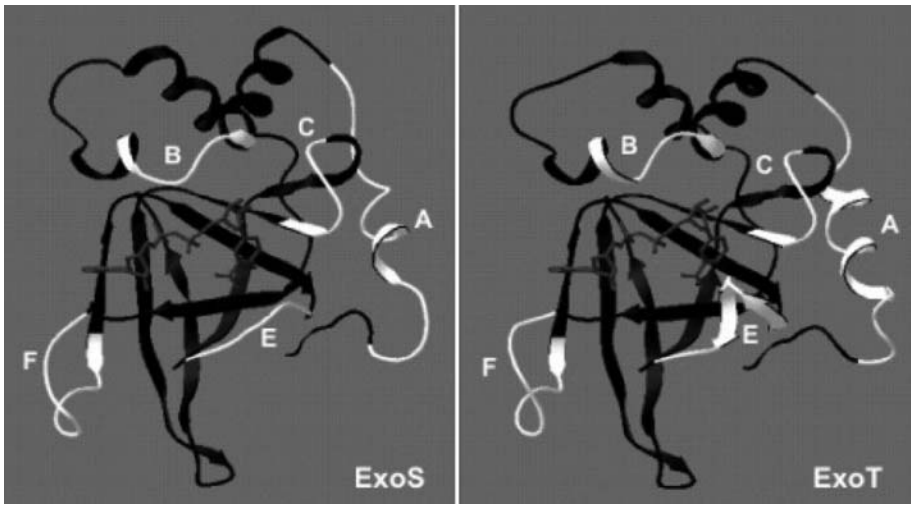

Fig. 6 Protein modeling of the ADP-ribosylation domains of ExoS and ExoT**.** Using the structures of C3 exoenzyme, Vip2 and Iota as templates, the 3-D structures of ExoS and ExoT ADP-ribosylation domains were generated by SWISS-MODEL. The regions that share low homology between ExoS and ExoT are highlighted and labeled as *A*, *B*, *F*, *C*, and *D*, respectively. Note that Regions A, B, C, and E are equivalent to the positions of helix α1, "active site loop," "ARTT" motif, and "PN loop," respectively

proteins. This indicated that an additional region was required for recognition of Crk. Subsequently, a specific Crk recognition motif on ExoT was defined as region A (Helix α1). The calculated electrostatic potentials of the substrate recognition surfaces on ExoS and ExoT displayed significant differences, where ExoS was a mixture of basic, acidic, and neutral charged residues, while ExoT was primarily composed of acidic residues. The electrostatic properties of the substrate recognition surfaces of Ras and Crk are complementary to ExoS and ExoT, respectively (Sun and Barbieri 2004).

ExoS recognizes a wide variety of genetically or structurally diverse proteins for ADP-ribosylation, including the monomeric GTPases, vimentin, and other undefined host proteins. ExoS also undergoes auto-ADP-ribosylation. Even with a one protein substrate, ExoS has multiple alternative sites for ADP-ribosylation. Arg41 of Ras is the preferred site for ExoS ADP-ribosylation, but when Arg41 is either ADP-ribosylated or mutated Arg128 becomes the preferred site of ADP-ribosylation (Ganesan et al. 1998). Arg41 and Arg128 are localized at the opposite sides of Ras molecule, indicating that ExoS could recognize multiple sites on Ras. While ExoS has a broad range of substrates relative to ExoT, it is not a nonspecific enzyme. In vitro and in vivo ExoS ADP-ribosylated a subset of proteins in mammalian cells. ExoS is a polysubstrate specific ADP-ribosyltransferase.

Polysubstrate specificity has been observed in several protein families, such as multidrug transporters, soluble multidrug recognizing proteins, and shark antibodies (Marchalonis et al. 1998). While the mechanism underlying polysubstrate specificity is not well understood, current biochemical and structural studies of multidrug transporters and their drug ligands indicate that polysubstrate specificity is due to a combination of hydrophobic effect, electrostatic attraction, and conformational complementarity, rather than a precise network established by hydrogen bonds and other specific interactions between proteins and ligands (Zheleznova et al. 1999, 2000; Neyfakh 2002). The hydrophobic interaction between proteins and ligands excludes interference of water molecules, which is in turn augmented by the electrostatic interaction surrounding the hydrophobic residues.

For the multidrug transporters, ligand property is not a strict factor in the binding mechanism, as molecules that contain hydrophobic and charged residues on the surface and physically fit into the binding site without including water molecules can be ligands. Different ligands interact with different subset of residues in the binding pocket. Compared to multidrug transporters, ExoS displays similar properties, requiring hydrophobic residues and charged residues to ADP-ribosylate substrates. ExoS may recognize multiple substrates through a similar mechanism to the multidrug transporters.

Evolutionary relationship and relative roles of ExoS and ExoT

Although bacterial ADP-ribosylating toxins target diverse mammalian proteins, structural and biochemical studies suggest that bacterial ADP-ribosyltransferases have similar 3-D structural backbones, which include a conserved NAD binding pocket and a conserved catalytic glutamate residue (Han et al. 1999, 2001). This indicates that the ADP-ribosylating enzymes evolved from a common ancestral ADP-ribosyltransferase, where the mechanisms of NAD binding and catalysis are relatively conserved, while the mechanism of substrate recognition diverged under various selection pressures. The phylogenetic trees derived from primary amino acid sequences of C3 exoenzyme, Vip2, Iota, C2 exoenzyme, ExoS, and ExoT show that ExoT is more closely related to the C2- or C3-like ADP-ribo-

sylating toxins than ExoS. ExoT and ExoS are probably the products of gene duplication, where the original gene product maintained protein function and the duplicated gene product diverged to attain a second independent function.

There are arguments for either ExoT or ExoS being the original gene product. Based upon prevalence among clinical isolates, *exoT* can be argued as the original gene, since *exoT* was more ubiquitous in strains of *P. aeruginosa* than *exoS.* Lomholt and Kilian et. al. screened clinical isolates of *P. aeruginosa* (145 strains) for the distribution of virulent factors (Lomholt et al. 2001); 59% of the 145 strains possess both *exoT* and *exoS*, 34% possess only *exoT*, and 6% possess only *exoS.* In another survey, 49% of *s*trains from patients with keratitis possessed only *exoT*, while 80%–100% strains from urine, lungs, wounds, and feces possessed a combination of *exoS* and *exoT*. Thirty-four percent of the 145 isolates contain *exoU*, another type-III cytotoxin recently characterized as a lipase (Sato et al. 2003). The presence of both *exoS* and *exoU* were essentially mutually exclusive. Based upon prevalence, *exoT* appears to be the more stable genotype and thus one can argue that *exoS* may have evolved and diverged from *exoT* to attain a novel property for polysubstrate recognition through gene duplication. In contrast, in environmental isolates ExoS is a common type-III cytotoxin, which could implicate ExoS as the progenitor gene product (Lanotte et al. 2004).

Biochemical studies can argue for the designation of either *exoT* or *exoS* as the progenitor. The biochemical studies that support the divergence of ExoS from ExoT through gene duplication include the determination that region A of ExoT is the Crk-specific recognition motif, while region A of ExoS does not play a role in substrate recognition. C3 and C3stau2 have slightly different substrate specificity, and show different molecular electrostatic surfaces on region A and E, indicating that region A of C3 ADP-ribosylating toxins also plays a role in substrate recognition. Therefore, upon duplication ExoS may have first become a nonfunctional duplicon that had limited affinity for Crk proteins due to mutations in region A and then evolved the polyspecificity of substrate recognition making ExoS a unique ADP-ribosylating enzyme relative to ExoT and the ADP-ribosylating enzymes from different species. This further indicates that ExoT and other orthologous ADP-ribosylating enzymes evolved at an earlier age, while ExoS evolved from ExoT paralogously through gene duplication. While paralogous proteins have dramatically different functions, they usually maintain similar 3-D structures. An important question is: can ExoS gain the polysubstrate specificity during evolution from ExoT without dramatic structural alterations? When only a limited number of residues of ExoT were replaced with residues from ExoS, the chimeras gained the ability to ADP-ribosylate different subsets of ExoS targets. These mutations do not appear to have dramatic structural alterations, since they have similar NAD glycohydrolase activity. Current studies from multi-drug transporters also indicate that the polyspecificity has arisen independently many times in protein evolution, without dramatic structural changes (Neyfakh 2002). Therefore, ExoS could gain the polysubstrate specificity during evolution from ExoT without dramatic structural alterations.

Biochemical properties that argue for the designation of ExoS as the progenitor and that exoT originated from gene duplication include the limited capacity for ExoT to catalyze the NAD glycohydrolase reaction, a common activity of other ADP-ribosylating proteins, where ExoT catalyzes the NAD glycohydrolase reaction at ~2% of the rate of ExoS. Second, ExoS and ExoT diverge in the primary amino acid sequence within the ARTT motifs, where ExoS has an aromatic residue that is conserved in most ADP-ribosylating

enzymes, while ExoT has a glutamate residue. Third, ExoS shows a limited capacity to ADP-ribosylate Crk, the preferred target of ExoT, while ExoT does not recognize the preferred targets of ExoS. Finally, a chimera that had the α-1 of ExoT engineered into ExoS efficiently ADP-ribosylated both ExoS targets and ExoT targets. This ExoS/ExoT chimera could represent an intermediate in the evolution of ExoS to ExoT, where α-1 evolved into an efficient Crk binding site and subsequently lost the ability to recognize ExoS targets through mutations within the ARTT motif (region C) and active site loop (region B).

Initially, since ExoT appeared to contain a "defective" ADP-ribosyltransferase domain relative to ExoS, the roles of ExoS and ExoT in bacterial pathogenesis and the selection for *P. aeruginosa* to maintain a "defective" gene, *exoT*, was unclear. The ADP-ribosylation of different eukaryotic proteins indicates that ExoS and ExoT play distinct roles in bacterial pathogenesis. While ExoS and ExoT are involved in antiphagocytosis, ExoT appears to be the major contributor for antiphagocytosis in most *P. aeruginosa* strains (Fleiszig et al. 1997; Cowell et al. 2000; Garrity-Ryan et al. 2000). While the RhoGAP domains of ExoS and ExoT stimulate reorganization of the actin cytoskeleton through inactivation of Rho, Rac, and Cdc42, which contributes to antiphagocytosis, this modulation is a reversible transient process. Moreover, the RhoGAP activity of ExoS and ExoT may be downregulated by ADP-ribosylation at their RhoGAP domains after delivery into host cells (Riese et al. 2002). Therefore, the ADP-ribosylation of Crk proteins by ExoT may represent the major contributor for antiphagocytosis. The ADP-ribosylation domain of ExoS is cytotoxic to mammalian cells. The mechanism of cell death caused by ExoS ADP-ribosyltransferase is not clear, but may be in part due to its polysubstrate specificity. The identification of new targets of ExoS ADP-ribosyltransferase will facilitate our understanding of ExoS-induced cell death. Recent studies show that the ADP-ribosylation activity of ExoS induces apoptosis of host cells (Frithz-Lindsten et al. 1997; Kaufman et al. 2000; Jia et al. 2003). Therefore, ExoS and ExoT are two type-III virulence factors that play distinct roles to disarm host defense, allowing the establishment of bacterial colonization.

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Pasteurella multocida toxin as a tool for studying G_q signal transduction

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Abstract *Pasteurella multocida* toxin (PMT) stimulates and subsequently uncouples phospholipase C (PLC) signal transduction through its selective action on the $G\alpha_q$ subunit. This review summarizes what is currently known about the molecular action of PMT on G_q and the resulting cellular effects. Examples are presented illustrating the use of PMT as a powerful tool for dissecting the molecular mechanisms involving pertussis toxin (PT)-insensitive heterotrimeric G proteins.

Introduction

Protein toxins have long been known to constitute important virulence determinants for pathogenic bacteria. The anthrax bioterrorism events of 2001 and the emergence of antibiotic-resistant, toxin-producing bacteria have provided strong impetus to increase our understanding of toxin-mediated disease processes (Wilson and Salyers 2002). The growing relevance of toxin-mediated effects on host cells in developing alternative antitoxin strategies heightens the need to better understand the structure-function relationships of protein toxins produced by pathogenic bacteria. Over the past couple of decades, our understanding of toxin action has expanded tremendously. Information learned from these efforts has enabled scientists to exploit their noxious properties for beneficial applications in studying problems in cell biology, physiology, and pharmacology.

Many protein toxins share the common feature of being highly specialized enzymes, capable of entering eukaryotic cells and catalyzing reactions that interfere with normal signal transduction and physiological processes, often resulting in morphological changes, cellular damage or cell death. Because of their highly specific action in cells, bacterial protein toxins can be used as selective and efficient tools for studying molecular mechanisms controlling signal transduction and other physiological processes (Schiavo and Van Der Goot 2001). For example, a number of toxins, including the clostridial toxins C2, C3,

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Table 1 Signaling of heterotrimeric G-proteins and their modulating toxins

Subfamily	Gα	Signaling[a]	Modulating toxin	Toxin effect on Gα	References[b]
G_i subfamily	G_{i1}	↓AC	PT	Inhibition	(a) (b)
	G_{i2}	↓AC	PT	Inhibition	(a) (b)
	G_{i3}	↓AC	PT	Inhibition	(a) (b)
	G_o	↓Ca^{2+} channels	PT	Inhibition	(a) (b)
	G_t	↑cGMP-PDE	PT	Inhibition	(a) (b) (c)
			CT	Activation	(a) (b) (d)
	G_{gust}	↑PDE	PT	?	(e)
	G_z	↓AC	?	?	(f) (g)
G_s subfamily	G_s	↑AC	CT	Activation	(a) (b)
	G_{olf}	↑AC	CT	Activation	(h)
G_q subfamily	G_q	↑PLCβ, ↑Rho	PMT	Activation/Inhibition[c]	(i) (j) (k)
	G_{11}	↑PLCβ, ↑Rho	?	?	(k)
	G_{14}	↑PLCβ	?	?	
	G_{15}	↑PLCβ	?	?	
	G_{16}	↑PLCβ	?	?	
G_{12} subfamily	G_{12}	↑Rho	?	?	
	G_{13}	↑Rho	?	?	

[a] AC: adenylate cyclase; PDE: phosphodiesterase; PLC: phopholipase C

[b] (a) (Fields and Casey 1997), (b) (Casey and Gilman 1988), (c) (Van Dop et al. 1984a), (d) (Van Dop et al. 1984b), (e) (Gilbertson et al. 2000), (f) (Casey et al. 1990), (g) (Ho and Wong 2001), (h) (Jones et al. 1990), (i) (Wilson et al. 1997), (j) (Zywietz et al. 2001), (k) (Vogt et al. 2003)

[c] Initial activation, followed by uncoupling of G_q signaling. Prolonged treatment with PMT results in inhibition

toxA and toxB, and the *E. coli* toxins CNF1 and CNF2, among others, have been used as selective modulators of cytoskeletal function through their action on small GTPases (Aktories et al. 2000). The clostridial neurotoxins have been enormously beneficial in uncovering molecular mechanisms of neurotransmitter release and essential aspects of neuronal physiology (Lalli et al. 2003; Schiavo et al. 2000). These neurotoxins are increasingly being put to beneficial use in medicine for the treatment of human diseases characterized by hyperfunction of nerves (Rossetto et al. 2001).

Heterotrimeric G proteins constitute a large family of pivotal regulatory GTPases that are responsible for transducing external (e.g., hormonal) signals from ligand-bound receptors to intracellular responses. They are made of α, β, and γ subunits and are distinguished into four main classes: G_s, G_i, G_{12}, and G_q. The first toxins used to define the molecular mechanisms of heterotrimeric G proteins and adenylate cyclase-mediated signaling pathways were cholera toxin (CT) and pertussis toxin (PT): CT activates G_s proteins, while PT inhibits $G_{i/o}$ proteins (Casey and Gilman 1988). PT was used to further distinguish a group of PT-insensitive G proteins (see Table 1) (Fields and Casey 1997). Of these G proteins that are refractory to PT treatment, a subgroup, later identified as the G_q family, were found to play critical roles as regulators of phospholipase C (PLC) signaling (Fields and Casey 1997; Rhee and Choi 1992; Sternweis and Smrcka 1992).

Receptors for many hormones, neurotransmitters and growth factors are coupled to G_q proteins. Stimulation of G_q-coupled receptors results in transient elevation of intracellular Ca^{2+} and increased levels of inositol trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers control vital cellular processes, including fertilization, cell growth, transformation, secretion, muscle contraction, metabolism and sensory perception

(Berridge 1993). IP_3 and DAG are generated from phosphatidylinositol 4,5-bisphosphate (PIP_2) through the action of PLC, of which γ-isoforms are activated through tyrosine kinase-linked receptors and β-isoforms are activated through G-protein-coupled receptors (Rhee and Choi 1992; Sternweis and Smrcka 1992). Ligands and G-protein-coupled receptors that activate PLCβ (Deckmyn et al. 1993; Quick et al. 1994; Rebecchi and Pentyala 2000; Rhee and Choi 1992; Sternweis and Smrcka 1992) can be distinguished by their sensitivity to PT (Berstein et al. 1992a, b; Camps et al. 1992; Wu et al. 1993). The $\beta\gamma$ subunits (but not α subunits) of PT-sensitive $G_{i/o}$ proteins preferentially stimulate PLCβ2>PLCβ3>PLCβ1, whereas the α subunits of the PT-insensitive G_q proteins stimulate PLCβ1≧PLCβ3≫PLCβ2 (Hepler et al. 1993; Park et al. 1993; Rhee and Choi 1992; Smrcka and Sternweis 1993).

Until recently, there had been no specific modulating reagent available for studying the role of G_q proteins in hormonal communication and signal transduction. The dermonecrotic toxin produced by *Pasteurella multocida* (PMT) can now be added to the list of bacterial protein toxins that modulate G proteins (Table 1). PMT stimulates Ca^{2+} and IP_3 signaling by activating G_q-dependent PLCβ1 (Wilson et al. 1997). PMT facilitation of Gα_q-protein coupling to PLCβ1 causes the same cellular responses elicited by G_q-protein-linked receptors, such as the muscarinic (M_1, M_3, M_5), bombesin, vasopressin, endothelin, thyrotropin-releasing hormone (TRH), and adrenergic receptors (α_1AR). Understanding the biochemical mechanism of PMT action may thus afford unique insight into G_q-mediated molecular signaling events and enables the use of PMT as a tool for studying these processes.

Cellular effects of PMT

PMT is the major virulence factor produced by *P. multocida* that is responsible for atrophic rhinitis, pneumonia-like respiratory disease, and dermonecrosis (Foged 1992). PMT is secreted as a monomeric, 1285-amino acid protein (M_r 146-kDa) (Lax and Chanter 1990; Petersen 1990; Petersen and Foged 1989). PMT binds to ganglioside-type receptors and enters mammalian cells via receptor-mediated endocytosis (Dudet et al. 1996; Pettit et al. 1993; Rozengurt et al. 1990) and acts intracellularly to initiate a number of signaling pathways leading to DNA synthesis and cellular proliferation (Higgins et al. 1992; Rozengurt et al. 1990; Seo et al. 2000; Wilson et al. 1997, 2000). Purified PMT alone is sufficient to experimentally induce progressive atrophic rhinitis in swine and symptoms of pneumonia in rabbits (Chrisp and Foged 1991; Foged 1992; Lax and Chanter 1990). Recombinant PMT is indistinguishable from native toxin, and both are equally potent at picomolar concentrations (Lax and Chanter 1990; Rozengurt et al. 1990; Wilson et al. 1997). Vaccination against PMT protects against challenge with *P. multocida* and development of atrophic rhinitis (Foged 1992).

PMT stimulates osteoclastic bone resorption in vitro (Felix et al. 1992; Kimman et al. 1987) and increases osteoclast cell number in vivo (Martineau-Doize et al. 1993). PMT appears to stimulate the differentiation of preosteoclasts into osteoclasts (Jutras and Martineau-Doize 1996) and promotes osteoclast proliferation leading to bone resorption, while apparently inhibiting bone regeneration by osteoblasts (Mullan and Lax 1998; Sterner-Kock et al. 1995). PMT acting on these multiple cell types, including activating mature osteoclasts and inducing preosteoclast proliferation, as well as proliferation/differentiation of periosteal (fibroblastic, osteogenic, and adipogenic) cells, may contribute to the symptoms of atrophic rhinitis (Mullan and Lax 1998; Rozengurt et al. 1990). Recent evidence

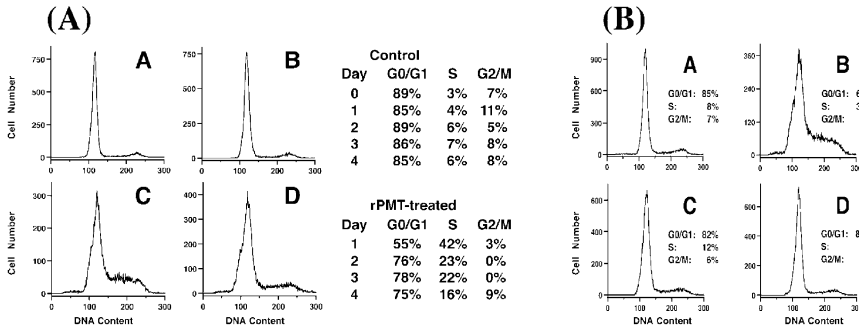

Fig. 1 Effect of PMT treatment on cell cycle progression in Swiss 3T3 cells. Shown are results from flow cytometry analyses of confluent Swiss 3T3 cells with or without single or multiple PMT treatments. **A** Representative DNA histograms from a time course of untreated (*a* and *b*) or PMT-treated (*c* and *d*) cells from day 1 (*a* and *c*) and day 4 (*b* and *d*). To the *right* is the summary of the percentages of untreated (*top*) or PMT-treated (*bottom*) cells found in G_0/G_1, S, and G_2/M phases of the cell cycle during the time course. **B** Representative DNA histograms from multiple PMT treatments: (*a*) untreated cells analyzed on day 5; (*b*) cells treated with PMT on day 5 and analyzed on day 6; (*c*) cells treated with PMT on day 0 and analyzed on day 5; (*d*) cells treated with PMT on day 0 and again on day 5 and analyzed on day 6. The percentages of cells found in G_0/G_1, S, and G_2/M phases of the cell cycle are also shown. (Reprinted with permission from Wilson et al. 2000.)

suggests that immunomodulation of the host may be an additional function of the toxin important in pathogenesis (Jordan et al. 2003).

In cultured epithelial cells, such as calf or monkey (Vero) kidney cells, calf testis, or bovine embryonic lung (EBL) cells, PMT causes primarily morphological changes and cytotoxic effects (Pennings and Storm 1984; Pettit et al. 1993; Rutter and Luther 1984). In cultured mesenchymal cells, such as murine, rat, or human fibroblasts, and in osteoblasts, PMT action is mitogenic and initiates DNA synthesis and cell division (Mullan and Lax 1998; Rozengurt et al. 1990). PMT has also been shown to induce anchorage-independent cell growth of fibroblasts (Higgins et al. 1992), as evidenced by colony formation in soft agar, suggesting that it has the ability to promote a transformed phenotype and leading to the speculation that it could promote tumor formation and cancer (Lax and Thomas 2002). Flow cytometry analysis of cells treated with PMT showed that PMT stimulates cells to move from the G_1 phase into and through the S phase, but it does not trigger apoptosis (Wilson et al. 2000). PMT-treated confluent quiescent Swiss 3T3 cells formed dense monolayers over the course of 4–6 days, with a concomitant increase in cell number up to threefold (Wilson et al. 2000). However, cell cycle analysis revealed that after the initial mitogenic response to PMT, cells subsequently arrested primarily in G_1 and became unresponsive to further PMT treatment (see Fig. 1), indicating that the mitogenic response was not sustained (Wilson et al. 2000).

Western blot analysis of the effect of PMT on the expression of a number of cell cycle markers, including the proto-oncogene c-Myc; cyclins D1, D2, D3, and E; p21; PCNA; and the Rb proteins, p107 and p130, showed that PMT initially upregulated these markers and stimulated cell cycle progression in Swiss 3T3 cells, yet continued expression of these markers, and hence continued proliferation, was not sustained (Wilson et al. 2000). However, PMT exhibited a differential effect on epithelial-like cells. Confluent Vero cells underwent rapid, dramatic morphological changes upon toxin exposure, but a mitogenic ef-

fect was not evident, based on the lack of a PMT-induced increase in cell numbers or in the rate of DNA synthesis, which was further substantiated by flow cytometry analysis. Furthermore, PMT failed to upregulate PCNA or cyclins D3 and E, which is critical for driving cells from G_1 into S phase, and hence, little or no cell cycle progression occurred in Vero cells (Wilson et al. 2000).

PMT effects on signal transduction

PMT and G_q-PLC signaling

PMT activates inositol phosphate pathways, Ca^{2+} mobilization and PKC-dependent phosphorylation in cultured fibroblasts and osteoblasts (Mullan and Lax 1998; Staddon et al. 1990, 1991). PMT also potentiates G protein-coupled receptor responses to bombesin, vasopressin, and endothelin (Murphy and Rozengurt 1992). These effects suggested the involvement of a cellular phosphatidylinositol-specific phospholipase C (PLC) in PMT action (Murphy and Rozengurt 1992). Wilson et al. (Wilson et al. 1997) subsequently demonstrated direct PMT-mediated stimulation of PLCβ1 activity and IP_3-induced intracellular Ca^{2+} mobilization by using voltage-clamped *Xenopus* oocytes as a model system to monitor the transient Ca^{2+}-dependent Cl^- current evoked upon microinjection with PMT. To identify the intracellular targets involved in the PMT-induced IP_3 signaling pathway, they examined the effects of specific antibodies against various G-protein and PLC signaling molecules on the PMT-induced Cl^- currents in the oocyte system. As showed in Fig. 2, only antibodies directed against PLCβ1, Gα_{pan}, and the C-terminus of G$\alpha_{q/11}$ completely blocked the PMT-mediated response in oocytes. In addition, GDPβS, a known inhibitor of

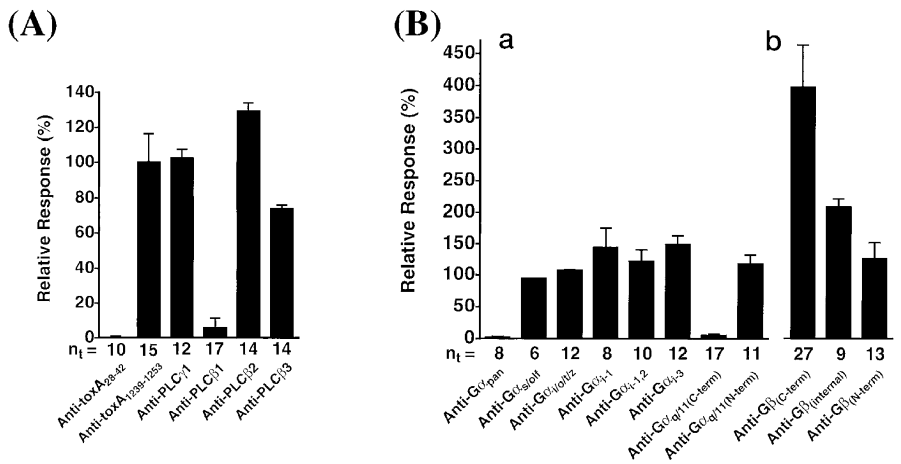

Fig. 2 Identification of the intracellular target of PMT as $G_{q/11}$ by using specific antibodies against key signaling proteins to block the PMT response in *Xenopus* oocytes. The peak inward Cl^- current was used to measure the effect of specific antibodies on the PMT-induced Ca^{2+}-dependent Cl^- currents in oocytes voltage-clamped at a holding potential of –80 mV. Antibodies against PMT (N-terminus or C-terminus) and various PLC isoforms (**A**) or against various Gα or Gβ subunits (**B**) were microinjected into the oocytes 3 h prior to microinjection with PMT. (Reprinted with permission from Wilson et al. 1997.)

Gα subunit-mediated signaling pathways, blocked the PMT-induced response. Furthermore, overexpression of mouse Gα_q in *Xenopus* oocytes increased the PMT-induced response, whereas treatment with antisense Gα_q mRNA reduced the response. These results established the direct involvement of Gα_q protein in PMT-activation of PLCβ1 (Fig. 2).

Antibodies against Gβ subunit did not block, but rather enhanced the PMT-induced response (Fig. 2), distinguishing that PMT action did not involve G$\beta\gamma$ subunit activation of PLCβ2 by a $G_{i/o}$-dependent pathway. Rather, by binding to Gβ, the antibodies caused the dissociation of Gα_q subunit from the heterotrimeric complex, which could then be acted upon by PMT to give an enhanced response. The PMT-induced response was likewise enhanced by the release of Gα_q subunit through sequestration of G$\beta\gamma$ subunits by using PT (Wilson et al. 1997). From these studies, the researchers concluded that the monomeric Gα_q subunit is the preferred target of PMT action, which subsequently activates PLCβ1. Mouse knockout cell lines were used to confirm that PMT-induced formation of inositol phosphates was exclusively dependent on Gα_q, and not closely-related G_q family members, such as G_{11}, G_{12}, or G_{13} (other G_q-family members, G_{14} or $G_{15/16}$, were not examined) (Zywietz et al. 2001).

A recent study using a series of chimeras between Gα_q and Gα_{11} in G$\alpha_{q/11}$-knockout cells identified a region of the helical domain of Gα_q that is important for PMT-induced activation of PLCβ (Orth et al. 2004). Exchange of Glu-105 or Asn-109 of Gα_{11}, each of which is located in the helical domain of the Gα subunit, with the corresponding His residues of Gα_q resulted in a mutant Gα_{11} that was now capable of mediating PMT-induced activation of PLCβ. However, the converse was not true, in that the reciprocal exchange of either His in Gα_q with the corresponding Gα_{11} amino acid did not prevent PMT activation of PLCβ. Whether this differential interaction is due to a difference in PMT recognition of Gα_q versus Gα_{11}, due to a difference in Gα_q versus Gα_{11} recognition of PLCβ, or due to a difference in recognition of Gα_q versus Gα_{11} by another unidentified PMT mediator has yet to be determined.

PMT was shown to stimulate tyrosine phosphorylation of Gα_q, but a mutant of PMT that does not activate G_q was also found to cause tyrosine phosphorylation of G_q, suggesting that this phosphorylation is not a prerequisite for Gα_q activation by PMT (Baldwin et al. 2003). Consequently, although tyrosine phosphorylation of $G_{q/11}$ has been reported to regulate $G_{q/11}$ activation (Umemori et al. 1997, 1999), the role of tyrosine phosphorylation in PMT action on G_q is not clear.

Repeated microinjection of IP_3 into oocytes reproduced transient Ca^{2+}-dependent Cl^- currents, indicating that the IP_3 pathway is not readily desensitized. After the initial transient response to PMT, additional injection of IP_3 still gave a response, but additional injection of PMT had no further response (Wilson et al. 1997). These results confirmed that the target of PMT action is upstream of IP_3 release and that PMT uncouples the signaling between G_q and PLCβ1. A model for the intracellular action of PMT based on all of these results is shown in Figure 3.

PMT and downstream signaling

Some of the intracellular events that occur upon exposure to PMT are: enhanced hydrolysis of inositol phospholipids to increase the total intracellular content of inositol phosphates (Staddon et al. 1991); increased production of DAG (Staddon et al. 1990); mobili-

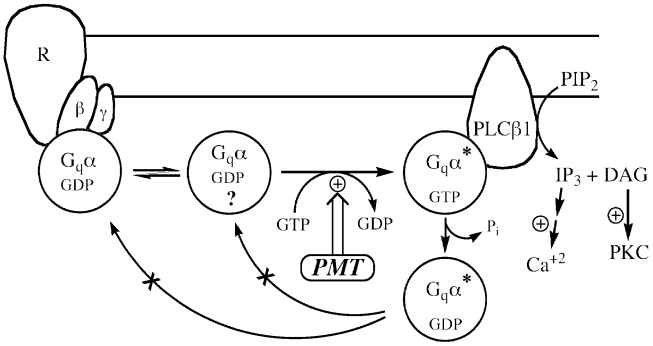

Fig. 3 A proposed model for PMT action on G_q-coupled PLC signal transduction. In this model, PMT acts on free, monomeric $G\alpha_q$, most likely in the GDP-bound form, and converts it into an active form, presumably GTP-bound, which stimulates PLCβ1. The PLCβ1 hydrolyzes PIP_2 into IP_3 and DAG, leading to Ca^{2+} mobilization that results in the Ca^{2+}-dependent Cl^- current. The PMT-induced response is transient due to GTPase activity of $G\alpha_q$, which is still intact and is stimulated by interaction with PLCβ1. The presumably modified GDP-bound G_q can be neither acted upon again by PMT nor reassociated with the G$\beta\gamma$-receptor complex.

zation of intracellular Ca^{2+} pools (Staddon et al. 1991); interconversion of GRP78/BiP (Staddon et al. 1992); and activation of protein kinase phosphorylation (Lacerda et al. 1996; Staddon et al. 1990). It has been suggested that activation of the small Rho GTPase mediates PMT-induced tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$) and paxillin, which results in actin stress fiber formation and focal adhesion assembly (Lacerda et al. 1996; Ohnishi et al. 1998; Thomas et al. 2001). Yet, this tyrosine phosphorylation appears to be independent of PKC activation and Ca^{2+} mobilization (Lacerda et al. 1996; Ohnishi et al. 1998).

How PMT-mediated activation of the PLC-IP_3 signaling pathway promotes cytoskeletal rearrangement is, as of yet, not clear. One hint toward this may be the recent finding that PMT can associate with vimentin (Shime et al. 2002), a component of intermediate filaments in cells. Another possibility is for PMT to act on the actin cytoskeleton through its indirect action on Rho via G_q (Chikumi et al. 2002; Dutt et al. 2002; Katoh et al. 1998; Vogt et al. 2003). Although the stimulation of inositol phosphate signaling by PMT did not occur in $G\alpha_q$-deficient or $G\alpha_q/G\alpha_{11}$-deficient cells, PMT could still stimulate other cellular effects in those knockout cells, including Rho activation, Rho-dependent actin rearrangements and focal adhesions, as well as JNK and Erk mitogenic signaling (Zywietz et al. 2001). These results indicate that certain effects of PMT action may also occur through other signaling pathways, independent of G_q or G_{11}. One possibility is that PMT acts on G_{12} or G_{13}, both of which are known to activate Rho protein (Fukuhara et al. 1999; Gratacap et al. 2001; Hart et al. 1998; Kozasa et al. 1998; Kurose 2003). $G\alpha_{12/13}$ can induce Rho-dependent responses by interaction with Rho-specific guanine nucleotide exchange factors (Sah et al. 2000). Although it is not known if PMT acts on $G\alpha_{12/13}$, this may provide a mechanism by which PMT could activate Rho in the absence of G_q and G_{11}.

PMT as a tool for studying signal transduction

PMT as a tool for studying G_q-PLC signaling

A number of investigators have used PMT as a pharmacological tool to study G_q-coupled PLC signaling along the lines of that shown in Figure 3. For example, PMT has been used as a selective activator of G_q-coupled PLC effectors. G_q-coupled adrenergic receptor signaling in cardiomyocytes differs significantly between even closely related animal species, such as mice and rats. In the rat cardiomyocytes, α_1-AR and endothelin receptors selectively activated PLC through G_q protein, but these receptors were not functional in mouse cardiomyocytes. PMT was used to show that G_q-PLC signaling pathway was still functional in the mouse cardiomyocytes (Sabri et al. 2000).

In discriminating PT-insensitive G-protein coupling of noradrenaline-induced α_{1A}AR activation in neonatal rat cardiomyocytes, α_{1A}AR was found to couple specifically to $G_{q/11}$ and not $G_{12/13}$ proteins by showing that overexpression of $G_{q/11}$-specific RGS4, but not $G_{12/13}$-specific Lsc-RGS blocked α_{1A}AR activation of both PLC and phospholipase D (PLD) (Gosau et al. 2002). In addition, this study showed that PLD activation occurred subsequent to G_q-activation of PLCβ and novel, Ca^{2+}-independent PKC isoforms δ and ϵ. The importance of G_q and not G_{11} in α_{1A}AR activation of both PLC and PLD was further demonstrated by using PMT, which mimicked the α_{1A}AR response.

Histamine induces catecholamine secretion from bovine adrenal chromaffin cells. PMT treatment caused a substantial additive increase in basal and histamine-stimulated inositol phosphate levels, but did not increase or prevent basal or histamine-stimulated secretion of the catecholamines, adrenaline and noradrenaline (Donald et al. 2002). This study showed that the secretion occurs through a PLC-independent membrane depolarization. The results obtained with PMT were consistent with other data, which showed that the PLC inhibitor ET-18-OCH3 blocked inositol phosphate formation without inhibiting catecholamine secretion. This histamine-induced catecholamine secretion also does not involve Ca^{2+} mobilization, since IP_3-receptor inhibitors, such as 2-aminoethoxydiphenylborate (2-APB) or ryanodine plus caffeine, or thapsigargin-depletion of intracellular Ca^{2+} stores, had no effect.

PMT was used to discriminate between G_q-dependent and G_q-independent signaling induced by saccharin in isolated rod taste cells from frogs (Okada et al. 2001). Data had suggested a role for G-protein-mediated release of IP_3 rather than cAMP in the saccharin-induced cationic conductance. However, treatment with PMT did not induce a response in the frog taste cells, suggesting that it may be a G$\beta\gamma$-coupled PLCβ2 isoform rather than a Gα_q-coupled PLCβ1 or PLCβ3 isoform that is involved in saccharin taste transduction. Although this conclusion has since been substantiated by others (Imendra et al. 2002), a cautionary note might be warranted here, regarding interpreting negative results with the use of PMT as a reagent. The investigators had found that PMT did not elicit any current response in the cells (Okada et al. 2001); however, unlike the previous studies mentioned above, they had not verified as a control that PMT in their system was still able to evoke an IP_3 response.

PMT has been used to explore the pathways that mediate interaction between endogenous Gα_q and Rho signaling. In COS-7 cells, Gα_q coimmunoprecipitated with the Rho guanine nucleotide exchange factor (Lbc), and Gα_q and Lbc synergistically activated serum response element (SRE.L)-dependent gene expression in a PLC- and Rho-dependent

manner (Sagi et al. 2001). In this study, the ability of PMT to synergize with Lbc in stimulating SRE.L-mediated gene expression confirmed that $G\alpha_q$ at endogenous levels also interacts with Rho-GEF-regulated pathways. However, it is still not clear how $G\alpha_q$ activation leads to Rho activation since, unlike for $G_{12/13}$, expression of $G\alpha_q$ alone did not activate Rho, but did enhance Rho-dependent responses.

PMT has also been used to help define the signaling processes that control maturation of dendritic cells (DCs) (Bagley et al. 2004). In these studies, the investigators were interested in determining the role of PLC and Ca^{2+} signaling in activation of monocyte-derived DCs. To show the involvement of Ca^{2+} signaling in DC maturation, they used a number of different agonists, including lipopolysaccharide, CT, dibutyryl-cAMP, prostaglandin E2, and the Ca^{2+} ionophore A23187, all of which induced maturation. PMT was employed as a control to validate the involvement of PLC signaling in DC maturation. They further showed that this activation by PMT was inhibited by xestospongin, an inhibitor that blocks Ca^{2+} release from IP_3-gated intracellular stores.

PMT as a tool for studying circadian rhythms

PMT has recently been used as a tool to study cholinergic regulation of the circadian clock in the hypothalamic suprachiasmatic nucleus (SCN) of the rat brain. In a brain slice model, the SCN clock is subject to muscarinic regulation through the G_q-coupled M_1 mAChR, with sensitivity exhibited only during the night phase of the clock's 24-h cycle (Gillette et al. 2001). It was found that the effect of 1-h treatment with PMT on circadian clock resetting mimicked the advance of the clock phase induced by carbachol-stimulated M_1 mAChR signaling and IP_3-mediated Ca^{2+} release (L. Artinian, W. Yu, B.A. Wilson, E. Gratton, M.U. Gillette, unpublished data). Inhibitors of IP_3-mediated Ca^{2+} release, such as xestospongin, blocked this PMT-induced phase shift. Thus, PMT-mediated activation of the G_q-PLC-IP_3-induced Ca^{2+} release resets the clock in the same direction as activation of muscarinic receptors and cGMP signaling in early night.

PMT as a tool for studying endothelial permeability

PMT has been used to show that Rho activation and resulting cell retraction plays an important role in increased endothelial permeability (Essler et al. 1998). Disruption of endothelial integrity by PMT involves Rho-dependent activation of Rho kinase (ROKα), which in turn inactivates myosin light chain (MLC) phosphatase PP1 and thereby increases MLC phosphorylation and actin reorganization, followed by cell retraction and concomitant rise in endothelial permeability (Fig. 4). PMT-induced actin rearrangement could be blocked by microinjection of the Rho GTPase inhibitor C3 transferase from *C. botulinum* or microinjection of the Rho-binding domain (RBD) or pleckstrin homology (PH) domain of ROKα, which interfere with ROKα interaction with its regulators. These results have led to the speculation that PMT-mediated Rho activation is responsible for the observed vascular effects of PMT in bite wounds (Aepfelbacher and Essler 2001).

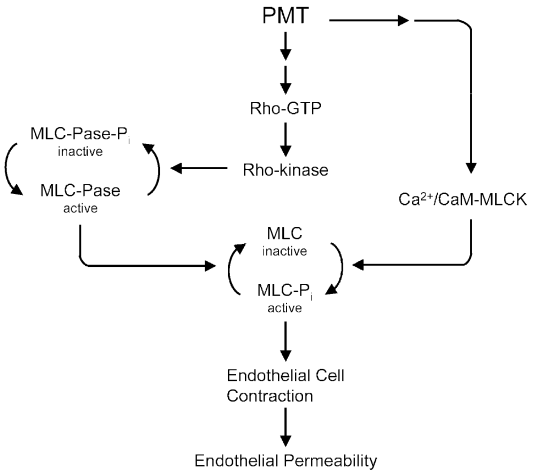

Fig. 4 A proposed model for PMT action on Rho-dependent vascular permeability. In this model, PMT stimulates the conversion of Rho protein into its active GTP-bound state. Activated Rho then stimulates Rho kinase to phosphorylate and thereby inactivate myosin light chain phosphatase (*MLC-Pase*), which in turn prevents the dephosphorylation of MLC, keeping it in its active phosphorylated state. PMT concomitantly causes the release of Ca^{2+} and activation of Ca^{2+}/calmodulin-dependent MLC kinase (*Ca^{2+}/CaM-MLCK*), which phosphorylates and activates MLC, resulting in endothelial cell contraction and consequent endothelial permeability. (Adapted from Essler et al. 1998.)

PMT as a tool for studying GIRK signaling

After the initial stimulatory response, PMT effectively uncouples G_q-PLC signaling and prevents any further activation through G_q (Wilson et al. 1997, 2000). Several investigators have demonstrated the effectiveness of using prolonged treatment with PMT to down-regulate G_q-mediated signaling. In heart tissue and in various neuronal and endocrine cells, cellular excitability is regulated by G-protein-coupled inward rectifying K^+ (GIRK) channels through selective hormonal stimulation (Sadja et al. 2003). As illustrated in Figure 5, GIRKs are activated in a PT-sensitive manner by receptors coupled to G-proteins of the $G_{o/i}$ family, such as the G_i-coupled A_1 adenosine receptor. This activation results from binding of the G_i-protein $\beta\gamma$ subunits to the channel. On the other hand, GIRK currents can be inhibited in a PT-insensitive manner either by $G_{i/o}$-coupled receptors, such as M_2/M_4 mAChR, or by G_q-coupled receptors, such as α_{1A}AR or M_1/M_3 mAChR. However, it was not clear whether the inhibition of GIRKs by these two different receptor types occurred through the same mechanism (Fig. 5).

To address this question, PMT was used to demonstrate the existence of two different regulatory pathways for PT-insensitive inhibition of GIRK channels, one involving PMT-insensitive $G_{i/o}$ and the other involving PMT-sensitive G_q (Fig. 5) (Buenemann et al. 2000). The researchers examined the effect of prolonged PMT pretreatment on GIRK inhibition in human embryonic kidney HEK293 cells transfected with GIRK1/4, A_1 adenosine receptors, and G_q-dependent or $G_{i/o}$-dependent inhibitory receptors. They found that pretreatment with PMT did not prevent the G$\beta\gamma$-mediated GIRK activation by stimulatory A_1 adenosine receptors. Likewise, PMT treatment did not affect the $G_{i/o}$-coupled M_2 mAChR-mediated inhibition of GIRK. In contrast, PMT completely blocked the inhibition of GIRK by the Gα_q-coupled α_{1A}AR receptor.

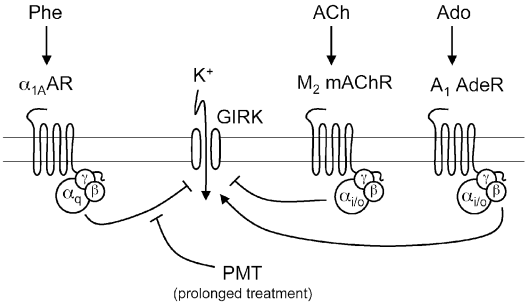

Fig. 5 A proposed model for PMT action on GIRK channel inhibition by $G_{i/o}$- and G_q-coupled receptors. In this model, opposing sets of G protein-coupled receptors modulate GIRK channels. Stimulation of $G_{i/o}$–coupled receptors, such as A_1 adenosine receptors, M_2 mAChR muscarinic acetylcholine receptors, or serotonin ($5\text{-}HT_{1A}$) receptors, causes the release of $G\beta\gamma$ subunits, which directly interact with and activate GIRK channels to increase K^+ currents. Rapid desensitization of the GIRK channel results from subsequent inhibition via the $G\alpha_{i/o}$ subunits. Inhibition of GIRK currents by the G_q-coupled receptors, such as α_{1A}-AR, ET_AR, M_1/M_3 mAChAR, or thyrotropin-releasing hormone (*TRH*) receptors, involves activation of PLCβ1 by the released $G\alpha_q$ to cause depletion of PIP_2 from the membrane, which results in inactivation of the GIRK channel. Prolonged treatment with PMT uncouples the G_q-signaling pathway and prevents inhibition of GIRK by G_q-coupled receptors, but not desensitization through $G\alpha_{i/o}$. *ACh*, acetylcholine; *Phe*, phenylephrine; *Ado*, adenosine.

PMT has also been used to mediate uncoupling of G_q signaling in cardiomyocytes to specifically block GIRK inhibition induced by phenylephrine and endothelin-1 (Meyer et al. 2001). In these studies, the investigators provided strong evidence for use of PMT as a superior tool to the commonly used aminosteroid PLC inhibitor U73122 for demonstrating the involvement of G_q-coupled PLCβ activity in mediating GIRK inhibition through PLCβ-mediated depletion of PIP_2. These conclusions were further substantiated by results in which PMT was used to examine GIRK channel regulation in HEK293 cells coexpressing GIRK1/4 with the $G_{i/o}$-coupled $5\text{-}HT_{1A}$ serotonin or G_q-coupled thyrotropin-releasing hormone (TRH) receptors (Lei et al. 2001). Both TRH and constitutively active $G\alpha_q$ inhibited GIRK. On the other hand, the inhibition of GIRK by TRH was shutdown by prolonged pretreatment with PMT, as well as by treatment with other known inhibitors of G_q signaling, RGS2 and PLCβ1-ct, which bind to $G\alpha_q$ and interfere with $G\alpha_q$-effector interaction, or by treatment with agents known to lower plasma membrane PIP_2 levels via cleavage of PIP_2 with 5'-phosphatidylinositol-phosphatase or via sequestration of PIP_2 with PLCδ-PH.

PMT as a tool for studying mitogenic signaling

Because PMT acts on G_q, PMT can now be used to study the role of G_q-mediated signaling in hormonal-stimulated mitogenesis. PMT stimulation of Erk signaling was shown to occur via G_q-dependent transactivation of the epidermal growth factor (EGF) receptor in a Ras-dependent manner in some cells, but via a PKC-dependent, Ras-independent pathway in other cells (Fig. 6). In one study (Seo et al. 2000), the mechanism of PMT-mediated Erk activation was compared to that of endogenous $G_{q/11}$-protein-coupled α-thrombin receptors in HEK-293 cells. Both PMT and the endogenous G_q-coupled receptors were

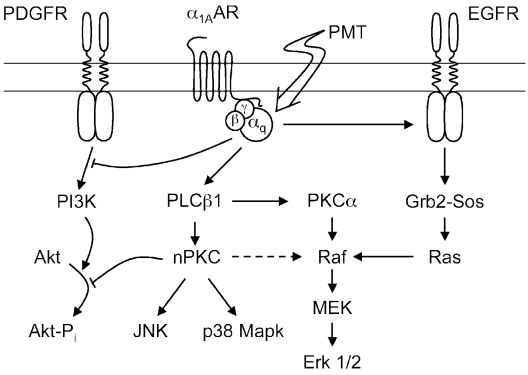

Fig. 6 A proposed model for PMT action on G_q-mediated mitogenic signaling. In this model, activation of G_q protein by either PMT or G_q-coupled receptors, such as α_{1A}-AR or α-thrombin receptor, results in activation of PLCβ1, as well as transactivation of the EGF receptor via Gα_q subunit and inhibition of the PDGF receptor. In HEK-293 cells and cardiac fibroblasts, subsequent activation of the Erk1/2 cascade is mediated predominantly via Gα_q transactivation of the Ras-dependent EGF receptor tyrosin kinase pathway, with no significant contribution derived from the PLCβ1-dependent activation of the PKC pathway. In cardiomyocytes, PMT stimulates via the Gα_q-PLCβ1 pathway both Ca^{2+}-dependent PKCα and novel nPKCs (*PKC*δ and *PKC*ϵ), which in turn lead to activation of the Erk1/2, as well as p38 MAPK and JNK cascades. PMT both inhibits PDGF receptor-dependent PI 3-kinase (*PI3K*) activation and prevents subsequent activation of the Akt-dependent survival pathway.

found to induce Ras-dependent Erk activation via a PKC-independent transactivation of the EGF receptor. For both PMT and the α-thrombin receptor, expression of two inhibitors of G_q signaling, a dominant-negative mutant of the G-protein-coupled receptor kinase (GRK2) and a C-terminal peptide of Gα_q (G$\alpha_{q305–359}$), blocked Erk activation. Erk activation by PMT was insensitive to a PKC inhibitor (GF109203X), but was blocked by an EGF receptor-specific inhibitor tyrphostin (AG1478), as well as by dominant-negative inhibitors of mSos1 and Ha-Ras. The results suggested that PMT-activated Gα_q transactivates the EGF receptor. In the other study involving cardiac fibroblasts, PMT was also found to stimulate Erk activation via EGF receptor transactivation (Sabri et al. 2002). However, in cardiomyocytes novel PKC isoforms mediated PMT activation of Erk1/2, as well as p38-MAPK, and JNK, and the EGF receptor appeared to have no role in this activation (Fig. 6).

Another example of PMT-mediated transactivation of tyrosine kinase signaling via G_q activation was reported for the generation of inositol phosphoglycans, second messengers of insulin signaling (Sleight et al. 2002). In rat liver membranes, PMT stimulated the production of inositol phosphoglycans, as measured by release of myo-inositol and chiro-inositol after acid hydrolysis, in a manner similar to what occurs upon insulin stimulation. Interaction between the G_q signaling pathway and the insulin receptor tyrosine kinase pathway was further supported by immunogold-labeling experiments showing colocalization of the insulin receptor β subunit (IRβ) and G$\alpha_{q/11}$ in partially purified rat liver membranes, enriched in PLCβ1, clathrin, and caveolin-1 (Sleight et al. 2002). Furthermore, direct interaction of G$\alpha_{q/11}$ with IRβ was demonstrated in another study through coimmunoprecipitation (Imamura et al. 1999).

PMT as a tool for studying apoptosis

In neonatal rat cardiomyocytes, PMT induced cardiac hypertrophy (i.e., cardiomyocyte enlargement, sarcomeric organization, and atrial natriuretic factor expression) in a manner similar to that which occurs upon norepinephrine stimulation (Sabri et al. 2002). PMT also activated Erk, and to a lesser extent p38 MAPK and JNK, via activation of PLC and novel PKC isoforms. PMT decreased basal Akt activation by preventing Akt phosphorylation through the activation by EGF or insulin-like growth factor-1 (IGF-1), and consequently enhanced cardiomyocyte susceptibility to apoptotic agents such as H_2O_2. PMT initially stimulates cardiac hypertrophy in a manner similar to moderate G_q stimulation, yet inhibits the Akt survival pathway and thereby enhances cardiomyocyte susceptibility to apoptosis in a manner similar to what occurs under intense, prolonged stimulation of G_q-coupled receptors (Adams et al. 1998). This suggests that hypertrophy and apoptosis may represent two phases of the same process leading eventually to cardiac decompensation and heart failure and further suggests that PMT might serve as an excellent tool to study this process.

A connection between G_q-coupled α_{1A}AR receptor activation and augmented UV-induced apoptosis through inhibition of phosphatidylinositol 3-kinase (PI3K) and Akt in response to platelet-derived growth factor (PDGF), as well as insulin and insulin-like growth factor 1 (IGF-1), has also come to light (Ballou et al. 2000, 2001). In Rat-1 fibroblasts, PI3K signaling induced by PDGF was also inhibited by treatment with PMT in a manner similar to α_{1A}AR (Fig. 6) (Lin et al. 2003). In this study, PMT pretreatment reduced the amount of phospho-Tyr751 on the PDGF receptor β subunit, thereby eliminating the docking site for the p85 subunit of PI3K. In addition, PMT pretreatment significantly inhibited the PDGF-induced Akt phosphorylation at Ser473.

Conclusions and future prospects

Discriminating among the various G-proteins involved in signal transduction processes has always been a challenge for cell biologists, physiologists, and pharmacologists. The discovery that certain toxins produced by bacteria can selectively act on different G-proteins has provided researchers with a growing repertoire of agents that can be used to manipulate signaling pathways for elucidating cellular functions of various signaling molecules. Until recently, studying the signaling of G_q GTPases was relatively intractable due to the lack of effective molecular tools that were specific for them; in fact, this family was referred to simply as the PT-insensitive G-proteins. While we do not yet know the precise biochemical basis for PMT action on G_q, there is considerable and convincing evidence that PMT can be used as a highly selective agent that targets G_q protein-coupled PLC signal transduction. Because initial exposure to PMT results in activation of G_q signaling, but prolonged treatment subsequently uncouples G_q-dependent PLC signaling, PMT can be used as both an activator and a downregulator of G_q-PLC signaling, depending upon the length of toxin treatment. PMT has already been shown to decipher a number of important signaling pathways involving G_q signaling. With the introduction of PMT as a G_q-selective molecular tool for studying PLC-mediated signaling, we can now begin to discern the different roles that G_q family members play in other signal transduction pathways and physiological processes. An important question that still remains to be answered is whether the other G_q family members, which are not coupled to PLC, might also be targets of

PMT. Indeed, there is strong evidence for other PMT effects that are independent of G_q-PLC signaling in G_q- and $G_{q/11}$-knockout cells.

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Cytolethal distending toxins

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Abstract The cytolethal distending toxins (CDTs) constitute the most recently discovered family of bacterial protein toxins. CDTs are unique among bacterial toxins as they have the ability to induce DNA double strand breaks (DSBs) in both proliferating and nonproliferating cells, thereby causing irreversible cell cycle arrest or death of the target cells. CDTs are encoded by three linked genes (*cdtA, cdtB* and *cdtC*) which have been identified among a variety of Gram-negative pathogenic bacteria. All three of these gene products are required to constitute the fully active holotoxin, and this is in agreement with the recently determined crystal structure of CDT. The CdtB component has functional homology with mammalian deoxyribonuclease I (DNase I). Mutation of the conserved sites necessary for this catalytic activity prevents the induction of DSBs as well as all subsequent intoxication responses of target cells. CDT is endocytosed via clathrin-coated pits and requires an intact Golgi complex to exert the cytotoxic activity. Several issues remain to be elucidated regarding CDT biology, such as the detailed function(s) of the CdtA and CdtC subunits, the identity of the cell surface receptor(s) for CDT, the final steps in the cellular internalization pathway, and a molecular understanding of how CDT interacts with DNA. Moreover, the role of CDTs in the pathogenesis of diseases still remains unclear.

A brief history of CDT

The first cytolethal distending toxin (CDT) was reported in 1987 as a novel type of toxin activity produced by pathogenic strains of *Escherichia coli*. The major hallmark of the observed cytotoxic effect was a remarkable cell distension, evident 3–5 days after addition of bacterial culture supernatants to cells growing in vitro, and resulting after a few more days in cell death (Johnson and Lior 1987a). The same authors later identified a similar activity in *Shigella* (Johnson and Lior 1987b) and *Campylobacter* spp (Johnson and Lior 1988a), and they designated this putative toxin as ‘cytolethal distending toxin’.

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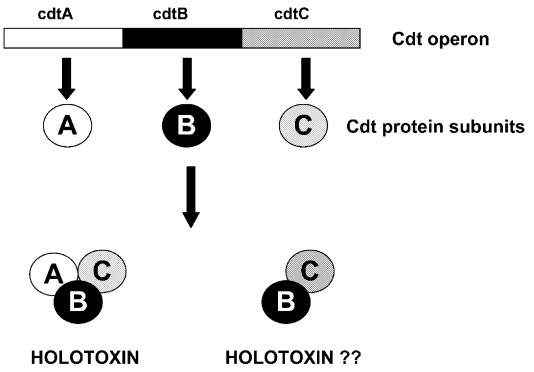

Fig. 1 Schematic representation of the three linked CDT genes and the respective protein subunits. Although the tripartite holotoxin (CdtA + CdtB + CdTC) has been found to be the most potent inducer of cellular intoxication, still some doubts exist whether this is the only possible subunit combination

CDTs from various *E. coli* strains were first cloned in 1994 by two American groups (Scott and Kaper 1994; Pickett et al. 1994) and in 1997 by French researchers (Peres et al. 1997). The CDTs turned out to be encoded by three linked genes, which were designated *cdtA*, *cdtB* and *cdtC*. Related CDT genes were identified in *Shigella spp* (Okuda et al. 1995), *Campylobacter jejuni* (Pickett et al. 1996), *Haemophilus ducreyi* (Cope et al. 1997), *Actinobacillus actinomycetemcomitans* (Mayer et al. 1999; Sugai et al. 1998), and several *Helicobacter* spp (Chien et al. 2000; Kostia et al. 2003; Taylor et al. 2003; Young et al. 2000a). The CDTs had no immediate resemblance to any other known proteins and thus, a new family of toxins rapidly emerged. So far no CDT has been identified from any Gram-positive bacterium. Since CDT variants are produced by many different bacterial species we previously proposed a CDT-nomenclature to avoid a confusing flora of different names in the future literature on CDTs (Cortes-Bratti et al. 2001a). A particular CDT is specified exactly by indicating the initials of the producing bacterium before CDT and, if necessary, the strain number or other common designation after CDT (e.g., HdCDT: *H. ducreyi* CDT).

The *cdtA, cdtB* and *cdtC* genes were found to encode proteins (CdtA, CdtB, CdtC) (Fig. 1) with predicted molecular masses of 23, 29 and 21 kDa and isoelectric points of 5.7, 8.3, 6.3, respectively, for the HdCDT. The purified HdCDT subunits show a normal migration pattern when subjected to SDS-PAGE. The CDT subunits were purified from some of the producing bacteria during the late 1990s and more recently in recombinant forms. The HdCDT was shown to enter sensitive target cells by receptor-mediated endocytosis (Cortes-Bratti et al. 2000) – a process likely to occur also with the other CDTs. In 2000 a deoxyribonuclease I (DNase I)-like enzymatic activity was identified in the CdtB component of CDTs from *E. coli* and *C. jejuni*, respectively (Elwell and Dreyfus 2000; Lara-Tejero and Galan 2000). Mutation of the catalytic residues abolished all cytotoxic effects. The proposed DNA damaging effect was consistent with our parallel observations on the cellular responses to HdCDT. They were identical to the cell cycle check point responses evoked by ionizing radiation (IR), a well known DNA damaging agent (Cortes-Bratti et al. 2001b). Finally, the HdCDT was reported in 2003 to induce DNA double strand breaks (DSBs) upon natural intoxication of mammalian cells (Frisan et al. 2003), thus giving direct proof that CDT attacks DNA. The crystal structure of the HdCDT holotoxin has been recently solved, and it reveals that indeed this toxin consists of an enzyme belonging to the DNase I family (CdtB), bound to two ricin-like lectin domains (CdtA and CdtC). HdCdtB shows the characteristic fold of the DNase I family: a central 12-stranded

β-sandwich packed between outer α-helices and loops on each side of the sandwich (Nesic et al. 2004).

This review deals mainly with the action of CDTs on mammalian cells. The possible roles of these toxins in pathogenesis, and their potential uses in the biomedical field will also be briefly discussed.

Actions of CDTs on mammalian cells

Morphological effect of CDT

The most conspicuous morphological effect of CDT on cells growing as an adherent monolayer is the cell distension leading within 72 h to a three- to fivefold increase of the cell size. Along with this slowly developing distension, the actin cytoskeleton is strongly promoted noted mainly as the appearance of prominent actin stress fibers (Fig. 2). This has been reported for *E. coli* CDT (EcCDT-II) in Chinese hamster ovary (CHO) cells (Aragon et al. 1997), for HdCDT in HEp-2/HeLa cells, in Don hamster lung fibroblasts and human foreskin fibroblasts (Cortes-Bratti et al. 1999; Frisan et al. 2003), and recently also in HeLa cells treated with CDT from *Helicobacter cinaedi* (Taylor et al. 2003). After several days of toxin exposure cells round up, show membrane blebbing in some cases, and then deteriorate completely. In contrast to fibroblasts and epithelial cells, T and B lymphocytes and dendritic cells do not distend but rather become rapidly apoptotic and fragmented after exposure to CDT (Cortes-Bratti et al. 2001b; Li et al. 2002; Shenker et al. 2004).

CDTs act on several cell types

As already evident from the morphological effect, CDTs affect various cell lines with differing outcomes depending on the cell type. Sensitive cells include HEp-2, HeLa, CHO, Vero and Don fibroblasts (reviewed in (Cortes-Bratti et al. 2001a; Pickett and Whitehouse 1999) as well as human embryonic intestinal epithelial cells (INT407) (Hickey et al. 2000), a human colon carcinoma cell line (Caco-2) and a human keratinocyte cell line (HaCat) (Cortes-Bratti et al. 1999). Human $CD4^+$ and $CD8^+$ T cells are highly sensitive to the *A. actinomycetemcomitans* CDT (AaCDT) (Shenker et al. 1999), which also inhibits proliferation of the murine B-cell hybridoma cell line HS-72 (Sato et al. 2002) as well as human periodontal ligament cells and gingival fibroblasts (Belibasakis et al. 2002). HdCDT affects normal human endothelial cells (HUVEC) (Svensson et al. 2002), foreskin keratinocytes and fibroblasts, embryonic lung fibroblasts, human B cells and dendritic cells (Figs. 3, 5) (Cortes-Bratti et al. 2001b; Li et al. 2002). Primary human fetal fibroblasts (IMR-90) (Hassane et al. 2003), Cos-7 cells (McSweeney and Dreyfus 2004), Jurkat and MOLT-4 cells (Ohara et al. 2004) are also sensitive to CDT intoxication.

In conclusion, most tested cells are sensitive to CDT. The only cells so far reported to resist CDTs are the Y-1 adrenal cells and 3T3 fibroblasts of mouse origin (Cope et al. 1997; Cortes-Bratti et al. 1999; Johnson and Lior 1988b). The reason for their resistance is not known but these cells might lack a cell surface receptor needed for binding of the toxin.

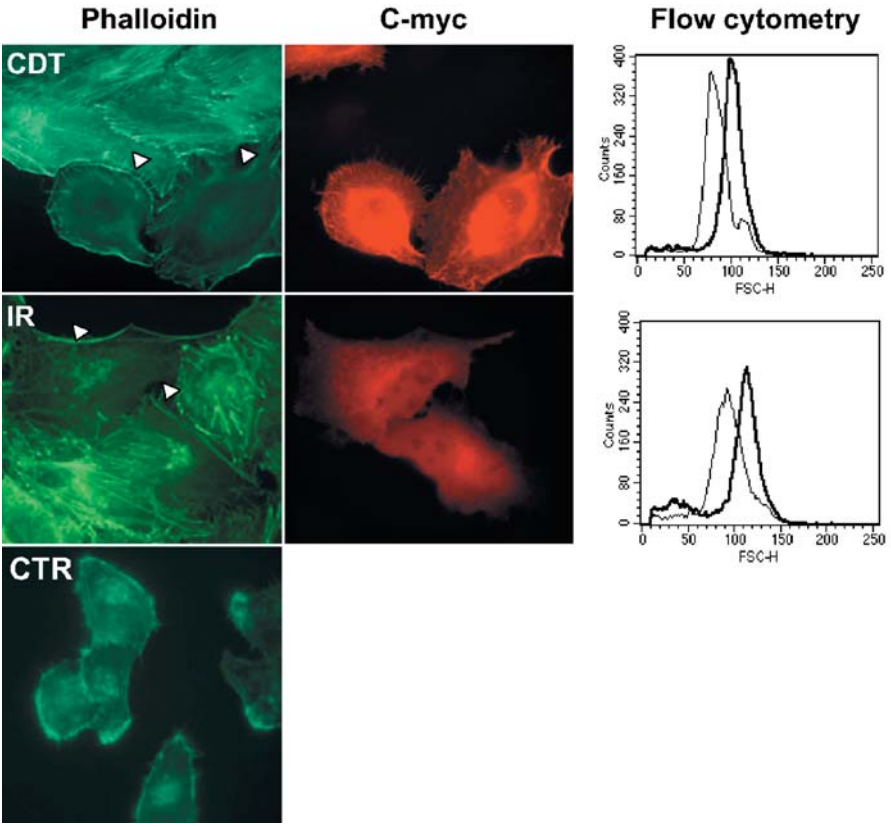

Fig. 2 CDT intoxication induces cell distension and promotion of actin stress fibers. HeLa cells were transfected with dominant negative RhoAN19, and 24 h after transfection cells were either left untreated (CTR) or exposed to HdCDT (2μg/ml) or irradiated (20 Gy) and incubated for additional 24 h in complete medium. Transfected cells were visualized by immunofluorescence using anti-Myc 9E10 antibody and F-actin was stained with FITC-phalloidin. *Arrows* indicate RhoAN19 transfected cells. The cell size of untreated (*thin line*) or treated (*thick line*) cells was analyzed by flow cytometry (FSC-H: forward scatter)

CdtB is a DNase

The CdtBs from several bacteria were shown to be able to cleave DNA in vitro. Indeed, all CdtBs on a close analysis turned out to have structural and functional homology to mammalian DNase I. The first paper describing position-specific homology between the CdtB subunit from EcCDT-II and mammalian DNase I was published by Elwell and Dreyfus (Elwell and Dreyfus 2000). The homology pattern was found at specific residues involved in enzyme catalysis (Glu86, His154, Asp229, His261), DNA binding (Arg123, Asn194) and metal ion binding (Glu62, Asp192, Asp260). EcCdtB-II also contained a pentapeptide sequence (aa 259–263: Ser-Asp-His-Tyr-Pro) found in all DNase I enzymes. Shortly after, similar conserved residues were described for the *C. jejuni* CdtB (CjCdtB) and in fact identified in all known CdtBs (Lara-Tejero and Galan 2000).

The position-specific homology is associated with functional activity, since crude Ec-CDT-II preparations have DNase activity as detected by in vitro digestion of the coiled

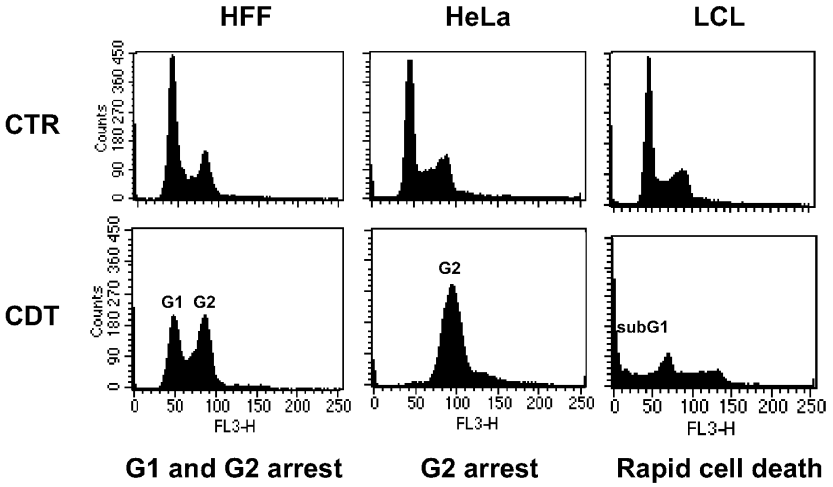

Fig. 3 Cell type-dependent effect of CDT. Human foreskin fibroblasts (*HFF*), HeLa cells and an EBV transformed lymphoblastoid cell line (*LCL*) show different patterns of cycle arrest or cell death upon CDT treatment. Cells were treated with HdCDT (2μg/ml) and cell cycle distribution was assessed by DNA staining with propidium iodide and flow cytometry analysis 24 h after treatment. The G_1 peak was arbitrarily set on the mean fluorescence intensity value of 50

pGEM-7zf plasmid (Elwell and Dreyfus 2000), and transfection of HeLa cells with CjCdtB was shown to induce a slowly appearing nuclear fragmentation and a marked chromatin disruption (Lara-Tejero and Galan 2000). Microinjected CjCdtB was also able to produce changes in the chromatin and enlargement of the nucleus. The DNase activity, as well as the cytotoxicity, was abolished by point mutations of conserved residues required for catalysis or for magnesium binding. Thus, the catalytic DNase activity appeared to be crucial for the cytotoxic activity of CDTs (Elwell and Dreyfus 2000; Lara-Tejero and Galan 2000).

CDT induces DNA double-strand breaks

We recently provided direct evidence that intoxication of HeLa cells with the HdCDT holotoxin induces DNA DSBs similarly to IR (Fig. 4) (Frisan et al. 2003). This first demonstration of CDT-induced DSBs in naturally intoxicated mammalian cells was made with a sensitive pulsed field gel electrophoresis (PFGE) method. It contradicts an earlier report showing that EcCDT did not induce DNA strand breaks, as measured with the alkaline single cell gel electrophoresis (Comet) assay (Sert et al. 1999). However, our findings agree with those of Hassane and co-workers who previously observed that yeast cells transfected with CjCdtB exhibited DSBs, also detectable by PFGE (Hassane et al. 2001). Furthermore, this effect on DNA is fully consistent with the DNase activity of CdtB and the widely reported observations that CDT-treated cells exhibit cell cycle arrest and/or undergo apoptosis (see below).

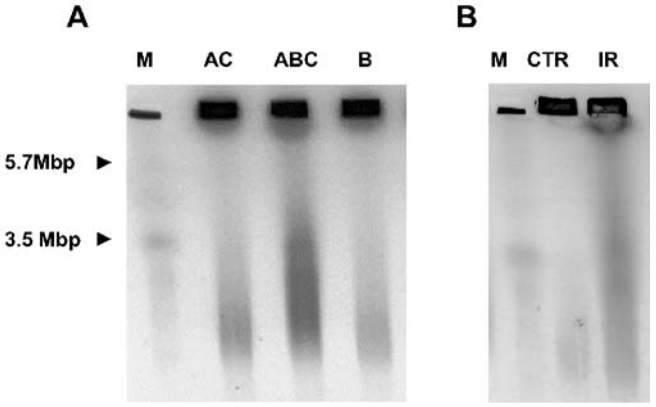

Fig. 4A, B CDT intoxication induces DNA DSBs. Twenty-five thousand HeLa cells per well were grown in 12-well plates in complete medium containing 4000 Bq [methyl-^{14}C]thymidine for 48 h. The cells were then washed three times in phosphate buffered saline and chased for 2 h in complete medium. **A** Cells were treated with (*AC*) CdtAC bacterial supernatant; (*ABC*) CdtAC bacterial supernatant incubated with purified *H. ducreyi* CdtB (20μg/ml); (*B*) purified *H. ducreyi* CdtB alone (20μg/ml) for 7 h, and processed for pulsed-field gel electrophoresis analysis. **B** HeLa cells were left untreated or irradiated (20 Gy, *IR*) and immediately processed for analysis by pulsed-field gel electrophoresis. *M*, molecular weight marker

Cellular responses to DNA damaging agents

It is well known that cells exposed to agents causing DNA damage activate checkpoint responses that arrest the cell cycle until the DNA damage has been repaired. These checkpoint responses can block cells in the G_1, S or G_2 phases of the cell cycle (Elledge 1996; Hartwell and Weinert 1989). The protein kinase 'Ataxia telangiectasia mutated' (ATM) and its homolog 'ATM and Rad3 related' (ATR) play a central role in sensing DNA damage. ATM is activated in response to DNA DSBs induced by IR, and can in turn trigger all the different checkpoints. ATR activation is mainly induced by other DNA damaging agents, such as UV irradiation (reviewed in Rotman and Shiloh 1999). Arrest in G_1 is mediated by the tumor suppressor gene p53, which is stabilized in an ATM dependent manner via phosphorylation on serine 20 by the chk2 protein kinase (Chehab et al. 2000; Siciliano et al. 1997). The G_2 arrest depends on inactivation of the cdc2 complex. Activation of cdc2 is achieved via dephosphorylation at Thr14 and Tyr15 by the Cdc25C phosphatase (Jackman and Pines 1997). The ATM-dependent protein kinases chk1 and chk2, activated in vivo in response to DNA damage, are both able to inactivate Cdc25C in vitro (Matsuoka et al. 1998; Sanchez et al. 1997), leading to accumulation of the inactive phosphorylated cdc2 and arrest of cells in the G_2 phase of the cell cycle.

Cellular checkpoint responses to CDTs

Cell cycle arrest

Already before the toxin was known to damage DNA, cells exposed to various CDTs had been reported to accumulate the phosphorylated (inactive) form of cdc2, implying G_2/M arrest (Comayras et al. 1997; Cortes-Bratti et al. 1999; Peres et al. 1997; Whitehouse et al.

1998). The inactive cdc2/cyclin B complex resulting after intoxication with EcCDT-III could be reactivated in vitro with recombinant Cdc25C (Sert et al. 1999). Furthermore, overexpression of Cdc25B or Cdc25C could override the G_2 arrest induced by this toxin, causing progression to a mitosis which, however, was abnormal (Escalas et al. 2000). Both results suggested that CDTs do not target specifically cdc2 but rather some upstream component, leading to inactivation of Cdc25C and consequent lack of cdc2 dephosphorylation as a secondary effect.

The cellular checkpoint responses induced by HdCDT were then shown to closely resemble those induced by IR. It became clear that the CDT-induced cell cycle arrest is not limited to the G_2/M phase, but that the checkpoint responses depend on the cell type (Cortes-Bratti et al. 2001b). A functional ATM protein was needed for rapid intoxication as demonstrated by the delayed responses in ATM deficient lymphoblastoid cell lines. In human fibroblasts, both IR and HdCDT treatment induced early activation of the p53 gene and the cyclin-dependent kinase inhibitor p21, which correlated with arrest in G_1 (Fig. 3). In epithelial cells both treatments induced chk2 kinase activation, accumulation of phosphorylated cdc2, and G_2 arrest. The checkpoint responses were detected already 4 h post-intoxication and therefore occurred much earlier than the morphological changes of intoxicated cells. Lymphoblastoid cell lines did not arrest in either G_1 or G_2 at the concentrations of HdCDT used, but rapidly underwent apoptosis (Cortes-Bratti et al. 2001b) (Fig. 3).

CDT-induced apoptosis

This aspect has been studied in some detail only in human T cells and T cell lines. Treatment of activated human T cells with the AaCDT (purified B subunit or recombinant holotoxin) was found to induce DNA fragmentation in 72–96 h, and FACS analysis showed reduction in cell size and increased nuclear condensation (Shenker et al. 2001). Mitochondrial changes were evident as a decrease in transmembrane potential and an elevation of reactive oxygen species, and the caspases 8, 9 and 3 were activated after the G_2 arrest. Overexpression of Bcl-2 decreased the CDT-induced apoptosis, but did not inhibit the CDT-induced G_2 arrest (Shenker et al. 2001). A more recent report on AaCDT-induced death of human peripheral T lymphocytes and the Jurkat and MOLT-4 cell lines, however, suggested that AaCDT has the ability to induce human T-cell apoptosis through activation of caspase-2 and caspase-7 (Ohara et al. 2004). In conclusion, the exact molecular responses on the way to apoptotic cell death have not been fully elucidated and can be expected to depend on the particular type of target cell.

CDTs activate sensors of DNA damage

We demonstrated that HdCDT induces ATM-dependent phosphorylation of the nuclear sensor of DNA damage, histone H2AX, as early as 1 h after intoxication. Moreover, the DNA repair complex Mre11 in the nuclei of HeLa cells was relocalized with kinetics similar to those observed upon IR (Li et al. 2002). Also the CjCDT was reported to induce both G_1 and G_2 arrest in primary human fetal fibroblasts (IMR-90 cells), and Rad50 foci were formed in the nuclei of the CjCDT-treated fibroblasts (Hassane et al. 2003). Thus, DNA damage-associated molecules are activated as an early response to the DNA DSBs induced by CDTs.

CDTs act on nonproliferating cells

It is noteworthy that CDTs also attack nonproliferating cells. The focus formation assays mentioned above enabled the discovery that human dendritic cells (DCs) (Li et al. 2002) as well as serum starved primary human fetal fibroblasts (Hassane et al. 2003) are sensitive to CDT. This contradicts previous notions that cells need to pass through the S phase in order to become intoxicated (Alby et al. 2001; Lara-Tejero and Galan 2000; Sert et al. 1999). The cell cycle analysis of HdCDT-treated human DCs shows that all control cells are in G_0 whereas the toxin-treated cells after 24 h either remain in G_0 or become apoptotic The short-term effect after 4 h toxin treatment of DCs is seen as a phosphorylation, i.e., activation, of H2AX (Fig. 5) (Li et al. 2002).

CDT-induced stress fiber promotion depends on ATM-mediated activation of RhoA

The reason that actin stress fibers are strongly promoted in certain CDT-treated cells was not understood in the early experiments (Aragon et al. 1997) although the possible involvement of small GTPases controlling the actin cytoskeleton was hypothesized (Cortes-Bratti et al. 1999). Later on it became feasible to investigate this aspect of CDT intoxication with new tools that allow biochemical assay of the activated small GTPases Rho, Rac and CDC42 (Benard et al. 1999; Ren et al. 1999). Thus, we could show that the HdCDT-activated stress fiber promotion in fibroblasts and HeLa cells depends on activation of RhoA, but not Rac or CDC42 (Frisan et al. 2003). This was observed both directly as activation of RhoA upon intoxication, and as an inhibition of the stress fiber formation in CDT-treated cells transfected with a dominant negative mutant of RhoA (see Fig. 2).

A cell distension and stress fiber promotion was observed also in irradiated cells. Interestingly, the increased size of CDT-treated or irradiated cells was not connected to the stress fiber formation, since dominant negative RhoA inhibited the formation of stress fibers without affecting the distension of the treated cells (Fig. 2). The distension phenomenon instead appears to depend on activation of PI3-kinase and its downstream effector mTOR, whereas this enzyme had no clear effect on induction of stress fibers (Frisan et al. 2003). The activation of RhoA upon intoxication or irradiation was ATM-dependent, thereby connecting the stress fiber promotion to the damaging action on DNA. Toxin-treated cells expressing a dominant negative form of RhoA detached and consequently died faster than cells expressing a functional RhoA (Frisan et al. 2003). Thus, the activation of RhoA was associated with prolonged cell survival, and it seems to represent an attempt of intoxicated cells to maintain cell adherence in order to stay alive while the DNA is supposed to be repaired. This ATM-dependent activation of RhoA constitutes a previously unknown type of cell survival response to induction of DNA DSBs, which seems to occur regardless of the agent inducing the DSBs.

Fig. 6 summarizes the intracellular responses activated by CDT intoxication.

Cellular internalization and nuclear localization of CdtB is required for cytotoxicity

Since CDT acts on DNA it is conceivable that this toxin, like most other intracellularly acting toxins, has to be internalized in target cells before it can act. Indeed, the HdCDT has been reported to enter HEp-2/HeLa cells by endocytosis via clathrin-coated pits

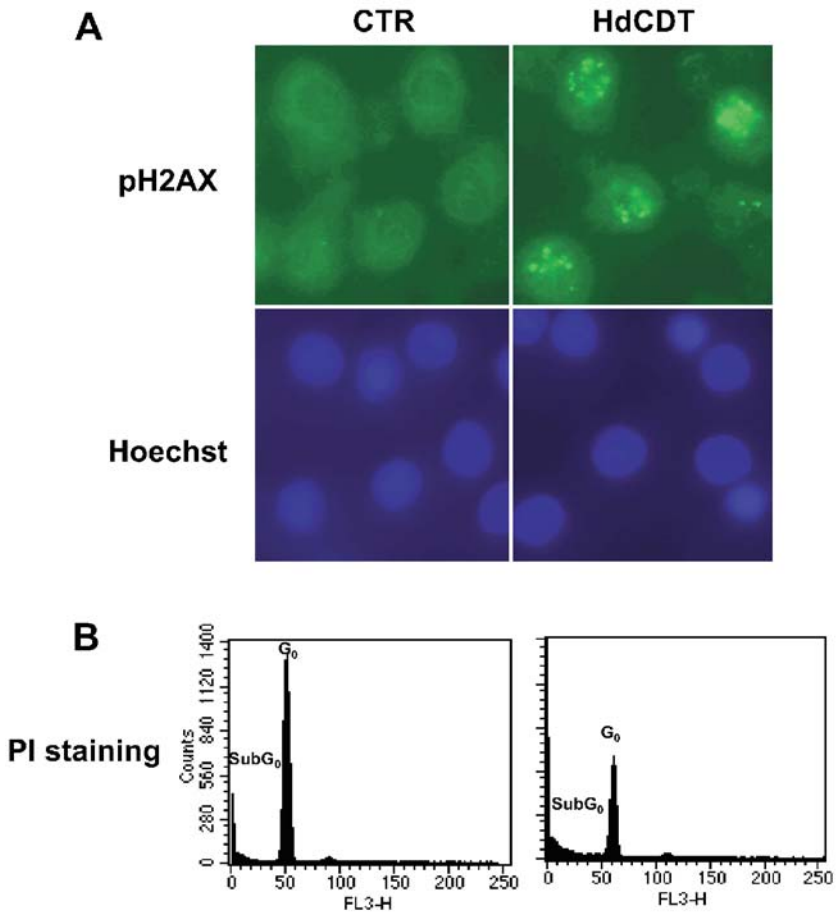

Fig. 5A, B CDT induces DNA damage and cell death in nonproliferating dendritic cells. **A** Five-day immature DCs were incubated with HdCDT (2μg/ml) for 5 h and stained with histone H2AX phospho-specific rabbit serum (*pH2AX*). Nuclei were counter-stained with Hoechst 33258. **B** Cell cycle distribution was assessed by DNA staining with propidium iodide (*PI*) and flow cytometry. The G_0 peak was arbitrarily set on the mean fluorescence intensity value of 50

(Cortes-Bratti et al. 2000). The cellular intoxication was completely inhibited at conditions that block the fusion of early endosomes with downstream compartments or after treatment of cells with agents that disrupt the Golgi complex. Apparently the toxin, after uptake via clathrin-coated pits, requires transport in vesicles at least to the Golgi complex before its activity can be expressed. From the Golgi it may be retrogradely transported to the endoplasmic reticulum (ER) and from there delivered either to the cytosol or directly to the nucleus (Cortes-Bratti et al. 2000).

CdtB contains no known conventional nuclear localization signal (NLS). However, microinjection experiments in HeLa cells showed that a 76-amino acid stretch (residues 48–124) in the AaCdtB constitutes an atypical NLS (Nishikubo et al. 2003). After microinjection, His-tagged CdtB-GFP entered the nucleus in 3–4 h. A lack of effect of leptomycin B on the speed of nuclear entry suggested that the relatively slow entry of the fusion protein

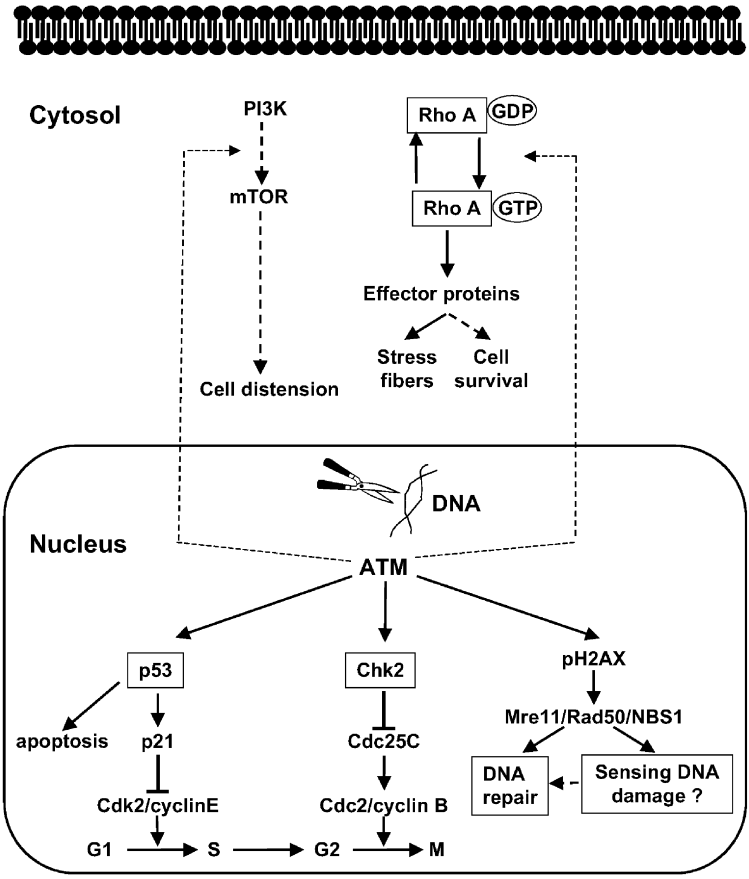

Fig. 6 Summary of the cellular responses activated by the CDT-induced DNA damage. *Dotted arrows* indicate pathways discovered using CDT as a tool for which intermediate effectors have not yet been completely elucidated

was not due to CRM1-dependent nuclear export of the protein. An in vitro transport assay demonstrated that the nuclear localization of CdtB was mediated via active transport requiring ATP and physiological temperature. Moreover, cells treated extracellularly with a holotoxin containing mutant CdtB, with an 11-amino acid truncation in the identified NLS, were unaffected. This observation suggested that the identified NLS may be functional for nuclear localization of the toxin also when mammalian cells are naturally intoxicated with the extracellularly added holotoxin (Nishikubo et al. 2003). Subsequently, two NLS sequences, designated NLS1 and NLS2, have been identified in the carboxy-terminal region of EcCdtB-II. Cell cycle arrest and nuclear localization were impaired in cells treated with CDT containing EcCdtB-II-ΔNLS mutants, while the in vitro DNase activity and the cell surface binding of the mutant holotoxins were not affected. Interestingly, fluorescence microscopy analysis showed a diffuse cytoplasmic distribution of EcCdtB-II-ΔNLS2 in cells intoxicated with the CDT holotoxin containing the EcCdtB-II-ΔNLS2 mu-

tant, while the EcCdtB-II-ΔNLS1 subunit localized preferentially in a perinuclear region of the intoxicated cells (McSweeney and Dreyfus 2004).

The studies cited above demonstrate beyond any doubt that at least the active CdtB subunit has to be endocytosed and undergo intracellular transport to the nucleus before it can damage cells. However, it is still not known whether in natural intoxication the nuclear entry of CdtB takes place from the cytosol or directly from the Golgi/ER. Nothing has yet been reported regarding the cellular localizations and fates of the other two toxin subunits.

Functions of the different CDT subunits

Early genetic studies demonstrated that all three genes need to be expressed in the producing bacterium for the generation of active (cytotoxic) CDT (Cope et al. 1997; Pickett et al. 1994, 1996; Sugai et al. 1998; Young et al. 2000a). However, some confusion was created by the difficulty to obtain highly purified subunits from bacteria, which possess the entire *cdt* operon and thus produce all three subunits. Although studies today are usually performed with recombinant subunits of CDT some discrepancies still remain as we shall see below. However, clarifying the roles of the three different subunits in cellular intoxication and other responses to CDT constitutes one of the most important frontiers in current CDT research.

CdtB. As stated already CdtB is definitely the most important active subunit showing DNase activity in vitro and in vivo. CdtB of various origins is cytotoxic after microinjection (Lara-Tejero and Galan 2000) or electroporation/facilitated entry into cells (Elwell et al. 2001; Mao and DiRienzo 2002). In contrast, CdtB added extracellularly alone is not able to bind to most target cells and consequently it is not cytotoxic by itself. T cells may be an exception as initial studies on the AaCDT suggested that its CdtB subunit alone was sufficient to cause G_2 arrest in phytohemagglutinin-activated human T cells (Shenker et al. 1999, 2000). However, the same authors demonstrated more recently that although their AaCdtB alone could bind to the Jurkat cell surface and was sufficient to induce G_2 arrest in human lymphocytes, the presence of both the CdtA and CdtC subunits was required to achieve maximum cell cycle arrest (Shenker et al. 2004). Indeed, a holotoxin comprised of CdtABC was >50,000-fold more toxic to Jurkat cells than toxins composed of either CdtAB or CdtBC. Today there is general consensus that all three subunits must be added together for extracellularly induced maximal intoxication (Lara-Tejero and Galan 2001; Lee et al. 2003).

CdtA. Shenker and coworkers (Shenker et al. 2004) report that immunoprecipitation of the CDT holotoxin from *A. actinomycetemcomitans* extracts, with a monoclonal antibody against CdtC, co-precipitated CdtA and CdtB. Interestingly, only a truncated form of CdtA (18 kDa) was immunoprecipitated with this holotoxin, suggesting that CdtA may undergo processing during the assembly of the holotoxin. The expression of two immunoreactive CdtA proteins corresponding to 25 kDa and 18 kDa has been observed also for the HdCDT (Frisk et al. 2001). CdtA seems to be able to bind to target cells, but alone it lacks cytotoxic activity (Lee et al. 2003; Mao and DiRienzo 2002). Also, the combination of only CdtA and CdtB was without cytotoxic effect on HeLa cells (Lara-Tejero and

Galan 2001; Lee et al. 2003). It is unclear whether the CdtA in these cases was in the truncated form or not.

CdtC. Besides the apparent maturation of CdtA in the presence of the two other subunits, also CdtC has been reported to be somehow processed in the holotoxin environment. The isoelectric point (pI) of the HdCdtC component was 1.5 pH units higher in recombinant strains expressing all three components than in recombinant strains expressing the CdtC protein alone (Frisk et al. 2001). A similar change of pI occurred after mixing the three individual recombinant components in vitro. Thus, it was suggested that HdCdtA/B may exert some kind of processing activity on HdCdtC, rendering it active (Deng et al. 2001; Frisk et al. 2001). Recently, however, the purified recombinant His-tagged AaCdtC alone, delivered to the cytosol with a lipid protein carrier, was found able to induce cell distension and eventually the death of CHO cells (Mao and DiRienzo 2002). The specific mechanism of this putative CdtC cytotoxicity has not been elucidated.

Cytotoxicity of combined CdtB and CdtC

Mao and DiRienzo (Mao and DiRienzo 2002) also found an additive cytotoxic effect exerted by the combination of AaCdtB and AaCdtC when delivered into the cytosol together. We previously observed cytotoxicity with an extracellularly added highly purified preparation of HdCDT in which we were not able to detect CdtA by Western blot analysis (Li et al. 2002). This is consistent with the observation that extracts from *H. ducreyi* producing CDT in which the *cdtA* gene was mutated still had some cytotoxic activity (Lewis et al. 2001). Also other workers found that the combination of purified HdCdtB with HdCdtC was slightly cytotoxic (Wising et al. 2002). Likewise, a mixture of recombinant AaCdtB and AaCdtC could induce G_2 arrest in HEp-2 cells, as measured by FACS analysis after a 72 h toxin exposure (Akifusa et al. 2001). Similarly, the extracellularly added combined recombinant subunits CjCdtB and CjCdtC were able to induce G2 arrest in HeLa cells (Lee et al. 2003). On a microgram basis, however, this combination was only about 25% as effective as the tripartite CjCDT holotoxin. It would be of interest to determine the toxin amount produced by the bacteria in vivo, in order to understand whether the CdtB/C combination could have any physiological relevance.

Cell surface binding of CDT subunits

Different CDTs have a documented ability to intoxicate cells even after exposure times as short as 2 to 15 min (Aragon et al. 1997; Cortes-Bratti et al. 1999) and HdCDT could be absorbed out from the medium by repeatedly passing the same toxin solution over fresh cultures of HEp-2 cells (Frisk et al. 2001). Such functional studies indirectly suggested that CDTs bind to cells rapidly and irreversibly, although no specificity of the binding could be demonstrated. It was earlier pointed out that a region in the CjCdtA, encompassing the amino acids 160 through 220, exhibits a lectin fold which is also present in the binding subunits of the plant toxins ricin and abrin (Lara-Tejero and Galan 2001). The recently determined crystal structure of HdCDT reinforced this observation and underscored the presence of a ricin-like lectin domain also in HdCdtC. However, no specific receptor for any CDT subunit has so far been identified.

When recombinant HdCdtA and HdCdtC were produced from two plasmids and expressed in the same *E. coli*, a noncovalent CdtA–CdtC complex was formed in the absence of CdtB (Deng and Hansen 2003). This CdtA–CdtC complex after association with HeLa cells (30 min, 4°C) was able to bind subsequently added CdtB, leading to cell killing within 72 h. Moreover, HeLa cells pretreated for 30 min at 37°C with the CdtA–CdtC complex became resistant to the cell killing induced by high concentrations of subsequently added HdCDT holotoxin (*H. ducreyi* culture supernatant). However, such treated cells were not resistant to the cell distension induced by lower amounts of the holotoxin (Deng and Hansen 2003), suggesting that cellular intoxication had indeed occurred. Thus, a more detailed analysis of the molecular markers of CDT intoxication should be performed.

Another recent piece of work considerably advanced our knowledge about binding of the CjCDT subunits to the HeLa cell surface (Lee et al. 2003). By competition experiments Lee and coworkers demonstrated that both the CdtA and CdtC subunits, but not CdtB, could bind with specificity to the HeLa cell surface. In unlabeled form these subunits were able to compete out binding of the corresponding biotinylated subunit. Interestingly, they could also compete with each other, suggesting that CdtA and CdtC were binding to the same receptor on the cell surface (Lee et al. 2003). Indeed, these two subunits of the CjCDT share about 40% sequence similarity with each other. McSweeney and Dreyfus recently noted the same binding competition between the CdtA and CdtC subunits of the EcCDT-II (McSweeney and Dreyfus 2004).

Lee and coworkers (Lee et al. 2003), in addition, produced in-frame deletion mutants of CdtA and CdtC from which 43 and 22 amino acids, respectively, were deleted. As the highest homologies within the CdtA and CdtC subunits from different bacteria are found in distinct regions, the deletions were made in one such region in CjCdtA (amino acids 126–168 deleted) and in the region of the highest homology within the CjCdtC subunit (amino acids 115–136 deleted). Both of these mutant subunits in biotinylated form were still able to bind HeLa cells as detected by an enzyme-linked immunosorbent assay on living cells. Moreover, each mutant subunit was able to compete out the binding of both the corresponding and the other wild-type subunit, as well as that of the holotoxin. The binding of the CDT holotoxin decreased nearly fivefold in the presence of the CdtA mutant subunit and almost threefold in the presence of the CdtC mutant subunit. These interesting findings then suggest that the deleted regions in each Cdt subunit do not play any significant role in cell surface recognition. On the other hand, the mutant subunits were unable to interact with CdtB and/or each other to reconstitute an active holotoxin, indicating that the deleted regions were instead critical for effective holotoxin formation (Lee et al. 2003).

In conclusion

The most reasonable interpretation of the combined current data on the CDT subunits is that CDT functions as an AB-toxin where the DNase CdtB constitutes the active subunit, and a CdtAC heterodimer constitutes the optimal binding subunit. This tripartite holotoxin is the subunit combination with maximal activity in all tested cell systems. However, the CdtC subunit alone can also function as a binding subunit, facilitating the cellular entry of CdtB, albeit with a much lower efficiency than the heterodimeric binding unit. That the AaCdtB alone can apparently act on Jurkat cells (Shenker et al. 2000, 2004) may have to

do with specific properties of T cells, which in contrast to epithelial cells or fibroblasts appear able to bind and take up CdtB.

Possible involvement of CDTs in bacterial infections

As CDTs are potent toxins produced by pathogenic bacteria they have to be regarded as potential virulence factors. CDTs are detected in an increasing number of clinical isolates but only few experiments with CDTs in animal model systems have been reported.

Occurrence of CDTs in clinical isolates from various geographic locations

Several studies on *E. coli* isolates have been performed, especially concerning the possible association of CDT with enteropathogenic (EPEC) serotypes. The general conclusion from these studies is that EcCDT is produced in some, but not all, EPEC serotypes (Ansaruzzaman et al. 2000; Ghilardi et al. 2001; Janka et al. 2003; Okeke et al. 2000; Pandey et al. 2003). For example, only 1 in 200 children in Brazil (0.5%) with acute diarrhea were infected with *cdt*-positive EPEC, while 1% of the nondiarrheic controls harbored *cdt*-positive isolates (Marques et al. 2003). CDT has also been identified in urosepsis *E. coli* isolates (Johnson and Stell 2000), in diarrhea isolates from dogs (Starcic et al. 2002), and from neonatal piglets (da Silva and da Silva Leite 2002), in extraintestinal human and animal urinary tract infection (UTI) and non-UTI strains (Toth et al. 2003), and in 83% of bovine isolates of necrotoxigenic *E. coli* (Mainil et al. 2003).

Pickett and coworkers recently investigated how the presence of *cdt* genes was related to a number of other virulence genes of *E. coli* (Pickett et al. 2004). Their study of a collection of 20 strains revealed that the CDT producers can be divided into three general groups with distinct differences in CDT type and in their complement of virulence-associated genes. Thus, *cdt* genes were found in a subset of uropathogenic *E. coli* strains, in additional extraintestinal isolates of uncertain clonal identity, and in certain strains with characteristics in common with Shiga-toxin producing *E. coli* and EPEC (Pickett et al. 2004). CDT and the *cdt* genes were also identified in 17 out of 340 non-O157 Shiga toxin-producing *E. coli* (STEC) of serotypes that were all *eae*-negative (Bielaszewska et al. 2004). Among these *eae*-negative STEC, *cdt* was proposed to be disease-associated as it was significantly more frequent in isolates from patients with hemolytic uremic syndrome (3 of 7) and in isolates from patients with diarrhea (14 of 138) than in isolates from asymptomatic carriers (0 of 65).

The production of CDT has been detected in few isolates of *Shigella dysenteriae*, *S. boydii* and *S. sonnei* (Okuda et al. 1995). In contrast, almost all investigated *C. jejuni* and *C. coli* strains have been reported to produce CDT or to possess the *cdt* genes. For instance, the *cdtB* gene was detected in all investigated isolates from chicken carcasses, i.e., the primary source of *C. jejuni* and *C. coli* in human infections (Eyigor et al. 1999a, 1999b). An in vitro assay showed that the *C. jejuni* isolates produced CDT at high toxin titers whereas *C. coli* produced little or no toxin. Likewise, 100/101 *C. jejuni* and 7 of 10 *C. coli* isolates from Danish broilers had the *cdt* genes. Again the *C. coli* strains produced lower amounts of the toxin (Bang et al. 2001). CDT production was also demonstrated from *C. fetus*, and PCR experiments suggested the presence of *cdtB* sequences in other species of *Campylobacter* (Pickett et al. 1996). A recent study on several virulence genes

including the *cdt* cluster in *C. jejuni* and *C. coli* isolates from Danish pigs and cattle demonstrated a high prevalence (83–95%) of *cdt* genes and CDT production (Bang et al. 2003).

In contrast to the situation in *Campylobacter* spp, the *cdt* genes and CDT activity is not present in all species of *Helicobacter*. The three genes have been identified in *H. hepaticus* (Young et al. 2000a), and the *cdtB* gene was detected in human clinical isolates of *H. pullorum* (Young et al. 2000b) and in canine isolates of *H. flexispira* (Kostia et al. 2003), but no CDT homology was found in *H. pylori* (Chien et al. 2000). Interestingly, CDT was recently reported as the first putative virulence factor present in *H. cinaedi*, which is the most commonly reported enterohepatic helicobacter in humans (Taylor et al. 2003). The *cdtB* gene detected by PCR, as well as CDT production detected by cytotoxicity, was present in all 11 investigated isolates, 10 of which were clinical isolates.

Out of 50 periodontitis strains of *A. actinomycetemcomitans*, 86% had the *cdt* genes and expressed cytotoxic activity (Ahmed et al. 2001). A majority (39/40) of clinical isolates from Brazil, Kenya, Japan and Sweden were also found to harbor the *cdt* genes and produce the AaCDT (Fabris et al. 2002). Although there was some variation in toxin production among the strains, no clear relationship between CDT activity and periodontal status could be found. In another study *A. actinomycetemcomitans* was detected in 106/146 subgingival plaque samples isolated from periodontitis patients, and among these only 13 sites were positive for the *cdt* genotype (Tan et al. 2002). However, 10 of these 13 positive sites were obtained from patients diagnosed with aggressive periodontitis, and the authors speculated that the cytotoxicity and immunosuppression by CDT may contribute to the pathogenesis of aggressive periodontitis (Tan et al. 2002). More recently CDT production and occurrence of the *cdtB* gene was studied in 73 strains of periodontopathogenic bacteria (Yamano et al. 2003) and CDT activity was found in 40/45 tested *A. actinomyctemcomitans* strains. The remaining 28 strains of other bacteria did not have CDT.

Finally, 89% of a group of isolates of *H. ducreyi* from different parts of the world showed CDT activity on HEp-2 cells (Purvén et al. 1995). A later study identified the *cdt* genes and CDT production in 83% of 29 isolates of *H. ducreyi* from chancroid (Ahmed et al. 2001). Likewise, 87% of 45 strains of *H. ducreyi* found in still more recent chancroid isolates from various parts of the world produced the toxin (Kulkarni et al. 2003).

In conclusion

A majority of strains of *C. jejuni, A. actinomyctemcomitans* and *H. ducreyi* appear to produce the CDT while the presence of the *cdt* genes in other bacteria is more variable. Table 1 summarizes the data reported in the previous paragraphs.

CDT-induced production of cytokines in vitro

CDT from *C. jejuni* has been shown to induce the release of the proinflammatory cytokine interleukin (IL)-8 from intestinal epithelial INT407 cells (Hickey et al. 2000). In contrast, a more recent study showed that while *C. jejuni* itself induced a number of proinflammatory cytokines, IL-8 among them, from the monocytic cell line THP-1, these responses did not depend on the presence of CDT (Jones et al. 2003). On the other hand, each of the purified recombinant subunits of AaCDT were found individually able to induce production

Table 1 Short summary of the CDT occurrence in clinical isolates

Toxin	Bacteria/disease	Species infected
E. coli CDTs	EPEC	Human
	Urosepsis *E. coli*	Human
	Diarrhea isolates	Dog
	Diarrhea isolates	Pig
	UTI strains	Human/animal
	Non-UTI strains	Human/animal
	Necrotoxigenic	Bovine
	STEC	Human
Shigella CDTs	Few isolates	
*Campylobacte*r CDTs	*C. jejuni*	Chicken/human
	C. coli	Chicken/human
	C. fetus	
Helicobacter CDTs	*H. pullorum*	Human
	H. flexispira	Human
	H. cinaedi	Human
	H. hepaticus	Human, mouse
AaCDT	Aggressive periodontitis	Human
HdCDT	Chancroid	Human

The references relative to the CDT distribution of clinical isolates are reported in the text

of the cytokines IL-1β, IL-6 and IL-8 but not tumor necrosis factor α, IL-12 or granulocyte-macrophage colony stimulating factor in human peripheral blood mononuclear cells (PBMC) (Akifusa et al. 2001). While CdtC was the most potent and CdtB had only minimal cytokine stimulating activity, the latter appeared to synergize with CdtA and CdtC to promote PBMC cytokine synthesis, and this synergy was most marked in inducing interferon (IFN)γ production (Akifusa et al. 2001).

Studies on CDTs in animal models

An *E. coli* strain expressing the *S. dysenteriae cdt* genes was able to induce watery diarrhea in the suckling mouse model, and the partially purified CDT had some effect also. In addition, the toxin caused a certain tissue damage in the descending colon of these mice (Okuda et al. 1997). When *cdtB* negative strains of *C. jejuni* were administered to severe combined immunodeficient mice no difference in enteric colonization was observed, but there was an impaired invasiveness of the bacteria into blood, spleen and liver tissues as compared to the wild-type bacteria (Purdy et al. 2000), implying that CDT might have a role in the pathogenesis (invasion) of *C. jejuni*. A more recent study of a *C. jejuni cdtB* mutant in wild-type and nuclear factor (NF)-κB deficient mice suggested that CDT may have pro-inflammatory activity in vivo, as well as a potential role in the ability of *C. jejuni* to escape immune surveillance (Fox et al. 2004). The *cdtB* mutant was less efficient than the wild-type *C. jejuni* in colonizing the wild-type (C57BL/129) but not NF-κB deficient mice. Despite 100% colonization of the NF-κB deficient 3X mice the *cdtB* mutant produced significantly less gastritis than the wild-type bacterium (Fox et al. 2004). The authors speculate that CDT may target the cells of the immune system in the lamina propria that influence the host's ability to clear bacterial pathogens. Young and coworkers recently provided further evidence for a potential pro-inflammatory activity of CDTs (Young et al. 2004). Strains of *H. hepaticus* expressing a functional CDT caused severe

colitis in a murine model of inflammatory bowel disease, using C57BL/6 IL-10 deficient mice. In contrast, the CDT-negative isogenic mutants were without effect (Young et al. 2004).

An isogenic *H. ducreyi cdtC* mutant was equally virulent as the parent strain when tested in the temperature-dependent rabbit model for experimental chancroid, despite the fact that it was not cytotoxic to HeLa cells and keratinocytes (Stevens et al. 1999). Isogenic *H. ducreyi cdtA* and *cdtB* mutants also proved to be as virulent as the wild-type strain with regard to lesion production in the same rabbit model (Lewis et al. 2001). In human volunteers, expression of CDT was also not required for pustule formation by *H. ducreyi*, although CDT might still be relevant for induction or persistence of the ulcer stage which is not testable in humans (Young et al. 2001). The purified HdCDT holotoxin induced dose-dependent pathologic skin reactions in rabbits (Wising et al. 2002). High levels of neutralizing antibodies against CDT were detected in only 22% and 2% of patients with chancroid and periodontitis, respectively. Moreover, a majority of healthy individuals also had HdCDT antibodies, and thus such antibodies may not be specific markers for chancroid infection (Mbwana et al. 2003).

In conclusion

The fact that CDTs are produced by diverse human pathogens suggests that these toxins might contribute to the development of different diseases. At the moment, however, there is no absolutely clear association between toxin and specific disease symptoms and one major problem is the lack of suitable animal models for this type of study. Conceivably, CDT could have a role in all instances where cell proliferation is required. Thus, CDTs produced by intestinal pathogens could possibly contribute to gastroenteritis by blocking the proliferation of crypt cells, although there are no studies yet on this particular aspect of the *E. coli*, *Campylobacter* or *Shigella* CDTs. The HdCDT could contribute to the very slow healing of chancroid ulcers. Furthermore, immunosuppression could be one important general effect of CDTs as suggested by the recent studies cited above and by the in vitro effect of CDT on DCs which have a central role for induction of both cellular and humoral-mediated immunoresponses (Li et al. 2002). Targeting DCs could represent a strategy developed by several pathogens in order to avoid or delay the onset of immunoresponses. In fact the AaCDT was originally isolated as the ‘immunosuppressive factor’ of *A. actinomycetemcomitans* and it might possibly play such a role in the pathogenesis of aggressive periodontitis and other infections caused by this bacterium (Shenker et al. 1999).

Future perspectives

In retrospect it is interesting to note that no toxicologist ‘jumped on’ the CDT directly after its discovery in the late 1980s, probably because it did not seem feasible to study something that requires several days to induce an effect on cells. However, during the 10 years after the CDT was first cloned this three-gene toxin has generated an increasing interest, and the development from ‘mysterious activity’ to well known toxin with defined mode of action, now even crystallized, has advanced rapidly. CDT turned out to be unique in that it is the only bacterial toxin known to target DNA as a primary action, subsequent-

ly activating a number of important cellular stress responses designed to save the attacked cell while it attempts to repair its DNA. Although—or perhaps because—we know today so much more than we did a decade ago there are many more questions waiting for their answers. In addition, there is reason to believe that CDT, like many other bacterial protein toxins, can be developed into a useful tool in cell biology and biomedicine.

Molecular mode of action of CDT

The receptor(s) for CDT has not yet been identified. This issue is complicated by the tripartite CDT structure with potentially two separate binding subunits, CdtA and CdtC, which might bind to separate receptor structures, or to the same as suggested by recent studies (Lee et al. 2003) and (McSweeney and Dreyfus 2004). The sequence homologies between the CdtA and CdtC subunits from different bacteria are much lower than between the different CdtB subunits. Thus, it is even possible that the receptor(s) for CDTs of different origin will turn out to differ slightly despite the fact that the active CdtB subunit apparently has the same DNase activity in all CDTs. That there are lectin-like regions in CdtA and CdtC (Nesic et al. 2004), similar to those found in the binding subunit of ricin, might point to a galactose-containing receptor, although Mao and DiRienzo (Mao and DiRienzo 2002) failed to inhibit the binding of AaCdtA to CHO cells with various galactosides and mannosides. Besides the binding to cell surface receptor(s) the three subunits must be able to interact with each other to form the fully active holotoxin. Obviously this interaction must depend on specific amino acid stretches, some of which have already been identified (Lee et al. 2003). It would also be of interest to combine subunits of CDTs from different bacteria to see whether they can fully complement each other. The binding of CdtB to T cells needs clarification; maybe there is a specific receptor for this subunit on these cells.

It is clear that CDT has to be internalized in cells so that the CdtB subunit can be transferred into the nucleus. Indirect functional studies suggested that HdCDT, or at least the active subunit, requires passage through an intact Golgi complex to intoxicate cells (Cortes-Bratti et al. 2000), and later observations in MDCK cells are fully consistent with these findings (T. Frisan and M. Thelestam, unpublished results). The CdtB subunit is able to enter the nucleus from the cytosol and this occurs via active transport (Nishikubo et al. 2003). However, the final steps in the transfer of the CdtB subunit, upon natural intoxication, from the vesicular uptake system to the nucleus are not clear. The cellular fates of CdtA and CdtC after fulfilling their putative roles to facilitate entry of CdtB have also not been clarified. Another unresolved issue is whether CdtC, if it really proves to be cytotoxic by itself, has an entirely different mode of action than CdtB.

CDTs as tools in cell biology

Bacterial toxins have been extremely useful in the study of different aspects of cell biology. So far, CDTs are the only bacterial protein toxins known to induce a subtle DNA damage. In the field of cell and tumor biology, regulation of the cell cycle is currently one of the major issues and CDT will probably be utilized as a tool in such studies. Toxins have also been very helpful in the study of endocytosis and intracellular transport in general. Studies with Shiga toxin showed for the first time that a molecule can be transported from

the cell surface to the Golgi apparatus and the ER (reviewed in Sandvig and Van Deurs 2000). Since the nucleus is its final destination CDT represents an interesting tool for studies of protein transport from the Golgi/ER to the nucleus. Finally, the study of CDT has already led to the discovery of a novel cellular signaling pathway transmitting survival signals from damaged DNA to the small GTPase RhoA. CDT will be useful in the continued efforts to clarify all the molecular details from DNA damage to RhoA activation, as well as from the latter event to the downstream components involved in protecting the cells from immediate death.

CDTs and disease

It will be interesting to see when CDTs will be more clearly demonstrated to cause, or at least to contribute to, the symptoms in infections caused by the bacteria producing them. Now that the mode of action of CDT is basically known this aspect will probably become more attractive for study even if the lack of really good animal models is still a problem. In any case CDTs are likely to affect the immune response as well as other situations in which cell proliferation is needed, such as the healing of chancroid or periodontic ulcers. Another potentially important aspect is that CDTs, being genotoxic, might possibly constitute a contributing factor in long term cancer development. Since CDT is produced by so many common pathogens this is a rather disturbing perspective, which has not yet been studied at all. On the other hand, the action of CDT on DNA makes it a potentially good candidate for an anti-tumor agent.

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Anthrax toxins

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Abstract *Bacillus anthracis*, the etiological agent of anthrax, secretes three polypeptides that assemble into toxic complexes on the cell surfaces of the host it infects. One of these polypeptides, protective antigen (PA), binds to the integrin-like domains of ubiquitously expressed membrane proteins of mammalian cells. PA is then cleaved by membrane endoproteases of the furin family. Cleaved PA molecules assemble into heptamers, which can then associate with the two other secreted polypeptides: edema factor (EF) and/or lethal factor (LF). The heptamers of PA are relocalized to lipid rafts where they are quickly endocytosed and routed to an acidic compartment. The low pH triggers a conformational change in the heptamers, resulting in the formation of cation-specific channels and the translocation of EF/LF. EF is a calcium- and calmodulin-dependent adenylate cyclase that dramatically raises the intracellular concentration of cyclic adenosine monophosphate (cAMP). LF is a zinc-dependent endoprotease that cleaves the amino terminus of mitogen-activated protein kinase kinases (Meks). Cleaved Meks cannot bind to their substrates and have reduced kinase activity, resulting in alterations of the signaling pathways they govern. The structures of PA, PA heptamer, EF, and LF have been solved and much is now known about the molecular details of the intoxication mechanism. The in vivo action of the toxins, on the other hand, is still poorly understood and hotly debated. A better understanding of the toxins will help in the design of much-needed anti-toxin drugs and the development of new toxin-based medical applications.

Abbreviations *CMG2:* Capillary morphogenesis protein 2 · *DTA:* Diphtheria toxin A chain · *EF:* Edema factor · *EFn:* N-terminal fragment of EF · *ETx:* Edema toxin · *GR:* Glucocorticoid receptors · *GSK3β:* Glycogen synthase kinase 3β · *I domain:* Integrin-like domain · *iNOS:* Inducible nitric oxide synthase · *LF:* Lethal factor · *LFn:* N-terminal fragment of LF · *LTx:* Lethal toxin · *MAPK:* Mitogen-activated protein kinase · *Mek:* MAPK kinases · *PA:* Protective antigen · PA_{20}: 20-kDa N-terminal fragment of PA · PA_{63}: 63-kDa C-terminal fragment of PA · *TEM8:* Tumor endothelial marker 8

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Introduction

Anthrax

Anthrax toxins are virulence factors of *Bacillus anthracis*, a gram-positive rod that causes anthrax. Anthrax is an epizootic disease affecting wild and domesticated herbivores (Mock and Fouet 2001; Turnbull 2002). The disease affects primarily livestock but can occasionally be transmitted to humans who come in contact with infected animals or animal products. It is often reported that anthrax was first described by Virgil as a plague affecting humans and cattle in a district of the eastern Alps (Sternbach 2003). It is also speculated that anthrax was the cause of two of the Egyptians plagues described in the Old Testament, the death of cattle and the appearance of boils. Anthrax played an important role in the history of bacteriology. *B. anthracis* was studied by Koch who used it in 1876 to prove his famous postulates. By growing the bacteria in vitro and challenging animals with the culture he was able to induce disease, disproving the theory of spontaneous generation and introducing the notion of germs. It was Koch too who observed the life cycle of *B. anthracis* for the first time and showed that the bacteria could form highly resistant spores. Anthrax was also studied by Pasteur who, by heat treatments, isolated an attenuated strain that in 1881 proved to be an effective vaccine.

Anthrax occurs when spores of *B. anthracis* gain access to host tissues. In animals this usually occurs by ingestion, whereas in humans, spores usually enter the host via a break in the skin barrier. The latter causes a cutaneous form of the disease with characteristic skin lesions (Friedlander 1999). First, a painless papule develops into a vesicle accompanied by an edema of varying intensity. After a few days the vesicle ruptures, yielding a black eschar. The coal-like appearance of the lesion gave its name to the disease, as anthrax comes from the Greek word for coal. On histology the lesion shows necrosis, lymphocyte infiltration, and edema. With antibiotic treatment the lesion usually heals spontaneously in a few weeks and leaves a limited scar. The main complication of this form of the disease is the systemic spread of the bacteria. The resulting septicemia is often fatal.

Septicemia is immediate when spores gain access to their hosts by inhalation. This causes the inhalation or pulmonary form of the disease, which is highly lethal. After a few days of initial mild symptoms including fever, cough, and malaise, inhalation anthrax takes an abrupt turn: increased respiratory distress, “shock-like” symptoms, coma, and death. A chest X-ray shows a characteristic widening of the mediastinum and pleural effusions. Inhalation anthrax is also often accompanied by acute meningitis.

Pathogenesis and role of the toxins

Regardless of the route of entry, spores are phagocytosed by macrophages where they germinate (Guidi-Rontani 2002). The bacteria, by an unknown mechanism, escape destruction and lyse the macrophages to gain access to host tissue. In its host, *B. anthracis* appears as a large encapsulated, non-motile rod often found in long chains. During infection the bacteria replicates to high titers of up to 10^9 cfu/ml of blood. This exceptional infectivity is due to two main virulence factors coded by genes present on two large plasmids, plasmids pXO1 and pXO2. Plasmid pXO2 carries genes directing the synthesis of a poly-D-glutamic acid capsule that was shown to inhibit phagocytosis of the planktonic bacteria (Makino et al. 1989). A bacteria cured of its pXO2 plasmid was isolated in the 1930s by

Sterne and shown to be avirulent and a good live vaccine (Sterne 1937). Plasmid pXO1 carries genes coding for toxins. The existence of a toxin was reported in the mid 1950s after it was observed that laboratory animals died when they were injected intravenously with clarified plasma from anthrax-infected guinea pigs dying from anthrax (Smith 2002; Smith and Keppie 1954). Subsequent intense research proved that the toxin consists of three polypeptides: protective antigen (PA), edema factor (EF) and lethal factor (LF). It was shown that these three proteins assemble into two distinct toxins with different physiological effects. A mixture of PA and EF forms edema toxin (ETx), which causes edema when injected subcutaneously in laboratory animals. A mixture of PA and LF forms lethal toxin (LTx), which causes death when injected intravenously in laboratory animals.

Several lines of evidence demonstrate that the toxins play a central role in the pathogenesis of anthrax: (1) highly purified preparations of toxins cause death and edema; (2) bacteria cured of their pXO1 plasmid or carrying a plasmid where individual toxin genes have been inactivated are dramatically less virulent (Pezard et al. 1991); (3) an immune response against toxin components and especially PA completely protects against intoxication as well as against anthrax infection; (4) during the course of infection a point is reached where antibiotics can clear bacteremia but are unable to prevent death, most likely due to the remaining circulating toxin that is unaffected by the drugs (Keppie et al. 1955). After the achievements of the 1950s and 1960s, work on anthrax toxin became dormant and was only revived in 1982 when Leppla showed that EF had adenylate cyclase activity (Leppla 1982). In the subsequent decades much work improved our knowledge of the structure, delivery, and cellular activity of the toxins (Ascenzi et al. 2002; Collier and Young 2003; Lacy and Collier 2002). Despite all this work, some molecular details of the intoxication remain unclear. Even more puzzling, the in vivo mechanism of action of the toxins is very poorly understood and subject to debate.

Anthrax as a bioweapon

In animals, once the host is dead and its carcass decays, bacteria come into contact with oxygen and sporulate. Anthrax spores are highly resistant to physical stresses and survive many years in the soil where they can infect their next host. The hardiness of the spores, the ease with which their dispersion can be achieved, and the mortality of the disease all suggest that *B. anthracis* could be used as a biowarfare agent. In 1941 the British government tested the release of anthrax spores on an island near Scotland (Christopher et al. 1997; Manchee et al. 1983). The island remained a hazard until 1986 when tons of seawater and formaldehyde were used to sterilize its soil. Militarization of anthrax was also pursued in the former Soviet Union. In 1979 an epidemic occurred near a Soviet microbiological military facility in Sverdlovsk (Abramova et al. 1993; Meselson et al. 1994). Ninety-six people were reported ill and 64 died. The authorities claimed that an outbreak in cattle was transmitted to humans by consumption of contaminated meat. However, it was later shown that an accidental release of an aerosol from the military facility was the culprit. Lastly, in October 2001 a new outbreak of inhalation anthrax occurred in the United States of America after the intentional release of spores through contaminated letters delivered to politicians and journalists. This attack resulted in five deaths from 11 confirmed cases of inhalation anthrax and 12 confirmed or suspected cases of cutaneous anthrax (Jernigan et al. 2001). Most of the deaths struck postal workers who did not open the tainted letters. This showed that the spores delivered during the attack had been weaponized and thus offered a chilling glimpse of the extent of the possibilities achieved in bioweapon engineering.

One of the challenges posed by an attack involving anthrax spores is the fact that victims who develop inhalation anthrax have symptoms that are difficult to distinguish from influenza-like illnesses. Once symptoms develop, it is often too late to undertake an effective treatment. Antibiotics can eradicate the bacteria, but the huge load of toxins can still ultimately cause death of the victim. In order to fight the toxin the only applicable course of action today is aggressive supportive care. The outbreak in 2001 showed that this approach reduced the mortality rate from nearly 100% to slightly below 50% (Jernigan et al. 2001). However, this approach would prove impossible in the case of a massive release of spores versus the limited occurrence of 2001.

Intoxication mechanism

Anthrax toxin is composed of three polypeptides: PA, EF, and LF. These three proteins are synthesized during the vegetative growth of the bacteria. In vitro expression of the genes coding for the toxin components is induced by addition of bicarbonate and a temperature of 37°C. A specific transcriptional activator, *atxA*, and a repressor, *pagR*, of the toxin genes have been isolated but a full picture of the regulation network has proved to be exquisitely more complex (Koehler 2002; Mock and Mignot 2003). Few specific details are known on the secretion mechanism of PA, EF, and LF. The polypeptides have standard signal sequences and are therefore thought to follow the general secretion pathway and be released in the extracellular milieu independently of one another.

Anthrax toxin belongs to the family of bacterial "AB" toxins. Schematically, these toxins work by the conjugated action of a cell binding B moiety, which allows the binding and translocation of an enzymatically active A moiety in the cytosol of a target cell. More specifically, anthrax toxins belong to the subgroup of binary toxins where the A and B moieties are carried by independent polypeptides. This group comprises adenosine diphosphate (ADP) ribosylating toxins like *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* Sb toxin, *C. difficile* ADP ribosyltransferase, and the vegetative insecticidal proteins from *Bacillus cereus*. However, anthrax toxin is (thus far) unique because it has one central B moiety, PA, which can associate with two enzymatic moieties EF and LF. The mechanism by which the toxins assemble and intoxicate cells is described below (Fig. 1).

Binding of PA to target cells

Mature PA is a 735-residue long, 83-kDa protein. The first step in the intoxication is the binding of PA to its cellular receptor. It was initially observed that a single class of proteinaceous receptors for anthrax toxin was located on the surface of many cell types. Using cross-linking experiments, an 80-kDa cellular protein was found to be associated with PA (Escuyer and Collier 1991). A decade later, a genetic approach was undertaken to find the gene coding for the receptor for the toxin (Bradley et al. 2001). First, Chinese hamster ovary (CHO)-K1 cells were chemically mutagenized and selected for resistance against a mixture of PA and a fusion protein consisting of the N-terminal domain of LF (LFn) and the catalytic domain of diphtheria toxin, DTA. This fusion protein is delivered to the cytosol by PA where DTA blocks protein synthesis. Resistant CHO cell lines were identified and found to lack a receptor for PA. Then, a complementing cDNA was identified by

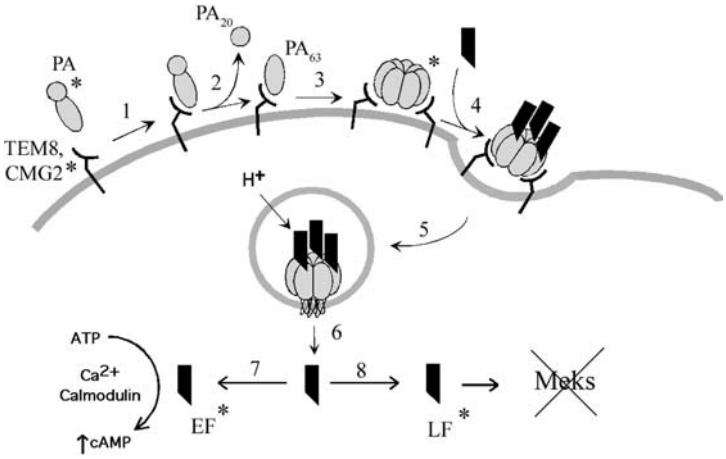

Fig. 1 Model of the intoxication mechanism of anthrax toxins. (*1*) The intoxication starts with the binding of PA to the cellular receptors tumor endothelium marker-8 (TEM8) and/or capillary morphogenesis protein 2 (CMG2). (*2*) Bound PA is cleaved by membrane endoproteases of the furin family, releasing a 20-kDa N-terminal fragment, PA_{20}. (*3*) The remaining 63-kDa C-terminal fragment, PA_{63}, oligomerizes into heptamers. (*4*) The heptamers can then associate with EF and/or LF. Alternatively, PA can be cleaved in the serum of infected hosts, possibly oligomerizing and even forming complexes with EF/LF before binding to cellular receptors (not shown). (*5*) The assembled toxic complexes are endocytosed and routed to an acidic compartment. (*6*) There, the low pH triggers a conformational change resulting in the formation of a cation-specific channel and translocation of EF/LF. (*7*) EF is a calcium- and calmodulin-dependent adenylate cyclase. (*8*) LF is a zinc-dependent endoprotease that cleaves the N-termini of Meks. The *asterisks* indicate the molecules for which a crystal structure has been solved

screening a library. The identified cDNA coded for a membrane protein named tumor endothelial marker-8 (TEM8), previously identified as an up-regulated protein in endothelial cells associated with colorectal cancer. TEM8 appears to be expressed in a broad range of tissues and apparently has three splice variants. The splice variant of TEM8 identified has a short cytoplasmic tail. The extracellular domain of TEM8 contains a von Willebrand factor type A domain, also called integrin-like domain (I-domain). These domains usually mediate protein–protein interactions involved in binding to cell adhesion molecules and extracellular matrix proteins. Consistent with this view, the extracellular domain of TEM8 was found to interact with collagen VI, a form of collagen that like TEM8 is preferentially expressed in tumor endothelium (Nanda et al. 2004). The I-domain of TEM8 is the PA-binding site (Bradley et al. 2001), and it was found that a metal ion-dependent adhesion site motif within the domain was critical for toxin binding. In integrins, this motif coordinates divalent cations like Mg^{2+} or Mn^{2+} that are important for ligand binding. The same type of binding and the implication of a carboxylate group in PA was reported (Bradley et al. 2003).

A second receptor for PA was found encoded by the capillary morphogenesis protein 2 (CMG2) (Scobie et al. 2003). Like TEM8, CMG2 is widely expressed and possesses an I-domain which is highly homologous to that of TEM8. CMG2 binds collagen IV and laminin and thus is also implicated in binding to extracellular matrix proteins. In CMG2 the binding of PA was found to be, like that of TEM8, dependent on the I-domain and its ion binding motif, although the cation specificity was slightly different between CMG2 and TEM8. The crystal structure of the I-domain of CMG2 has recently been solved (Lacy et al. 2004). The structure shows a typical I-domain fold, with a close homology to the

"open" or high affinity conformation of the αM integrin I-domain. Coordination of the metal ion was structurally similar in the two molecules.

Toxin assembly and endocytosis

Once bound to a receptor, PA can be cleaved by furin or a furin-like membrane endoprotease (Gordon et al. 1995). Furin belongs to a class of calcium-dependent serine endoproteases, the prohormone–proprotein convertases. Although furin is prominently found intracellularly, PA cleavage, however, occurs at the cell surface. On polarized cells, receptor and furin are both localized on the basolateral surface (Beauregard et al. 1999). The structure of furin with a substrate-derived inhibitor has recently been solved (Henrich et al. 2003). This structure helps to elucidate the stringent specificity of this protease towards the sequence R-X-K/R-R. In PA, this sequence is found in the N-terminus, and cleavage results in the release of a N-terminal 20-kDa fragment (PA_{20}). This fragment is not thought to play a further role in the intoxication. The remaining 63-kDa fragment (PA_{63}) oligomerizes quickly into a heptamer (Milne et al. 1994), a step that PA_{20} sterically prevented. Cleavage of PA at the normal furin cleavage site can also be achieved in solution using moderate amounts of trypsin (Novak et al. 1992). After cleavage in solution, however, PA_{63} will not oligomerize spontaneously as is the case on cells. Oligomerization in solution requires the addition of ligand or separation of PA_{63} from PA_{20} by running cleaved PA on an anion exchange chromatography column (Miller et al. 1999). Similarly, cleavage of PA has been reported to occur in vivo in the serum of animals (Brossier et al. 2000; Ezzell and Abshire 1992). In that case it is unclear, however, if PA_{63} is oligomerizing in the serum, and if so, whether this occurs with or without the presence of EF, LF, or both.

Once assembled into a heptamer, PA_{63} can associate with EF and LF. This yields the fully assembled toxic complex. It was found that the PA-binding domains of LF or EF were in the ~250 residue-long N-terminal domains termed LFn or EFn, respectively (Arora and Leppla 1993). It was demonstrated that the stoichiometry of the complex at saturating concentrations of enzymatic moieties (either EF or LF) is three ligand molecules per heptamer (Mogridge et al. 2002a). Moreover, PA_{63} mutants unable to oligomerize could not bind LFn, whereas a ternary complex of a PA_{63} dimer associated with one molecule of LFn could be purified (Mogridge et al. 2002b). These data suggest that there are seven binding sites for EF/LF binding on the heptamer located across the boundary between monomers. The unexpected stoichiometry is explained by the fact that binding to one site of the heptamer sterically blocks the adjacent ones, therefore only allowing a maximum of three molecules bound per heptamer (see below and Cunningham et al. 2002). It is still unclear, however, if both EF and LF molecules can bind to one heptamer of PA_{63}.

Regardless of whether it associates with EF and/or LF, it was shown that proteolytic cleavage of PA triggers and is absolutely necessary for the internalization of PA (Beauregard et al. 2000). This led to the hypothesis that the oligomerization process triggered a cell event leading to endocytosis. Consistent with this hypothesis it was observed that only PA_{63} oligomers could be endocytosed (Liu and Leppla 2003).

Prior to cleavage, PA bound to its receptor is found in detergent-soluble parts of the plasma membrane, but after cleavage it is rapidly relocalized to detergent-insoluble parts, also termed lipid rafts (Abrami et al. 2003). It was found that the clustering of anthrax toxin receptors, either via PA_{63} oligomerization or using an antibody, causes its relocalization into lipid rafts. As monomers, receptor molecules are taken up slowly, but upon clustering

the endocytosis is rapid, caveolae-independent, and clathrin-dependent. These molecules are therefore ideal receptors for PA. By associating with a receptor that is slowly endocytosed as a monomer, PA can stay at the cell surface until it is able to oligomerize. Once oligomerization is achieved, then it can rapidly proceed with the intoxication. Indeed, even a PA mutant that cannot be cleaved by furin can be internalized when clustering is triggered using an antibody. The mechanism for relocalization and triggering of endocytosis are unclear. The cytoplasmic tail of TEM8 was found not to be involved in this process, as even an anchored version of the extracellular domain of TEM8 was functional in an intoxication assay (Liu and Leppla 2003).

After endocytosis it was demonstrated by several approaches that the toxic complex is delivered to an acidic compartment. Treatment of cells with lysosomotropic amines can block LTx intoxication (Friedlander 1986). Similarly, treatment with chloroquine, which prevents endosome acidification, prevented ETx intoxication (Gordon et al. 1988). Lastly, treatment with bafilomycin, which inhibits the vacuolar ATPase proton pump and therefore acidification, blocks LTx action (Menard et al. 1996).

Translocation of EF/LF

It was observed that PA_{63} heptamers could form pores in planar lipid bilayers when shifted to a low pH (Blaustein et al. 1989).These channels were found to be cation selective. Indeed, a K^+ efflux could be measured from liposomes incubated with PA_{63} heptamers at low pH (Koehler and Collier 1991). Later, the ability to form channels was also observed on cells by measuring an efflux of $^{86}Rb^+$ when cells are incubated in the presence of PA_{63} heptamers at low pH (Milne and Collier 1993). In the latter experiments, the optimal pH for release was found to be approximately pH 5 and negligible above pH 6. However, in planar lipid bilayers, channel formation could be measured around pH 7. The reason for this discrepancy is unclear. Collectively these experiments suggest that once PA_{63} heptamers reach the endosome they form a channel.

Channel formation in the endosome is associated with translocation of EF/LF, but the mechanism is unclear. It was shown that LFn could direct the translocation of heterologous polypeptides fused to its N or C termini when cells were incubated with a mixture of PA and the chimera (Arora and Leppla 1993; Milne et al. 1995). Thus, LFn seems to encompass all the information necessary for translocation, and the remaining part of the polypeptide is passively transported along. Moreover, since both N-terminal and C-terminal fusions are equally translocated, there does not seem to be directionality in the translocation process.

By incubating cells on ice with trypsin-activated PA and radiolabelled LFn, complexes of LFn bound to PA_{63} heptamers can be formed but, because of the low temperature, remain on the cell surface. When those complexes are then exposed to low pH, it was shown that the radiolabelled ligand becomes inaccessible to a protease added extracellularly, and thus has been translocated into the cytosol (Wesche et al. 1998). Translocation is therefore observed in conditions triggering pore formation, whether in endosomes or on the cell surface. All known PA mutants specifically impaired in translocation are also deficient in pore formation (Mourez et al. 2003; Sellman et al. 2001b). These data led to the hypothesis that translocation occurs through the channel of the pores formed by PA_{63} heptamers. Strikingly, it was observed that pore formation on cells was blocked by addition of LF or LFn (Zhao et al. 1995). The size of the channel formed by PA_{63} heptamers was assessed

in planar lipid bilayers and shown to be around 12 Å (Blaustein and Finkelstein 1990). This implies that the ligands cannot be transported in a folded state. The translocation of fusions between LFn and dihydrofolate reductase (DHFR) or between LFn and DTA was studied in the cell surface assay described above. It was shown that translocation was blocked in the presence of the ligands of the heterologous parts of the DHFR and DTA chimeras, namely methotrexate and adenine, respectively (Wesche et al. 1998). This suggests that, indeed, stabilization of folding prevents translocation and that low pH must trigger some partial or complete unfolding of the ligands bound to the heptamers. Translocation of unfolded enzymatic domains via a cell-binding domain forming a pore has also been observed in other AB toxins like diphtheria toxin (Oh et al. 1999) and *C. botulinum* neurotoxin (Koriazova and Montal 2003). However, the nature of the "channel" in those cases is thought to be quite different from that of the heptamer of PA_{63}.

Since enzymatic moieties are translocated unfolded, refolding must occur in the cytosol where chaperones might be involved. In a recent report the role of such a cytosolic chaperone, Hsp90, in the toxicity of C2 toxin was tested (Haug et al. 2003). It was found that Hsp90 is necessary for translocation and activity of C2. It was also necessary for the activity of iota toxin but not for anthrax lethal toxin. The reason for the discrepancy is unclear but could be due to the use of a different cell type in the LTx assay or to true differences. It is interesting to note that diphtheria toxin, another AB toxin with ADP ribosyltransferase activity, was also shown to require Hsp90 (Ratts et al. 2003). Therefore, one possibility might be that Hsp90 is specifically involved in the translocation/refolding of ADP ribosyltransferase toxins.

There are, however, numerous questions challenging such a model for the translocation step. Since the lumen of the heptamer can only accommodate an unfolded polypeptide, or at the most a helix, one has to assume that only one polypeptide can be translocated at a time. The fact that up to three ligand molecules can be bound per heptamer raises the question as to how these three molecules can be sequentially translocated. It is easier to envision the translocation of multiple molecules in a model where the ligands are translocated on the sides of the heptamer instead of through its lumen. Consistent with this hypothesis, it was observed that LF and EF at low pH are able to destabilize and/or insert into lipid bilayers by themselves (Kochi et al. 1994a; Wang et al. 1996; Wang et al. 1997). This suggests that they might participate in their own translocation. Another experiment yielded an intriguing result about the translocation ability of PA: a polycationic tail fused to DTA could promote its translocation in the cytosol of cultured cells in a PA-dependent manner (Blanke et al. 1996). The efficiency of translocation was less than that of the LFn–DTA fusions, and the translocation was not inhibited by the presence of LFn. One model to explain this peculiar result would be that the polycationic tail allows DTA to interact with the anionic phospholipids of the membrane and get translocated at the interface with PA_{63} heptamers when pH becomes acidic. Alternatively, the cationic tail could target DTA to the lumen of the heptamers.

Enzymatic activity

After translocation, the enzymatic moieties have access to the cytoplasm, where they can exert their activity. Because the edema response induced by ETx was reminiscent of that induced by cholera toxin, it was hypothesized and then shown that EF, like cholera toxin, can increase the intracellular cyclic adenosine monophosphate (cAMP) concentration

(Leppla 1982). In fact, it was shown that EF is itself an adenylate cyclase strictly dependent on the presence of calcium and calmodulin. *Bordetella pertussis* also produces a highly homologous adenylate cyclase toxin. EF has a specific activity 1,000-fold higher than that of mammalian calmodulin-activated adenylate cyclases, but because bacteria lack calmodulin, this high activity is restricted to the cytosol of eukaryotic cells.

The enzymatic activity of LF remained a mystery for a long time. The first clue came when a zinc-binding motif, HEXXH, present in all zinc-dependent endoproteases was recognized in LF (Klimpel et al. 1994; Kochi et al. 1994b). The only substrates of LF identified to date are mitogen-activated protein kinase (MAPK) kinases (Meks) (Duesbery et al. 1998; Vitale et al. 1998). LF cleaves all known Meks (Mek 1 to Mek 7) except Mek 5 (Duesbery et al. 1998; Pellizzari et al. 1999; Vitale et al. 2000; Vitale et al. 1998). The cleavage site is located in the N terminus of Meks in a proline-rich region. As a consequence of the cleavage, Meks are unable to dock with their substrate MAPK. Furthermore, it was recently shown that this cleavage also reduces the intrinsic kinase activity of Meks (Chopra et al. 2003). The N-terminal region of Meks is not the only region recognized by LF, since point mutations in a conserved C-terminal region could prevent proteolysis without affecting kinase activity (Chopra et al. 2003).

Structural studies

The structure of PA was solved both as a monomer and as a trypsin-activated, purified soluble heptamer of PA_{63} (Petosa et al. 1997). The soluble heptamer structure is thought to be identical to that of the heptamer assembled on the cell surface prior to endocytosis. Hence it was termed "prepore" to differentiate it from the structure of the membrane-inserted "pore" at low pH. There is little structural difference between the two forms of PA_{63} in the monomer and the prepore. This confirms the idea that the only role of PA_{20} is to sterically prevent oligomerization of PA_{63}. PA has four domains (Fig. 2). Mutational studies have helped define the function of each of the domains which are described below. Recently, a global cysteine scanning study of PA_{63} has helped to give a global view of the structure–function relationships in PA, summarizing most of these studies (Fig. 3, Mourez et al. 2003).

Domain 4 (residues 595–735) starts with a hairpin and helix connecting this otherwise independent domain to the rest of the molecule. The rest of the fold of domain 4 is that of a β-sandwich with an immunoglobulin fold. Mutations in domain 4 affect the binding of PA to target cells (Brossier et al. 1999; Rosovitz et al. 2003; Varughese et al. 1999) and monoclonal antibodies binding to this domain affect PA binding (Little et al. 1996). This proves that domain 4 is the receptor-binding domain. To date the receptor and receptor-binding domain have not been implicated in any intoxication mechanism other than binding on cells (Bradley et al. 2001) and, possibly, triggering endocytosis (Abrami et al. 2003). It would be interesting to assess if the receptor and domain 4 are involved additionally in pore formation and translocation.

Domain 3 (residues 488–595) has a ferredoxin-like fold. In a random mutagenesis approach undertaken to elucidate the function of this domain, inactive mutants were found to be unable to form heptamers (Mogridge et al. 2001). The primary role of domain 3 therefore seems to be the oligomerization of PA_{63}. In this hypothesis, residues of domain 3 (centered on aspartate 512) on one face of PA_{63} would make one oligomerization interface. The complementary interface would be formed by a patch of residues from do-

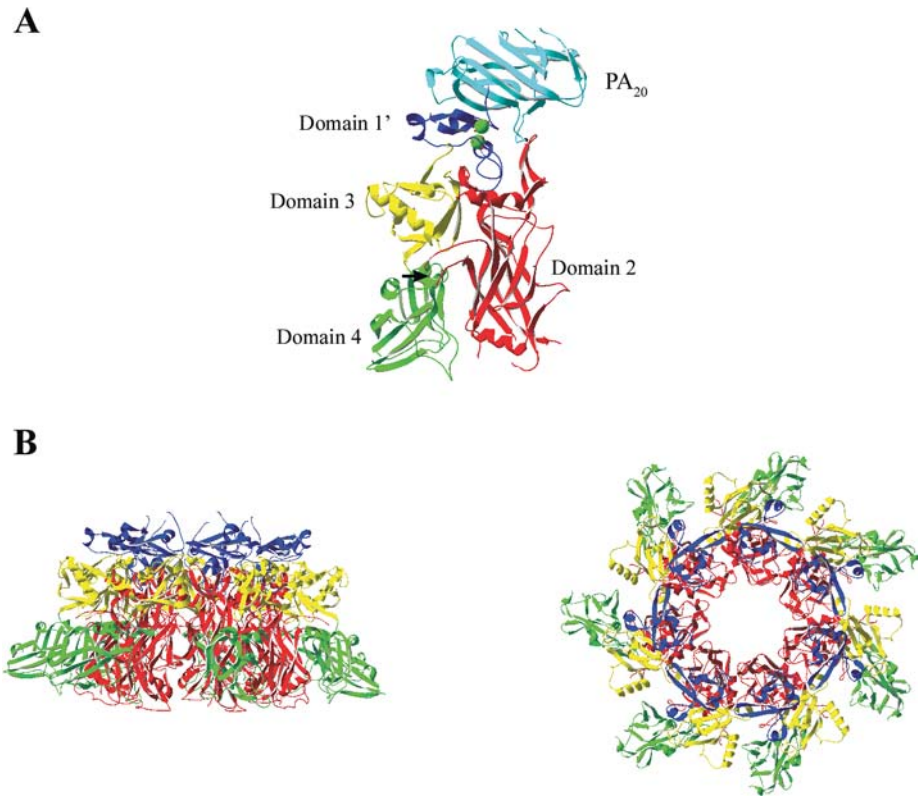

Fig. 2A, B Crystal structure of PA. Ribbon representation of the crystal structure of (**A**) PA and (**B**) the PA_{63}-soluble heptamer believed to represent the structure of the prepore (the membrane-bound heptamer before its encounter with low pH) (Petosa et al. 1997). The four domains are represented with different colors: domain 1 consists of PA_{20} in *cyan* and domain 1′ in *blue*; domain 2 in *red*; domain 3 in *yellow* and domain 4 in *green*. **A** The two calcium ions bound in domain 1′ are represented as *green spheres* and the *arrow* indicates the flexible loop of PA_{63} that inserts into the membrane at low pH. **B** Side view (*left*) of the prepore (the lipid bilayer would be at the bottom of this view) and top view (*right*) of the prepore (the lipid bilayer would be at the back of this view). The structure images in this figure and Figs. 3 and 4 were generated using Swiss-PDBviewer 3.7 (http://www.expasy.org/spdbv/)

mains 1 and 2 (centered on lysine 199 and arginines 468 and 470) on the other face of PA_{63}. Indeed, mutations of the latter residues also prevent oligomerization (Mogridge et al. 2002b). Furthermore, by mixing PA mutants where one or the other oligomerization faces have been mutated, stable dimers can assemble through the only intact interface and be purified (Mogridge et al. 2002b).

Domain 2 (residues 249 to 488) has a β-barrel core structure and makes up most of the lumen in the prepore structure. Domain 2 was shown to possess a chymotrypsin-sensitive region critical in the translocation process (Novak et al. 1992). The deletion of two phenylalanines in this region was also found to prevent translocation (Singh et al. 1994). This region, corresponding to the loop joining the $2\beta2$ and $2\beta3$ strands, is disordered and unresolved in the PA structures (Petosa et al. 1997), suggesting that it is highly flexible. By analogy with the structure of the pore-forming toxin α-hemolysin of *Staphylococcus aureus*, which is also a heptamer that forms channels in membranes (Song et al. 1996), it

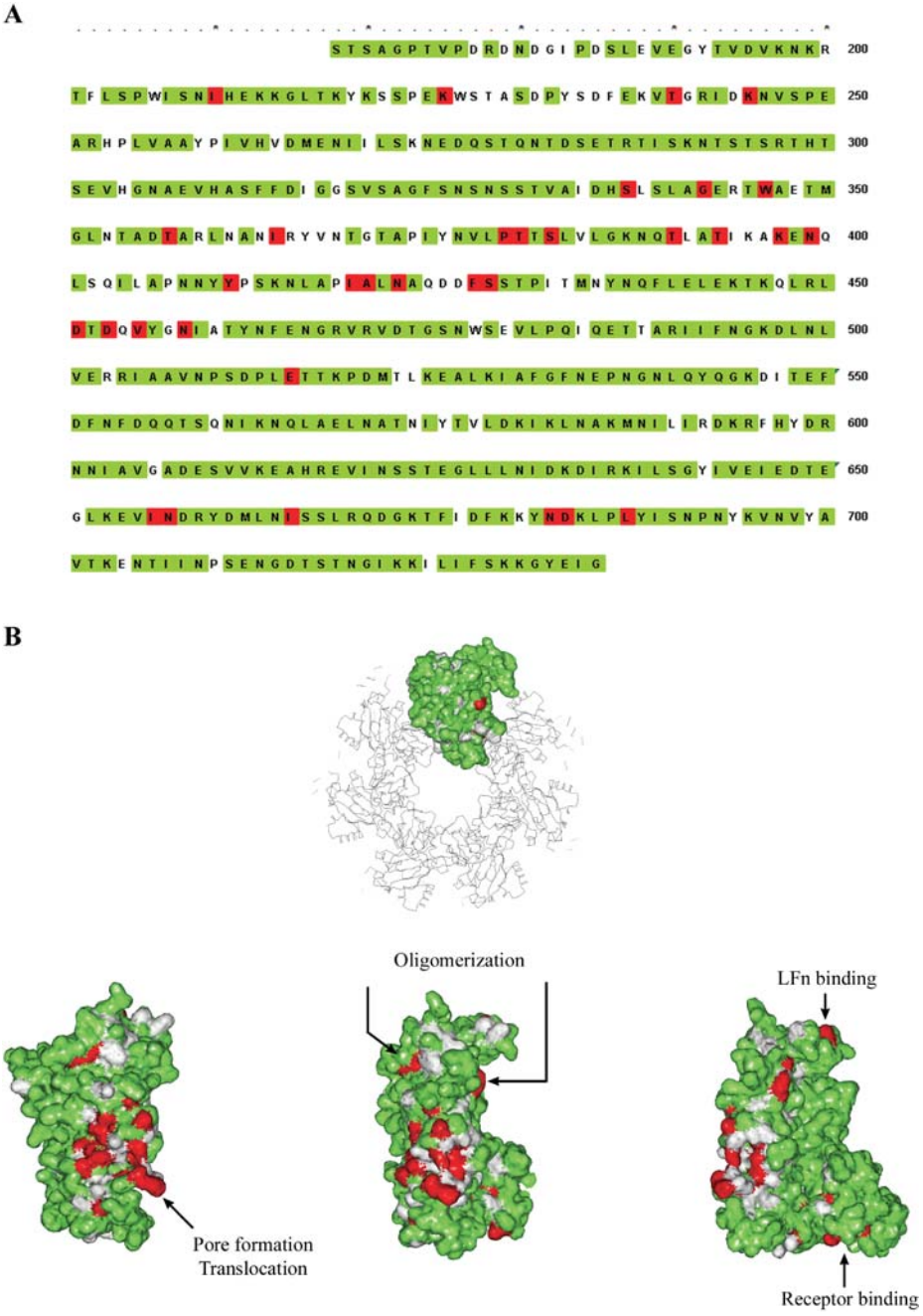

Fig. 3A, B Global structure–function study of PA_{63}. Summary of the results of the cysteine scanning experiment performed on PA_{63} (Mourez et al. 2003). **A** Sequence of PA_{63}. **B** Molecular surface of a PA_{63} monomer isolated from the heptamer, top view (*top*) of the heptamer with the highlighted PA_{63} monomer; and three views of the monomer: a left-side view (*bottom left*), a center view (*bottom middle*) and a right side view (*bottom right*), as viewed from inside the lumen of the heptamer. Both in **A** and **B**, the residues for which changes to a cysteine resulted in an unstable, active, or inactive mutant are colored *white*, *green*, and *red*, respectively. The functions affected by mutations of the red residues are indicated in **B**

was hypothesized that this flexible loop might insert into membranes at low pH (Petosa et al. 1997). The loops of all the monomers would then form a β-barrel channel in the membrane. The structure of the pore form of the heptamer remains elusive, but various approaches were developed to test this hypothesis. By replacing each residue of the flexible loop with cysteine the accessibility of the mutants at low pH can be tested with a thiol-reactive membrane impermeant reagent (Benson et al. 1998). The reagent used adds a charge, restricting the conductance of the channel that can be measured in planar lipid bilayers. This showed that the loop inserts into the membrane and structures itself as two β-strands of alternating hydrophilic and hydrophobic residues. This is the typical structure of the strands of a β-barrel.

The insertion of the flexible loops would require a major rearrangement in the rest of domain 2 and presumably the unfolding of a Greek-key motif consisting of four β-strands (Petosa et al. 1997). To test this hypothesis the cysteine mutagenesis and accessibility study was extended beyond the flexible loop (Nassi et al. 2002). This showed that the barrel structure extends beyond the flexible loop and confirms the existence of a major rearrangement in the rest of domain 2 upon insertion. The structural rearrangement taking place upon pore formation is likely responsible for the sodium dodecyl sulfate (SDS) resistance of the pore, whereas the prepore is SDS sensitive (Miller et al. 1999; Milne et al. 1994).

The identification of the residues that provoke this conformational change upon a drop in pH, the "pH trigger," remains elusive. It was postulated that histidine residues, which have a protonation state likely to change between pH 7 and pH 5, might be involved (Petosa et al. 1997). In domain 2, histidines are located at the top of the flexible loop or in a loop with a pH-sensitive mobility. No confirmation that these residues are indeed part of the pH trigger has been obtained.

In the cysteine-scanning study (Mourez et al. 2003), most of the mutations that abolished the activity of PA were found in domain 2 and clustered in the region forming the lumen of the prepore. This region seems therefore peculiarly important. Most of these mutants were unable to make pores and translocate LFn (M. Mourez and R.J. Collier, unpublished data; Mourez et al. 2003). Mutants in some charged residues in the lumen of the prepore mutations were shown to have similar effects (Sellman et al. 2001b). Some of these mutations were found to have a dominant-negative effect, with likely as little as one mutant molecule per heptamer being enough to block the activity of the whole heteroheptamer (Sellman et al. 2001a). In the cysteine scanning study, dominant-negative mutants were found only in domain 2 and clustered in the 2β6 strand, 2β7 strand, and the 2β10–2β11 loop (Mourez et al. 2003). All these residues are located in the lumen and are solvent accessible in the prepore structure. This suggests that the lumen, in addition to the flexible loop, is critically important in pore formation/translocation. The dominance of these mutations suggests that the pore formation process involves a structural rearrangement where some residues of the lumen of the prepore find themselves making contacts in the pore structure or in a transition state. This again suggests that the conformational change taking place upon lowering the pH is extensive.

The N-terminal domain, domain 1 (residues 1–249), contains the furin cleavage site at position 167. The domain has therefore two subdomains, PA_{20} (residues 1–167) and domain 1′ (residues 167–249). Domain 1′ has two calcium ions bound through a modified EF-hand motif. These calcium ions are tightly bound and most likely play a structural role (Gao-Sheridan et al. 2003; Gupta et al. 2003). Consistent with these results, most of the unstable cysteine mutants mapped to the calcium binding sites (Mourez et al. 2003).

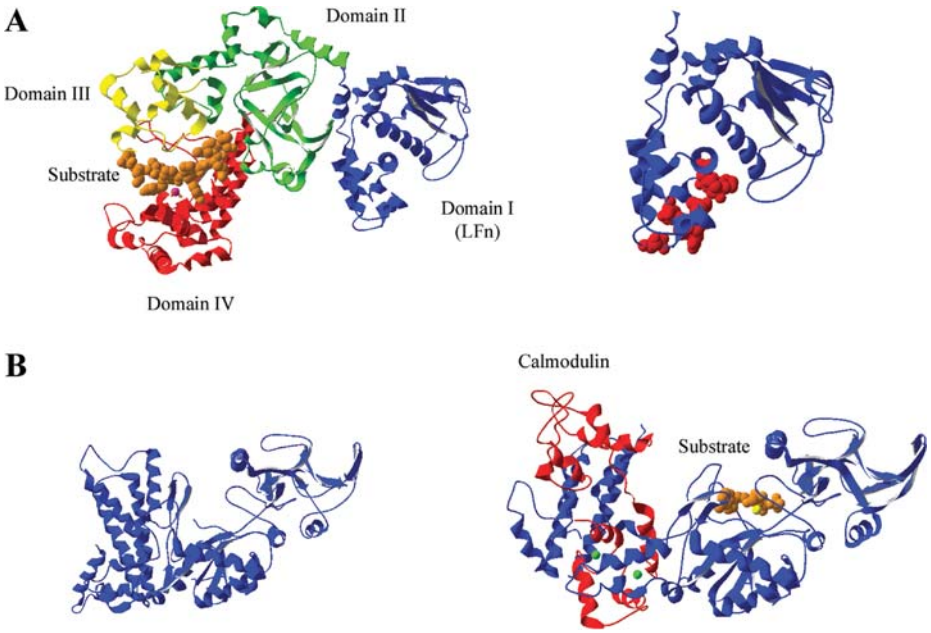

Fig. 4A, B Crystal structure of LF and the catalytic domain of EF. **A** Ribbon representation of the crystal structure of LF bound to an optimized peptide substrate (Turk et al. 2004): whole molecule (*left*) and close up view of the structure of LFn in the same orientation (*right*). The four domains are represented by different colors: domain I in *blue*, domain II in *green*, domain III in *yellow*, and domain IV in *red*. The zinc atom is represented as a *pink sphere* and the substrate as *orange spheres*. The residues that, when changed to an alanine, cause the resulting mutants to be unable to bind to PA (Lacy et al. 2002) are represented by *red spheres*. **B** Ribbon representation of the crystal structure of the catalytic domain of EF alone (*left*, Drum et al. 2002) or bound to calmodulin and a high-affinity inhibitor (*right*, Shen et al. 2004). EF is represented in *blue* and calmodulin in *red*. The calcium atoms bound to calmodulin are represented as *green spheres*, the divalent cation bound to EF as a *yellow sphere* and the inhibitor, adefovir diphosphate, as *orange spheres*

In addition to this structural role, domain 1′ is also important in PA_{63} oligomerization (Mogridge et al. 2002b) and is the site of interaction of PA with its ligand (Chauhan and Bhatnagar 2002; Cunningham et al. 2002). The release of PA_{20} uncovers a surface that was buried before. Critical residues in the binding of LFn were found by mutating this surface (Cunningham et al. 2002). By combining oligomerization mutants in order to make PA_{63} dimers together with the mutations in domain 1′ it was possible to define the residues involved in the unique ligand binding site of a PA_{63} dimer (Cunningham et al. 2002). This showed that the EF/LF binding sites are located across the monomer–monomer interface and are close to one another. Binding on one site will therefore likely sterically prevent binding on the adjacent ones, and gives a rationale for the odd stoichiometry of three ligand molecules bound per heptamer (Mogridge et al. 2002b).

The structure of LF was solved in the presence of a Mek 2-derived peptide substrate (Pannifer et al. 2001) as well as with an optimized substrate or inhibitors (Panchal et al. 2004; Turk et al. 2004). LF has four domains (Fig. 4). The N-terminal domain, domain I or LFn, corresponds to residues 1–263. The first 27 residues were not resolved in the structure, but truncation of up to residue 36 has no impact on binding or translocation (Lacy et al. 2002); therefore, this region is not believed to have major functional importance. LFn is composed of a bundle of 12 α-helices and 6 β-strands making two sheets on

one face of the molecule. Conserved residues between EFn and LFn could be mapped on the surface of LFn (Lacy et al. 2002). The conserved residues clustered to a patch of LFn that by mutagenesis studies was shown to be the PA binding site (Lacy et al. 2002). Domain II (residues 263–550, excluding residues 300–386) is the only part of LF having structural similarities with other proteins. It resembles the fold of the ADP ribosyltransferase of the vegetative insecticidal toxin of *B. cereus*. Domain III (residues 3000–386) is an α-helical bundle made up of four imperfect sequence repeats from a feature of domain II. It is inserted in domain II, and there is evidence that it has functional significance (Arora and Leppla 1993), possibly helping in substrate recognition. The C-terminal domain, domain IV (residues 551–777), has the zinc protease site. The active site is similar to that of the thermolysin proteases family with the HEXXH motif located in an α-helix neighboring a four-stranded β-sheet. The substrate binding site is a long cleft formed by domain II and parts of domains III and IV (Tonello et al. 2003; Turk et al. 2004). In the original structure the peptide substrate was not bound in a productive conformation, but a later structure shows a peptide substrate bound in a correct orientation (Tonello et al. 2003; Turk et al. 2004). Peculiarly, the folds of domains IV and I (LFn) are highly similar, except that catalytic residues (and especially the HEFGH motif) have been mutated in the LFn structure (to a YEIFGK sequence). This modularity based on duplication and mutations suggests an interesting evolution scenario (Lacy and Collier 2002).

The crystal structure of the catalytic C-terminal portion of EF (residues 291–800, which excludes EFn) was solved alone, in the presence of calmodulin, and in the presence of calmodulin and a noncyclizable analog of ATP (Fig. 4 and Drum et al. 2002). Since EFn and LFn have sequence homologies and are functionally identical, it is believed that they are structurally related, and therefore a complete picture of EF could be modeled. Contrary to expectations, there are no significant homologies with mammalian adenylate cyclases. The structures show the conformational changes arising upon binding of calmodulin. This is the first insight into how calmodulin modulates a biologically active substrate. An α-helical domain undergoes a 15-Å translation and 30° rotation leading to the activation of the catalytic site. The catalytic site has a single metal ion coordinating the substrate and positioning it for the catalytic action of histidine 351. This contrasts with the mechanism of mammalian adenylate cyclases, which do not possess catalytic histidines and use a second metal ion instead. Calmodulin itself is bound in an extended conformation that is also different from previous structures obtained with peptides derived from mammalian adenylate cyclases. These differences might explain the enhanced activity of EF compared to mammalian counterparts.

The structure of an assembled toxic complex has yet to be obtained. However, the structure of LF, the structure of the prepore, and the identification of the binding sites on those structures opens the possibility to compute molecular models of an assembled toxic complex. These models can then be tested by measuring molecular distances, for instance using fluorescence resonance energy transfer. One such study was recently published (Croney et al. 2003) and it is expected that more of these studies will yield a complete and possibly dynamic picture of the interactions between PA_{63} heptamers and their bound substrates.

In vivo effects of anthrax toxins

Targeting of macrophages by LTx and death by inflammatory shock

After many unsuccessful attempts at finding a cell line with a measurable phenotype when treated with LTx, it was noted that some murine macrophage-like cell lines were lysing after 2 h in contact with LTx (Friedlander 1986). It was also noted that the sensitivity of these macrophages was correlated to that of the mice strains from where the cell line originated (Hanna et al. 1993). This led to the hypothesis that macrophages play a central role in anthrax pathogenesis. Reports that sublytic doses of exposure to LTx triggered release of pro-inflammatory cytokines by sensitive macrophages offered a possible explanation for their role during infection (Hanna et al. 1993): low doses of LTx would induce in the macrophages a cytokine build up which would ultimately be released upon lysis and induce a shock. This is consistent with the observation of "shock-like" death observed in animals challenged intravenously by LTx (Smith et al. 1955). Consequently, the "macrophage hypothesis" has been cited in many reviews and focused the attention of research groups on macrophages and on cytokine expression modulation by LTx. However, conflicting reports soon emerged.

What is the relevance of macrophage lysis by LTx?

When the cellular targets of LF, Meks, became known, it also became apparent that there was no difference in their cleavage between "sensitive" and "resistant" macrophages (Salles et al. 2003; Watters et al. 2001), raising the possibility that macrophage lysis is due to unspecific mechanisms rather than the direct action of the toxin. Moreover, the susceptibilities of animals to LTx and that of their macrophages is not perfectly correlated as (1) strains of inbred mice that harbor "resistant" and "sensitive" macrophages are both sensitive to LTx with only relatively minor differences; and (2) some resistant macrophages come from species, such as rats or humans, that are sensitive to LTx and anthrax (Cui et al. 2004; Kim et al. 2003; Popov et al. 2002a).

Three linked loci, Ltxs1–3, have been implicated in the difference of resistance of macrophages from resistant (C57BL/6, DBA/2J) versus sensitive (C3H/HeJ, BALB/c) strains of inbred mice (McAllister et al. 2003; Watters et al. 2001). The only gene positively identified from these loci is *kif1C*, encoded in Ltxs1 (Watters et al. 2001). Kif1c is a ubiquitously expressed kinesin-like motor protein likely involved in the intracellular transport of some molecular cargo. A human homolog of Kif1C has been shown to be involved in the retrograde transport from Golgi to the endoplasmic reticulum, but the nature of the cargo is unknown. Consistent with a role of molecular shuttling in LTx sensitivity, brefeldin A treatment increased sensitivity of "resistant" macrophages to LTx and correlated with Kif1c relocalization (Watters et al. 2001). However, no direct link between Kif1c and LF activity can be made at this time, and therefore the role of Kif1c in the lysis phenomenon strengthens the idea that lysis is only a peripheral event during LTx action.

Recent reports have shown that destruction of macrophages might not be restricted to the sensitive cell lines. LTx was shown to induce the apoptosis of macrophages when these cells are activated by lipopolysaccharide (LPS) (Park et al. 2002). Another study also found the hallmarks of apoptosis in LTx-treated macrophages (Popov et al. 2002b). Such an event might occur in vivo as caspase inhibitors were reported to have protective

effects during infection of mice with *B. anthracis* (Popov et al. 2004). Another group reported that macrophage lysis by LTx could be triggered in "resistant" macrophages by addition of peptidoglycan or poly-D-glutamic acid capsule (Kim et al. 2003). The authors propose that the gain of sensitivity is due to tumor necrosis factor (TNF)-α release by these components and an autocrine effect. However, in this study lysis was found to be consistent with necrosis but not with apoptosis. Regardless of this discrepancy with the previous reports, collectively these studies revive the hypothesis that macrophage destruction is a relevant phenomenon in anthrax.

In order to understand the extent of the impact of LTx treatment on macrophages and how lysis/apoptosis/necrosis could be triggered, one group undertook a transcriptional profiling experiment using DNA array (Tucker et al. 2003). This approach showed the differential regulation upon exposure to toxin of a number of genes controlled by glycogen synthase kinase 3 β (GSK3β). This signaling pathway is not well studied in macrophages but is better understood in the context of embryonic development, especially that of the zebrafish. Thus, among other experiments designed to confirm LTx action on the GSK3β pathway, the authors challenged zebrafish embryos with LTx. Developmental abnormalities appeared which correlated well with what would be expected from impairment of GSK3β activity. More work is needed in order to ascertain the identities and roles of the genes regulated by GSK3β that could be implicated in LTx action on macrophages or other host cell types. It is interesting to note that among the genes identified in this study were genes coding for kinesins.

The picture of LTx action on macrophages is even more complex than previously thought. A recent report showed that sensitive macrophages can be "desensitized" to lytic challenge with LTx by prior incubation with sublytic amounts of toxin (Salles et al. 2003). This "toxin-induced resistance" might represent an example of the adaptability of macrophages during infections. On the molecular level the authors showed that despite proteolysis of Mek 1 and Mek 2, the sublytic challenge induced a recovery of downstream targets of these kinases by an unknown compensatory mechanism. The authors speculate that this compensation might account for the observed resistance. Indeed, the authors showed that the kinetic of Mek cleavage was much faster in sensitive cell lines compared to resistant ones. Thus, resistance to LTx might be due to a slower kinetic of Mek cleavage, giving more time to allow a compensatory mechanism to establish itself. Interestingly, this is consistent with a careful study of the kinetics of LF cleavage performed with a model substrate (Tonello et al. 2003). This study suggests that because of differences in the structure of their cleavage sites, Meks are likely to be cleaved non-uniformly, implying that LF will have a considerably variable activity in different cell types and that even in one cell type the activity is likely to differ, depending on environmental signals. These phenomena might contribute to the discrepancies between the studies performed with LTx and macrophages.

Does LTx induce or suppress inflammatory responses?

Contrary to the previous report described above (Hanna et al. 1993), in recent years many groups reported that LTx did not induce pro-inflammatory cytokines either in cells or in animals (Erwin et al. 2001; Moayeri et al. 2003; Moayeri and Leppla 2004; Pellizzari et al. 1999; Popov et al. 2002a). In fact, the induction of such cytokines and the release of nitric oxide in response to classical stimuli like LPS or interferon (IFN)-γ were inhibited

(Pellizzari et al. 1999). Furthermore, if the hypothesis of the release of pro-inflammatory cytokines were to be true, inhibiting or preventing the inflammatory response during LTx challenge should decrease the effects of the toxin. However, an irreversible inhibitor of the inducible nitric oxide synthase (iNOS), which decreases the inflammation response, increased LTx sensitivity of mice (McAllister et al. 2003); and strains of "knock-out" mice for the receptor of IL-1 or iNOS were shown to be more susceptible to *B. anthracis* (Kalns et al. 2002). Inhibition of pro-inflammatory cytokine release in human peripheral blood mononuclear cells stimulated by *B. anthracis* cell wall was also reported (Popov et al. 2002a). Recently, it was shown that LTx had an inhibitory effect on IFN-regulatory factor 3 activation by LPS (Dang et al. 2004). Since this regulatory factor is essential in type I interferon expression during LPS activation, this could explain the inhibitory effects of LTx on cytokine expression.

It was also shown that LTx could inhibit glucocorticoid receptor (GR) function in transfected cells and in BALB/c mice (Webster et al. 2003). Glucocorticoids play an important role in the regulation of the inflammatory response. The perturbation of the inflammatory response by the inhibition of GR could prevent the host from clearing an infection with *B. anthracis*. Little is known, however, about how LTx can affect the GR, other than the fact that a catalytically active LF is required. Moreover, this study raises the possibility that GR are not the only nuclear receptors affected by LTx. Indeed, the same study showed an effect on progesterone receptor and estrogen receptor α, but none on mineralocorticoid receptor and estrogen receptor β. The relevance of these observations is unclear.

Lastly, it should be noted that it is difficult to assess the role of the toxin on cytokine release in an infectious setting from the studies with purified LTx. Two reports by the same group show, on the one hand, LTx-mediated inhibition of pro-inflammatory cytokine release in vitro (Popov et al. 2002a), and, on the other hand, significant cytokine release in vivo in mice infected with *B. anthracis*, with some differences between LTx sensitive and resistant mice strains (Popov et al. 2004). The authors suggest that the cytokines are released by cells undergoing LTx-induced apoptosis, especially in the liver. The controversy over whether or not LTx induces cytokine release persists. It is unfortunately very important to solve this question, as cytokine-oriented therapies are being advocated to treat anthrax.

Are there other cellular targets of LTx?

Toxin production is induced very early after germination, while bacteria are still in the macrophages. Two studies have addressed the role of toxins in those early events of the infection by looking at germination of spores in macrophage cell lines. These studies yielded conflicting results. In one study, after germination the bacteria were found to escape the macrophages phagosomes, replicate in the cytosol, and get released from the macrophages, all events being independent of toxin production (Dixon et al. 2000). In another study, the newly germinated bacteria did not escape phagosomes, did not replicate, and escaped macrophages only thanks to toxin production (Guidi-Rontani et al. 2001). Few new data have helped resolve this contradiction, and it is therefore still unclear if the toxins play a role in the escape from macrophages. Another report showed that when using bestatin, an aminopeptidase inhibitor with some activity against LTx, spore-infected human mononuclear cells showed higher sporicidal, bactericidal, or both activities (Popov

et al. 2002a). This supports a role for LTx in early events of infection like macrophage escape.

It was recently shown that LTx has a profound impact on the function of dendritic cells by disrupting their interactions with T cells (Agrawal et al. 2003). Such interactions are a prerequisite for activation of the T cells and subsequent induction of an inflammatory response, production of antibodies by B cells, and differentiation of B and T cells into memory cells. These are main events in the establishment of acquired immunity. The authors propose that by its effect on dendritic cells, LTx could prevent the host from containing an infection by *B. anthracis*. Although this hypothesis is tempting, it remains to be tested. It is, for instance, unclear if the host has time to mount an effective response involving dendritic cells during an infection with *B. anthracis*.

It is puzzling that only immune cells seem to be affected by LTx. Since virtually all cell types have receptors for the toxins and Meks are so central in cellular physiology, it is likely that LTx can have consequences on other cell types. As a first example of such a possibility, an in vitro experiment showed that LTx could have a cytotoxic action on endothelial cells (Kirby 2004). This is highly significant because of the numerous observations that LTx action in animals is consistent with a vasculature collapse (see below). This study showed that LTx induced caspase-dependent apoptosis of different kinds of endothelial cells. In this cell type, LTx cleaved Meks and inhibited downstream pathways that the author suggests are responsible for the cytotoxicity observed.

Synergy with ETx and role of ETx

Strikingly, little work has been done on the mechanism of action and the role of ETx in an infection compared to all the reports with LTx. This is an unfortunate oversight. By inactivating the genes coding for EF or LF in an attenuated strain of *B. anthracis* still lethal to mice, it is clear that both genes are required for full virulence in a mouse model of infection (Pezard et al. 1991). This suggests that ETx and LTx act synergistically during infection. It is interesting to note that ETx was shown to down-regulate the production of TNF-α and IL-6 by monocytes stimulated with LPS (Hoover et al. 1994). These effects are consistent with the suppression of the inflammatory response of macrophages reported in studies with LTx, and could explain why there is a synergy between the two toxins.

Little is known about the mechanism of edema formation by ETx in vivo. It is usually assumed that ETx acts similarly to cholera toxin, causing an increase in cAMP in cells and an efflux of water. However, the nature of the cells affected by ETx during an infection is undetermined. A mouse adrenocortical cell line and a human polarized epithelial cell line have been shown to undergo macroscopic changes upon treatment with ETx (Beauregard et al. 1999; Soelaiman et al. 2003).

How do toxins cause “shock-like” death in animals?

The consensus that emerged from the original work on LTx is that the toxin caused death by inducing shock-like symptoms and vascular leakage (Beall and Dalldorf 1966; Smith and Stoner 1967). Before that consensus was reached, other theories had been put forth, such as blockage of the capillaries (Dalldorf and Beall 1967) or an effect on the central nervous system (Bonventre et al. 1967; Remmele et al. 1968; Vick et al. 1968). In one ex-

periment, primates challenged intrathecally with LTx died within 10 min of the injection, from respiratory blockade (Vick et al. 1968). Unfortunately, these experiments were never reproduced later and in our hands intracerebral challenge of Fisher 344 rats with ten times the minimum intravenous lethal dose of LTx did not cause any symptoms (M. Mourez, unpublished data). This could be due to inherent differences between animal models. Death of animals by shock is therefore the currently accepted hypothesis.

Several different events, however, can cause a shock. Because of the previously reported lysis of macrophages and induction of pro-inflammatory cytokine release, it was argued that in anthrax the shock could be induced by cytokine release (Hanna et al. 1993). However, the recent animal studies cast doubts on this hypothesis. Most notably, a careful pathological study was undertaken by Leppla and co-workers to look at the effect of LTx on "resistant" and "sensitive" strains of inbred mice (Moayeri et al. 2003). This study did not find any evidence of inflammation related pathology or any systemic release of pro-inflammatory cytokines. Depletion of circulatory mononuclear cells that could be attributed to LTx-mediated lysis was observed in sensitive mice strains and not in resistant ones but was not paralleled in fixed tissue macrophages and was not correlated with an alteration of the pathological findings. Ultimately, this study determined that animals died of pleural fluid accumulation and liver failure probably due to hypoxia. Both effects could be due to vascular permeability alterations. The hypoxic response induced by LTx is highly significant, since a severe hypoxia has often been reported in cases of anthrax. It is then interesting to note that the strain of mice more resistant to LTx is also more resistant to hypoxia. No vascular collapse, coagulopathy, or endothelial cell damage could be seen. Lastly, this study showed that LTx treatment induced a high neutrophilia and induction of KC chemokine expression, a potent neutrophil recruiter. Both mediators seem to be expressed by hepatocytes and therefore the neutrophils and hepatocytes might represent specific targets of LTx. The precise mechanism and order of the hypoxia and vascular collapse still have to be determined.

The effect of a continuous infusion of LTx in rats was also recently published by the same group and, again, no inflammation or cytokine release could be observed (Cui et al. 2004). In this model, however, no hypoxia was observed. Instead, hypotension and acidosis was noted. This is in contrast to the previous belief that the response of rats to LTx is a pulmonary change causing edema and cardiovascular collapse (Beall and Dalldorf 1966). In the uniquely sensitive Fisher 344 rat model, a pulmonary edema is evident as quickly as 60 min postchallenge with LTx (Ezzell et al. 1984).

As discussed above, LTx was recently shown to have an inhibitory effect on glucocorticoid receptors (Webster et al. 2003). The inhibition of GR might exacerbate the hypoxic shock. Interestingly, the Fisher 344 rat strain is known to have an aberrant glucocorticoid response that in some situations can cause death of the animals. Since the same strain of rats is exquisitely sensitive to LTx, it raises the possibility that GR inhibition by LTx is responsible for this sensitivity. Combining this result with the study of LTx effects in rats (Cui et al. 2004), one hypothesis could be that through inhibition of GR, LTx might affect vascular integrity or vasoconstrictor functions.

Conclusion: what are the roles of the toxins in an infection?

The effects and roles of the toxins in vivo are far from clear. With so many possibilities being discussed, it seems especially important at this stage to put the question back in the context of a *B. anthracis* infection. With that perspective it can be expected that the toxins possibly exert three kinds of effects: (1) As the spores germinate in the macrophages the bacteria start producing toxins; thus it is conceivable that the toxins have an intracellular effect that could ultimately result in the release of bacteria from macrophages. Once bacteria are released they continue to secrete the toxins, which can then have a (2) local as well as a (3) systemic effect.

With only two studies available yielding opposing results, the ability of the toxins to promote release of the bacteria from phagocytes is an interesting hypothesis, but it still awaits confirmation. The local effect of the toxins, on the other hand, seems to be reaching a consensus. It is likely that LTx and ETx act synergistically locally to promote bacterial infection by perturbing the host immune response. This is probably achieved through multiple actions including cytokine release inhibition and macrophage destruction. Edema is another local response mediated apparently solely by ETx. It is assumed that the adenylate cyclase activity on some cells can cause an electrolyte imbalance through a cAMP concentration increase, similarly to cholera toxin. Lastly, death is a systemic effect mediated apparently only by LTx. This "effect" remains mysterious, and this raises the question: Is it an aberration or a relevant pathological mechanism? One challenging observation, for instance, is that in vivo LTx sensitivity can be inversely correlated to the sensitivity to anthrax infection, i.e., mice more sensitive to LTx are more resistant to bacterial challenge (Welkos et al. 1986). This casts doubts about the real significance of death caused by LTx. Lastly, the prevailing hypothesis put forward to explain death has been the induction of shock by cytokine release. It seems likely that this hypothesis will come under more scrutiny and might even be replaced by another in which LTx causes shock through hypoxia and vascular collapse. These effects could, for example, be mediated by toxicity to endothelial cells or by an effect on glucocorticoids.

Fighting anthrax toxin

In most cases, cutaneous anthrax can be treated easily with antibiotics. In the case of inhalation anthrax, however, as detailed above, antibiotic treatments are not enough, presumably because the toxins are unchecked. The recent use of anthrax in an attack has therefore triggered an intense search for drugs that act against the toxin.

Even before the toxins were discovered, passive immunization was demonstrated to protect rabbits using sheep hyperimmune serum (Gladstone 1946). The source of antigen was in the serum of infected animals, and therefore it was stated that *B. anthracis* secreted a "protective antigen." It was later realized that this antigen is part of anthrax toxin. An attenuated strain of *B. anthracis* was discovered by Sterne and found to be a very effective vaccine in animals (Sterne 1937). This Sterne strain is not encapsulated but produces toxin and it was found that the main immune reaction was directed against the toxin components, and mainly PA. Consequently, a cell-free vaccine was prepared from supernatants of cultures of a Sterne strain adsorbed to aluminum hydroxide gel. This vaccine is the only licensed commercial vaccine used in humans (Friedlander et al. 2002). The immune response is believed to help clear the toxin from the serum of infected patients and is there-

fore, in a sense, an anti-toxin therapy. The acellular anthrax vaccine adsorbed is very effective, but the immunization schedule is cumbersome: it has to be administered at 0, 2, 4 weeks and 6, 12 and 18 months with yearly boosters.

Many new formulations (recombinant PA and/or LF, adjuvant) and delivery vectors (naked DNA, live bacteria, viruses) have been tested to try and improve the safety and immunization schedule (Friedlander et al. 2002). For instance, it was shown that additional antigens besides the toxins are needed for optimal immunization, like antigens from spores or capsules that could enhance phagocytosis (Brossier et al. 2002). Consequently, promising experimental vaccines, including capsule and PA antigens have been designed (Rhie et al. 2003; Schneerson et al. 2003).

A prophylactic approach might not be economically viable, however, or even accepted by the public. Therapeutics able to neutralize the toxin after the exposure to *B. anthracis* spores are therefore needed. There are a number of steps in the intoxication process that can be targeted and various types of inhibitory molecules that can be used to block them.

Inhibitors of receptor binding

Experiments showed that a polyclonal anti-PA antibody could save some guinea pigs from a lethal anthrax challenge (Little et al. 1997). However, neither anti-PA monoclonal antibodies nor anti-EF or anti-LF polyclonal antibodies had any effect (Little et al. 1997). Similar experiments showed that polyclonal anti-PA antibodies were effective in treatment of challenged guinea pigs (Kobiler et al. 2002) and in the treatment of challenged mice in conjunction with antibiotics (Karginov et al. 2004). Efforts are now being made to obtain antibodies that could be used in humans. For instance, recombinant single-chain antibodies have recently been obtained that showed inhibitory activities against LTx in vitro and in vivo (Maynard et al. 2002). In another approach, human antibodies fragments from phage-displayed libraries generated from immunized donors were screened against PA. Selected clones displayed inhibitory activity in vivo (Wild et al. 2003). There is some evidence that the protective epitopes are located in domain 4 of PA, and therefore it is likely that all these active antibodies prevent PA binding on cells (Flick-Smith et al. 2002; Rosovitz et al. 2003).

The discovery of receptors for anthrax toxin opened up the possibility to directly target these molecules. An inhibitor could be designed to bind on the metal ion-binding motif and compete with PA for binding. Alternatively, a soluble mimic of the receptor could function as a decoy preventing binding of PA on cells. Since the functions of TEM8 and CMG2 are not known, those approaches have potential toxicity risks. Still, soluble parts of TEM8 were shown to have potent inhibitory effects in cell cultures and in rats challenged with LTx (Bradley et al. 2001; Scobie et al. 2003).

Inhibitors of furin activation

Small molecules could be designed to block the furin-like proteases and prevent PA activation. Furin is a protease involved in many biological processes, and therefore inhibiting its activity might prove to have serious toxic consequences. Despite these concerns, a small peptide of hexa-D-arginine proved to be a stable inhibitor of furin and was shown to be able to inhibit anthrax toxin in vivo and in cell cultures (Sarac et al. 2004). The struc-

ture of furin (Henrich et al. 2003) will probably help in the design of specific small molecule inhibitors of this protease, which might prove effective.

Inhibitors of assembly

Some molecules could prevent the binding of EF/LF to PA_{63} heptamers. Such an inhibitor was developed based on peptides selected from phage-displayed libraries (Mourez et al. 2001). The peptides were selected in order to specifically bind to the surface involved in the interaction of PA_{63} heptamers with EF/LF. The authors assumed that each peptide could bind to one of seven sites on a heptamer. If true, then by grafting multiple copies of the peptides on a flexible polymeric backbone, a molecule could be designed that would bind PA_{63} heptamers at multiple sites simultaneously. This strategy allowed for the design of polyvalent molecules with dramatically improved efficacy compared to the peptide alone. The molecules were able to prevent the toxicity of LTx administered to rats. Peptidic inhibitors are, however, notoriously prone to degradation and the backbone used was polyacrylamide. Further improvements are therefore required before these molecules can be made into viable therapeutics.

Inhibitor of translocation

As stated above, some PA mutants unable to promote translocation were shown to be dominant negative and block in vitro the action of LTx (Mourez et al. 2003; Sellman et al. 2001a; Yan and Collier 2003). These mutants have also proved to be very effective in vivo. Compared to the inhibitors described above, the PA mutants have the advantage of being "optimized" for in vivo stability and targeting. In addition, PA is already administered to humans in a vaccine formulation. One disadvantage, however, is the fact that PA mutants will bind to the ubiquitous receptors and therefore are likely to be taken out of the systemic circulation quickly.

Inhibitors of enzymatic activities

With the solving of the structures of EF and LF and the understanding of their catalytic activities, the door has been opened for an intense search of small molecule inhibitors. There are already a huge number of commercial drugs active against proteases that could have an effect against LF. Since the discovery of the enzymatic activity of LF many groups have designed high-throughput screens and used the crystal structure of LF to look for small molecule inhibitors (Cummings et al. 2002; Panchal et al. 2004; Tonello et al. 2002). As a result, hydroxamate derivatives of substrate peptides have been designed and have in vitro inhibitory constants in the nanomolar range (Tonello et al. 2002). Non-peptidic inhibitors have also been obtained with an in vitro inhibitory constant in the micromolar range (Panchal et al. 2004; Turk et al. 2004).

Using the crystal structure of EF it became possible to screen a database of small molecule compounds that could specifically inhibit its enzymatic activity. This allowed the identification of a number of lead compounds with inhibitory constants around 20 μM (Soelaiman et al. 2003). Recently, the same group showed that adefovir dipivoxil, a drug already approved in the treatment of chronic infection with hepatitis B virus, was active

against EF (Shen et al. 2004). The inhibition constant was 27 nM, lower than the previous leads, and crystal structure of the inhibitor in complex with EF and calmodulin revealed a strong interaction of the active metabolite of the drug with EF. This tight contact explained a 10,000-fold higher affinity of EF for the drug over its natural substrate, ATP. Interestingly, these compounds are also effective against *B. pertussis* adenylate cyclase.

Unknown mechanism of action

A number of other drugs have been shown to have protective effects either in cell cultures or in animals: inhibitors of proteasome (Tang and Leppla 1999); intracellular calcium agonists (Bhatnagar et al. 1989; Shin et al. 2000); antioxidants like *N*-acetyl-L-cysteine and mepacrine (Hanna et al. 1994); phospholipase C and protein kinase C inhibitors (Bhatnagar et al. 1999); protein synthesis inhibitors (Bhatnagar and Friedlander 1994); and inhibitors of an unknown protein phosphatase by calyculin A (Kau et al. 2002) all showed some protecting effects against LTx challenge. These effects are difficult to interpret and/or transpose in a clinical setting.

Using anthrax toxin

Some investigators have decided to capitalize on the ability of anthrax toxins to be translocated in vivo in the cytoplasm of almost any cell type to design innovative therapeutics.

Epitope delivery system

As stated above, LFn was found to be required and sufficient for binding to PA_{63} and for delivery of recombinant polypeptides fused to its N or C terminus in the cytosol of target cells in the presence of PA. Although this system does not allow the delivery of all polypeptides (Wesche et al. 1998), it was shown to allow the in vivo delivery of a cytotoxic T lymphocyte epitope from an intracellular pathogen in mice (Ballard et al. 1996). This delivery stimulated a cytotoxic T lymphocyte response in the absence of any other co-stimulant, which could protect mice from a challenge with the pathogen. This showed that an engineered anthrax toxin could be used as a basis to design cellular vaccines effective in vivo. A cytotoxic immune response against human immunodeficiency virus (HIV) could also be achieved using this system (Lu et al. 2000).

Antitumor effect

Some tumors have elevated levels of active MAPK including breast carcinoma and glioblastoma. The idea that LTx could be used as an antitumor therapeutic was recognized when it was shown that LF inhibited MAPK signaling (Duesbery et al. 2001). LTx antitumor activity was tested in vitro on transformed cells and in vivo on transformed cells implanted in athymic nude mice. In both cases LTx was able to inhibit transformation and growth (Duesbery et al. 2001). The use of unmodified LTx raised safety concerns and subsequent attempts were made to specifically target tumor cells. To that end, engineered tox-

ins that can only be activated on tumor cells were designed. Recombinant PA molecules were constructed where the furin cleavage site was replaced with those of membrane proteases that are dramatically overexpressed on tumor cells (Liu et al. 2001, 2003). A mixture of the recombinant PA molecules with a fusion of LFn to the enzymatic part of *Pseudomonas exotoxin* A displayed a specific and dramatic cytotoxicity toward a range of transplanted tumors.

Further improvements can still be achieved by preventing recombinant PA binding to normal cells and redirecting it specifically towards tumor cells. In addition, instead of fusing an unspecific toxic moiety to LFn, one could envision grafting an enzymatic moiety with some specific activity against tumor cells. The high specificity against tumor cells that would be achieved by redirecting the binding, activation, and enzymatic activities of a single chimeric toxin toward those cells would make for a safe and efficient therapeutic.

Concluding remarks

The recent years have brought numerous advances in the anthrax toxin field: the structures of LF and EF have been solved; two receptors have been identified; and critical information has been gathered on the molecular details of the toxin's binding, assembly, endocytosis, pore formation, and the enzymatic activities of EF and LF. This progress has made possible the design of potent toxin inhibitors and the use of toxin-based therapies in cancer treatment and vaccines.

Much remains to be learned. The structure of the pore formed by PA_{63} heptamers upon exposure to low pH remains elusive. The same is true about the conformational changes taking place during the transition from prepore to pore. Now that they have been identified, the role of the toxin receptors in these and other events of the intoxication mechanism can also be tested. The fundamental question of how EF and LF are translocated is also unanswered. We know that EF and LF require some unfolding and that PA heptamers are making pores in the membrane. We do not know whether EF and/or LF use the channel as a conduit for their translocation or if they can cross the lipid bilayer around the channel. We also do not know if cellular proteins help in the translocation process or in the refolding of EF and LF. These questions remain a major challenge.

It is strikingly ironic that in vivo studies have been central to the identification of the toxins and yet it is the in vivo effects of the toxins that we understand the least. Edema and death caused by the toxins have been observed for decades; however, the mechanisms that have been suggested to cause these physiological effects are subject to debate. New hypotheses are required. We need to know which cells the toxin targets during an infection and what the effects of the toxin's ability to increase cAMP concentration and cleave Meks are on these cells. We need to keep in mind that effects other than death and edema could be more relevant to the infectious process. And, lastly, we must not forget that EF and LF act synergistically when assessing the roles and action of the toxins. The need for anti-toxin drugs to counter the use of *B. anthracis* spores as weapons and the potential applications of toxin-based new therapeutics has made this research even more essential.

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H. Barth

Uptake of binary actin ADP-ribosylating toxins

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Abstract The focus of this article is on the cellular uptake mechanism of the family of binary actin ADP-ribosylating toxins from clostridia. These toxins are special-type AB toxins, because they are composed of two nonlinked proteins, which have to assemble on the surface of eukaryotic cells to act cytotoxically. The enzymatically active component (A), ADP-ribosylates G-actin in the cytosol of target cells. This leads to a complete depolymerization of the actin filaments and, thereby, to rounding up of cultured cells. The second component of these toxins, the binding/translocation component (B), mediates the transport of the enzyme component into the cytosol.

Abbreviations *B.: Bacillus* · *C.: Clostridium* · *C2I:* Enzyme component of C2 toxin · *C2II:* Binding/translocation component of C2 toxin · *CHO:* Chinese hamster ovary · *DHFR:* Dihydrofolate reductase · *ER:* Endoplasmic reticulum · *Ia:* Enzyme component of iota toxin · *Ib:* Binding/translocation component of iota toxin · *MTX:* Methotrexate · *PA:* Anthrax protective antigen · *v-ATPase:* Vesicular-type H^+-ATPase · *VIP:* Vegetative insecticidal protein · *Hsp:* Heat shock protein

Introduction

Bacterial exotoxins represent well known pathogenic factors for several serious human diseases. By definition, these proteins act independently of the presence of the bacteria that produce them. After secretion by the bacteria, the toxins bind to eukaryotic target cells and are taken up into the cytoplasm by a series of steps including: (a) docking of the toxin to specific receptors on the cell surface; (b) receptor-mediated endocytosis; (c) translocation of the toxin into the cytosol, where they modify specific substrate proteins. This

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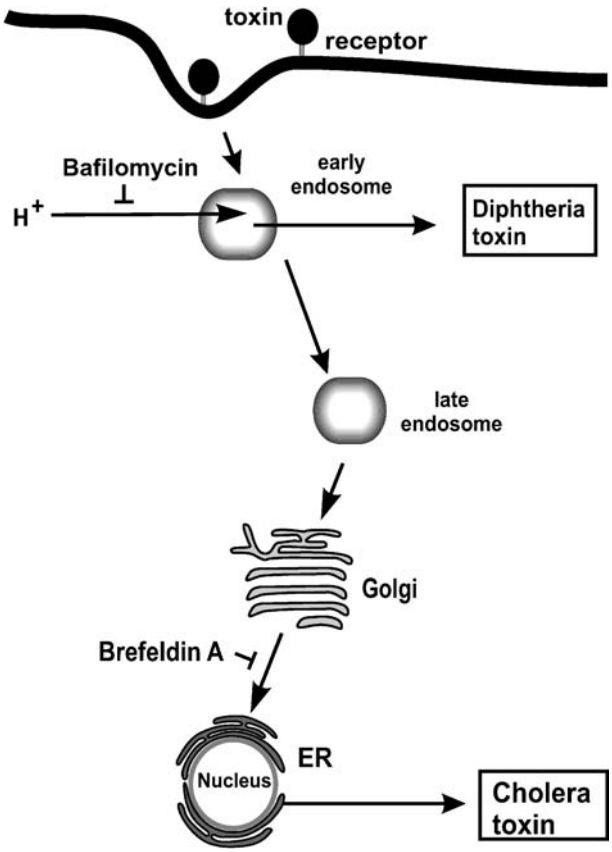

Fig. 1 Uptake pathways of bacterial toxins into eukaryotic cells. Following binding of the toxin to receptors on the surface of target cells and endocytosis, there are two classical intracellular traffic pathways, which are used by bacterial toxins to reach the cytosol. The so-called short trip toxins such as, for example, diphtheria toxin translocate from early endosomes into the cytosol. Therefore, acidification of the endosomal lumen by the v-type H^+-ATPase is essential. Acidification of endosomes and thereby translocation of the toxins can be blocked by the inhibitor bafilomycin A1. The second type, the long trip toxins, is delivered from early endosomes via late endosomes and the Golgi apparatus to the endoplasmic reticulum (*ER*). For this routing, a special sequence in the protein, the so-called KDEL-like sequence, is necessary. These toxins translocate from the ER into the cytosol

causes structural alteration and functional changes of these proteins and finally leads to various cellular effects, including cell death.

There are two commonly used routes by which toxins are taken up (Fig. 1) (for a review see Sandvig and Van Deurs 2002). In the first pathway, the toxins translocate from early acidic endosomal compartments into the cytosol. This pathway, which is used by diphtheria toxin, the anthrax toxins or the *Clostridium botulinum* neurotoxins, can be specifically blocked by the drug bafilomycin A1, which is an inhibitor of endosomal acidification (Werner et al. 1984). During the so-called retrograde uptake pathway, the toxins also reach acidic endosomes but then they are transported to the endoplasmic reticulum (ER) via late endosomes and the Golgi apparatus. These toxins translocate from the ER into the cytosol. This pathway can be inhibited by brefeldin A, an inhibitor of vesicle trafficking between the Golgi apparatus and the ER. However, to enter the cytosol of host cells, the toxins have to cross at least one cellular membrane. For this purpose, bacterial toxins are organized in different functional domains: an enzymatically active domain (A-domain) and a binding/translocation domain (B-domain). The B-domain mediates the transport of the A-domain across membranes into the cytosol of target cells. The A-domain enzymatically modifies substrate proteins, which have essential functions for the host cells. These proteins serve either as central elements in signal transduction pathways,

as for example GTPases, or the toxin substrate proteins represent important structure proteins for the cell. Here, the cellular uptake mechanism of a special family of bacterial toxins, the binary actin ADP-ribosylating toxins, is reviewed.

Binary actin ADP-ribosylating toxins

In most bacterial toxins, the A- and the B-domain are located on the same polypeptide chain, but in a special group of bacterial toxins, the binary toxins, the A- and the B-domain are located on two individual and nonlinked proteins. The binding/translocation component and the enzyme component have to assemble on the cell surface to exhibit their cytotoxic effects (for review see Barth et al. 2002a). To the group of binary toxins belong the lethal toxin and the edema toxin from *Bacillus anthracis* (for reviews see Leppla 1995 and Mourez et al. 2002) and the family of binary actin-ADP-ribosylating toxins. This family includes *C. botulinum* C2 toxin (Aktories et al. 1986), *C. perfringens* iota toxin (Perelle et al. 1993; Stiles and Wilkens 1986), *C. spiroforme* toxin (Popoff and Boquet 1988; Stiles and Wilkins 1986), *C. difficile* ADP-ribosyltransferase (Popoff et al. 1988; Gülke et al. 2001) and the vegetative insecticidal proteins (VIP) toxins from *B. cereus* (Han et al. 1999) (for overview see Table 1).

C. botulinum C2 toxin

C. botulinum is an anaerobic spore forming bacterium that produces various toxins. The extremely potent neurotoxins cause botulism in humans (Simpson 1981). Beside the neurotoxins C1 and D, *C. botulinum* type C and D strains produce the enterotoxic C2 toxin, an actin-ADP-ribosylating toxin, which causes necrotic and hemorrhagic lesions in the intestinal wall (Simpson 1984a; Ohishi and Odagiri 1984; Ohishi and DasGupta 1987; Ohishi 2000). Because the gene for the C2 toxin is located in the bacterial chromosome and the genes for the neurotoxins C1 and D are located on phage DNA, phage curing of such *C. botulinum* strains results in strains that still produce C2 toxin but no neurotoxins (Rubin et al. 1988). C2 toxin seems not to be involved in botulism but it is lethal when it is applied to animals. Between 60 and 90 min after application of 1–2 pmol of C2 toxin, mice, rats, guinea pigs and chicken died and for mice the LD_{50} (intravenously) is less than 50 fmol of C2 toxin (Ohishi and Odagiri 1984; Simpson 1984a). C2 toxin was purified by Ohishi and coworkers in 1980 (Ohishi et al. 1980). This toxin is composed of two individual and nonlinked proteins (see Fig. 2): the binding and translocation component C2II and the enzyme component C2I (Ohishi et al. 1980). The enterotoxic effects of the C2 toxin were observed only when both components were applied together to animals because C2I is translocated into the cytoplasm by the binding component C2II following receptor-mediated endocytosis (Simpson 1989).

The enzymatic component C2I possesses ADP-ribosyltransferase activity (Simpson et al. 1988; Simpson 1984b) and Aktories and co-workers identified C2 toxin as the first toxin to have actin as its cellular substrate (Aktories et al. 1986). Therefore, with the identification of C2 toxin the new family of binary bacterial ADP-ribosylating toxins was introduced. C2I exclusively ADP-ribosylates G-actin at arginine-177 (Vandekerckhove et al. 1988). ADP-ribosylated G-actin acts as a ‘capping protein’ at the fast growing ends (plus or barbed ends) of actin filaments with the consequence that no more G-actin molecules

Table 1 Binary actin ADP-ribosylating toxins

Components of the toxin	M_r (kDa)	Enzymatic activity
C. botulinum C2 toxin		
C2I	49 (Fujii et al. 1996)	ADP-ribosylation of actin
C2II	81 (Kimura et al. 1998)	
	60 (activated) (Blöcker et al. 2000)	
C. perfringens iota toxin		
Ia	45 (Perelle et al. 1993)	ADP-ribosylation of actin
Ib	94 (Perelle et al. 1993)	
	81 (activated) (Perelle et al. 1993)	
C. spiroforme toxin		
Sa	44 (Popoff et al. 1989)	ADP-ribosylation of actin
Sb	92 (Popoff et al. 1989)	
	76 (activated) (Popoff et al. 1989)	
C. difficile CDT		
CDTa	48 (Perelle et al. 1997)	ADP-ribosylation of actin
CDTb	99 (Perelle et al. 1997)	
	75 (activated (Perelle et al. 1997))	
B. cereus VIP		
VIP2	52 (Warren et al. 1996)	ADP-ribosylation of actin
VIP1	100 (Warren et al. 1996)	
	80 (activated) (Warren et al. 1996)	

are able to assemble to this end of the filaments (Weigt et al. 1989; Wegner and Aktories 1988). Because at the slow growing ends of the filaments the disassembly of actin monomers continues, actin filaments depoylmerize. ADP-ribosylation also affects the interaction of actin with various actin binding proteins as gelsolin (Wille et al. 1992). The cytotoxic effects of C2 toxin on cells is depicted schematically in Fig. 2A. In summary, these effects result in a complete breakdown of the actin cytoskeleton so that treatment of cultured cells with both components of C2 toxin leads to rounding up of the cells within about 2–3 h.

The enzymatic component C2I (49.4 kDa, 431 amino acids) from *C. botulinum* strain KZZ1577 (92-13) was cloned and the protein was characterized in detail (Barth et al. 1998b) (see Fig. 2B). The N-terminal domain of C2I serves as an adaptor for interaction with C2II (Barth et al. 1998a) and the C-terminal domain of C2I domain harbors the catalytic site, which was characterized by site directed mutagenesis (Barth et al. 1998b).

There are several highly conserved amino acid residues located in the active site in most ADP-ribosyltransferases. The so-called catalytic glutamic acid residue is essential for transferase activity and in all ADP-ribosyltransferases, which ADP-ribosylate arginine residues, there is a second essential glutamic acid residue upstream of the ‘catalytic’ glutamic acid (Carroll and Collier 1984; Jung et al. 1993). In C2I, Glu389 is the ‘catalytic’ glutamic acid and Glu387 is the second conserved glutamic acid residue (Barth et al. 1998b). Further conserved motifs in ADP-ribosyltransferases are the serine-threonine-serine sequence (‘STS’ motif, residues 348, 349, 350 in C2I) upstream of the glutamic acids and an arginine residue (R299 in C2I) upstream of the ‘STS’ motif (Barth et al. 1998b).

A detailed analysis of the N-terminal domain of C2I revealed that the minimal sequence necessary for interaction with C2IIa and/or for translocation of C2I into the cytoplasm covers residues 1–87 (Barth et al. 2002b). C2I shares high amino acid sequence identity with the binary actin-ADP-ribosylating VIP2 (Barth et al. 2002b). Recently, the crystal structure of VIP2 toxin from *B. cereus* was solved (Han et al. 1999) and it is sug-

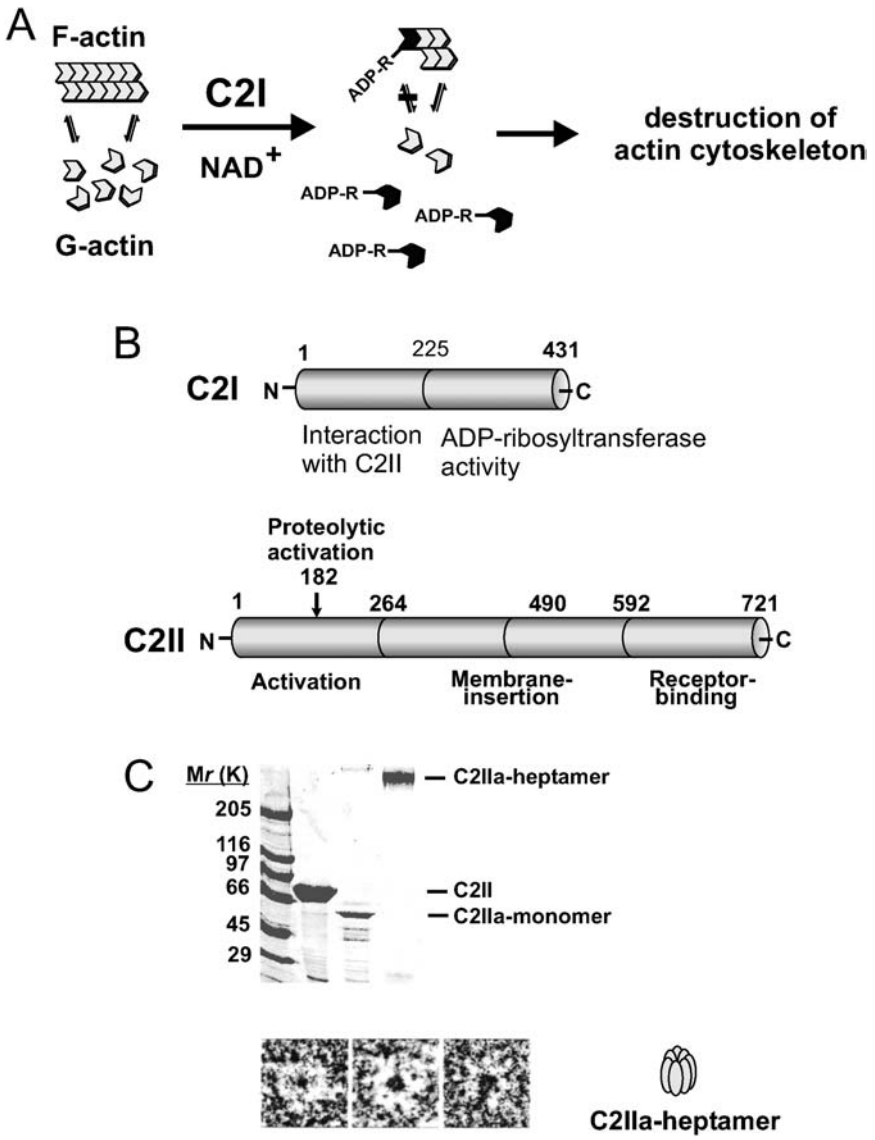

Fig. 2A–C The binary *C. botulinum* C2 toxin. **A** Intracellular mode of action of the C2I enzyme component. C2I ADP-ribosylates G-actin at position Arg177. ADP-ribosylated G-actin assembles to the fast growing end actin filaments but than acts as a capping protein and prevents further assembling of actin monomers. This leads to depolymerization of actin filaments and to a complete breakdown of the actin cytoskeleton. For further details see text. **B** The two components of C2 toxin. The enzyme component C2I has two functional domains, the N-terminal domain (residues 1–225) acts as an adaptor for interaction with the C2II binding component and mediates translocation of C2I across membranes. The C-terminal domain harbors the catalytic domain with the conserved amino acid residues. The C2II binding component can be functionally divided into four domains according to the protective antigen from *B. anthracis* (for details see text). C2II has to be cleaved by trypsin at position 182 within domain 1 to become activated. Domain 2 seems to be involved in membrane insertion and domain 4 is the receptor binding domain. **C** Activated C2IIa forms SDS-resistant heptamers in solution. The Coomassie blue-stained SDS–polyacrylamide gel is shown in the *upper panel*. Electron microscopy from C2IIa heptamers show ring-shaped structures (*lower left panel*), which represent the so-called pre-pore form of C2IIa. The C2IIa pre-pore is depicted in the *lower right panel*

gested that C2I has an identical folding. Amino acid alignment of C2I with VIP2 revealed that amino acids 1–225 of C2I correspond to the N domain of VIP2 (Barth et al. 2002b). Amino acids 1–87 of C2I, which are sufficient to mediate the uptake of a $C2I^{1-87}$–C3 fusion toxin into cells, are related to the N-terminal α-helices 1–4 in VIP2. The residues 12–29 of C2I correspond to the first α-helical structure (α1) in VIP2. After truncation of the N-terminal residues 1–29 of C2I, the resulting $C2I^{30-225}$–C3 fusion toxin is not transported into cells in the presence of the transport unit C2IIa (Barth et al. 2002b). However, residual binding of $C2I^{30-225}$–C3 to cells (via C2IIa) is still possible (Barth et al. 2002b).

Taken together, the N-terminal domain of C2I (residues 1–87 as the minimal essential part) acts as an adaptor, which interacts with C2IIa and to which foreign peptides can be fused. Recently it was demonstrated by Marvaud and colleagues that iota toxin also serves as a vehicle for transport of C3 transferase, when C3 transferase is fused to the Ib docking region of Ia (residues 129–257) (Marvaud et al. 2002).

C2I is delivered into the cytosol of target cells by the binding/translocation component C2II. C2II from *C. botulinum* strain KZZ1577 (92–13) is a protein of 721 amino acid residues with a molecular mass of 80.5 kDa (Barth et al. 1998b) (see Fig. 2B). Cloning of C2II revealed a high sequence similarity to the anthrax protective antigen (PA), the binding/translocation component of the anthrax toxins (Blöcker et al. 2000). Anthrax toxins are produced and secreted by *B. anthracis* which causes the disease anthrax. PA mediates cell entry of two different enzyme components, the lethal factor, a metalloprotease, which cleaves MAP-kinases (Duesbery et al. 1998) or the edema factor, which is an adenylyl cyclase (Leppla 1982, 1989). The crystal structure of PA and a detailed structure–function analysis of this protein was extremely helpful for the studies on C2II (Petosa et al. 1997). Reviews on the role of PA for cellular uptake of anthrax toxins are acknowledged (Leppla et al. 1995; Mourez et al. 2002, see also article by M. Mourez, this volume). Based on the crystallographic data, PA is divided into four functional domains: the N-terminal domain 1 with the activation site for the protease, domain 2 which is involved in membrane insertion, domain 3 of unknown function, and the C-terminal domain 4, which mediates receptor binding. Alignment of C2II and PA reveals significant sequence identity within domains 1–3 (about 50%) but no homology in domain 4, because PA and C2I bind to different cellular receptors (Blöcker et al. 2000). Thus, like PA, C2IIa can be divided into four domains, which have been studied for their role in the uptake of C2 toxin into cells.

To mediate cytotoxic effects, i.e., transport of C2I into cells, C2II has to be activated by proteolytic cleavage, which cuts off a ~20-kDa peptide from the N terminus (Ohishi 1987; Barth et al. 2000). For in vitro activation of C2II, the trypsin cleavage site was identified between amino acid residues Lys181 and Ala182 (Blöcker et al. 2000). The resulting active C2IIa fragment (59.8 kDa), but not C2II, immediately forms heat-labile and SDS-stable heptamers (~ 420 kDa) in solution (Barth et al. 2000). The C2IIa heptamers are annular structures with an inner diameter of about 2–4 nm and an outer diameter of about 11–13 nm (see Fig. 2C). C2II and activated C2IIa exhibit different abilities with respect to their role in mediating cytotoxicity. C2IIa, but not C2II, binds to cells and mediates cellular uptake of C2I (Barth et al. 2000). Moreover, only C2IIa forms ion-permeable channels in cell membranes as well as in artificial black lipid bilayer membranes (Schmid et al. 1994; Barth et al. 2000). Like C2II, PA (83 kDa) is activated by proteolytic cleavage. A 20-kDa fragment is removed from the N terminus by the cellular protease furin (Klimpel et al. 1992). The resulting PA-63 forms heptamers on the surface of cells (Milne

et al. 1994) and recently it was shown that lethal and edema factors bind only to the oligomeric form of PA-63 (Mogridge et al. 2002). Moreover, like C2II, PA-63 forms ion-permeable channels in artificial lipid bilayer membranes and in membranes of intact cells (Blaustein et al. 1989; Milne and Collier 1993). The role of the C2IIa heptamers in docking to the cellular C2 toxin-receptor, the function of C2IIa channels for the transport of C2I into the cytosol, the interaction of both components, and the involvement of host cell proteins in the cellular uptake mechanism of C2 toxin have been elucidated in past years.

The receptor for C2 toxin

The first step required for cellular uptake of C2 toxin is the binding of the C2IIa heptamers to specific receptor molecules on the surface of target cells. The receptor for C2 toxin was the first one identified within the family of binary actin-ADP-ribosylating toxins. Recently, a mutant Chinese hamster ovary (CHO) cell line (CHO-RK14) was used to demonstrate that C2 toxin binds to asparagine-linked complex and hybrid carbohydrates on the surface of cells (Eckhardt et al. 2000). This mutant CHO cell line, which was generated by chemical mutagenesis, was resistant to C2 toxin but still sensitive to *C. perfringens* iota toxin (Fritz et al. 1995). Moreover, CHO-RK14 cells were not able to bind C2IIa because they were deficient of the functional C2 toxin receptor. CHO-RK14 cells are mutated in the gene for N-acetylglucosaminyltransferase I. Therefore, these cells are lacking complex and hybrid N-glycans, which are essential for C2 toxin binding (Eckhardt et al. 2000) (see Fig. 3A). Transfection of these mutated cells with the intact N-acetylglucosaminyltransferase I gene recovered sensitivity of the cells against C2 toxin (Eckhardt et al. 2000). The receptor for C2 toxin was present on all vertebrate cell lines and primary cultured cells, which have been tested so far for sensitivity to C2 toxin, including cells from fish (Eckhardt et al. 2000). However, cells derived from insects have different carbohydrate structures on the cell surface and such cells are resistant to the toxin, suggesting that the described carbohydrate structure might be sufficient for binding of C2IIa. C2II binds with its C-terminal domain to the cell receptor. Recently, we reported that the C-terminal amino acids are structurally important for interaction with the receptor. When the seven C-terminal amino acid residues were deleted, C2II still forms heptamers, but these heptamers are no more able to bind to cells (Blöcker et al. 2000).

Cellular uptake of C2 toxin

Following binding of C2IIa to the cellular receptor, C2I assembles and the receptor–toxin complexes are taken up via receptor-mediated endocytosis (Simpson 1989). The endocytosed toxin reaches endosomal compartments (Barth et al. 2000). As mentioned above, from this point there are two intracellular pathways for trafficking of toxins which are commonly used by bacterial toxins to reach the cytosol. C2 toxin does not take the retrograde pathway, as intoxication of cells with C2 toxin is not inhibited by brefeldin A or nocodazole, drugs that block this pathway (Barth et al. 2000). C2 toxin translocates directly from early acidic endosomes into the cytosol. As mentioned above, this step can be blocked by bafilomycin A1, which specifically blocks the vesicular-type H^+-ATPase (v-ATPase) (Barth et al. 2000). This ATPase is located in the membrane of endosomes and drives the acidification of the endosomal compartment. Acidification of the endosomal lu-

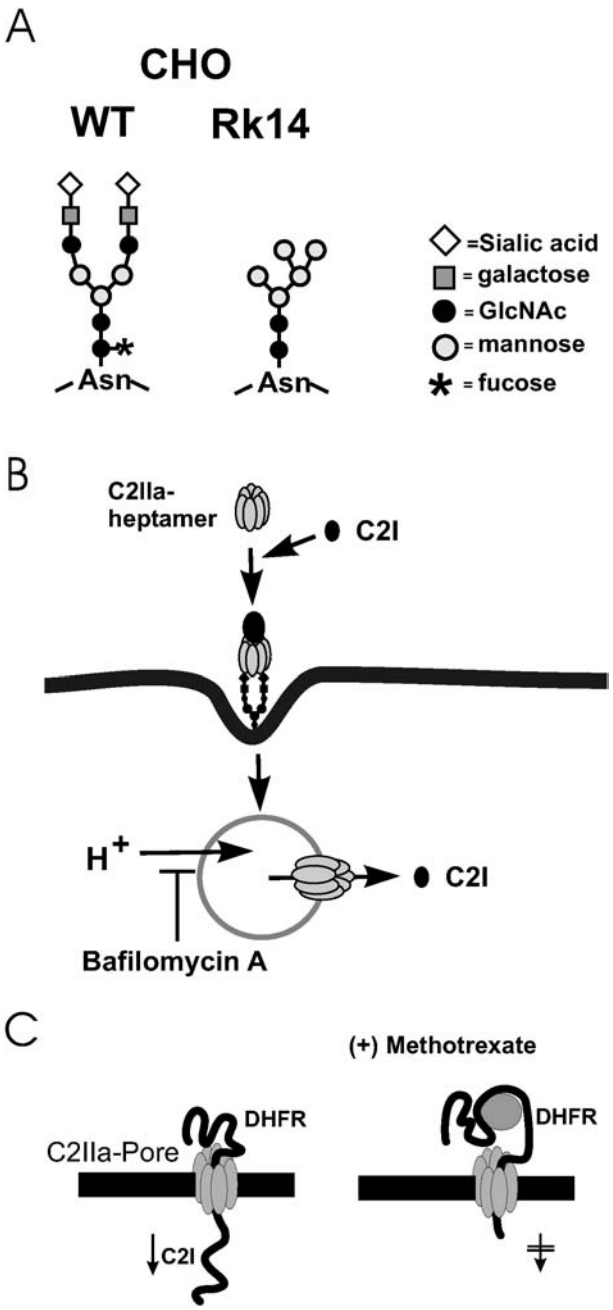

Fig. 3A–C Cellular receptor and uptake mechanism of C2 toxin. **A** C2 toxin binds to asparagine (*Asn*)-linked complex and hybrid carbohydrates. The receptor for the C2 toxin of Chinese hamster ovary (*CHO*) wild-type (*WT*) and of C2 toxin-resistant CHO-RK14 mutant cells is depicted. **B** Model of the cellular uptake mechanism of *C. botulinum* C2 toxin. The enzyme components C2I assemble with the C2IIa heptamers, which mediate binding to the receptor. The whole complex is taken up via receptor-mediated endocytosis and reaches early endosomes. After acidification of the endosomal lumen, C2IIa heptamers insert into the endosomal membrane and thereby form pores. C2I is translocated into the cytosol, most likely via the pores. Bafilomycin A inhibits the v-type ATPase in the endosomal membrane and prevents pore formation of C2IIa and translocation of C2I. **C** Translocation of C2I across endosomal membranes requires unfolding of the protein. The C2I-DHFR (dihydrofolate reductase) fusion protein was delivered across cell membranes by the C2IIa component. In the presence of the substrate methotrexate (*MTX*), the DHFR domain was stabilized in a folded state and its unfolding and translocation of the MTX-bound C2I-DHFR fusion toxin was prevented

men induces a conformational change of C2IIa and thereby leads to exposure of hydrophobic residues to the surface of the protein. As a consequence of acidification, membrane insertion and pore formation of C2IIa occurs and C2I translocates into the cytosol (see Fig. 3B).

The series of events involved in delivery of active C2 toxin into the cytosol can be mimicked at the surface of intact cells (see Fig. 3B) (Barth et al. 2000). This method allows monitoring of the single steps as membrane insertion, pore formation and translocation of the enzyme component into the cytosol. In brief, the components of C2 toxin were allowed to bind to the membrane receptors of cultured cells at 4°C, i.e., under conditions in which endocytosis cannot occur. Subsequently, a short pulse of acidic and warm (pH< 5.5 for 5 min) medium, which was given to the cells, induced delivery of the toxin into the cytosol, directly across the cell membrane (Barth et al. 2000). Pore formation of C2IIa can be determined by measuring the efflux of radioactive rubidium-86 from preloaded cells (Blöcker et al. 2003). Pore formation was observed only with the proteolytically activated C2IIa under acidic conditions and it was dependent on binding of C2IIa to its receptor. When the enzyme component C2I was present in such experiments, the efflux of rubidium from cells was reduced, suggesting that following acidification, C2IIa inserts into the membranes and forms pores and that C2I interacts with the inner part of the channel (Blöcker et al. 2003). It was possible to deliver C2I into the cytosol under the experimental conditions described but a precise timing and coordination of the individual steps was essential. C2I has to be bound to C2IIa prior to acidification in order to become translocated. This finding implies that once the C2IIa pore is formed in the membranes, it does not act as a nonspecific channel for trafficking free C2I molecules from the medium into the cytosol (Blöcker et al. 2003). With respect to the conditions in endosomes, this observation is important because it demonstrates that insertion of C2IIa into membranes, pore formation and translocation of C2I is a precisely coordinated process, which is initiated by an acidic milieu. Interestingly, intoxication of cells by C2 toxin is observed only under conditions in which pore formation of C2IIa can occur, indicating that these pores are absolutely essential for delivery of C2I into the cytosol (Blöcker et al. 2003). The central question is the role of the C2IIa pore for the translocation mechanism of C2I. There are two possibilities: (a) C2IIa pores are necessary for the translocation of C2I but not directly involved in this step or (b) the C2IIa pores are directly involved in the membrane transport of C2I. If this second hypothesis holds true, one should expect an interaction of C2I with C2IIa during the membrane translocation. It was shown experimentally by the use of different approaches that C2I interacts directly with the C2IIa pores. One approach was the efflux of radioactive rubidium through C2IIa pores in the membranes of intact cells, which was reduced in the presence of C2I, as described above (Blöcker et al. 2003). In parallel, it was found that C2I interacted with the lumen of C2IIa channels in artificial lipid membranes (Blöcker et al. 2003). However, there has still been the question of whether C2I is transported directly through the C2IIa channel from acidic endosomes into the cytoplasm. Because the inner diameter of the channel is only about 1–2 nm (Barth et al. 2000), it was an interesting question whether C2I needs to unfold for translocation. Unfolding of C2I during translocation across membranes was studied with a fusion protein consisting of C2I and dihydrofolate reductase (DHFR), which was C-terminally fused to C2I, as a tool (Haug et al. 2003b) (see Fig. 3C). The C2I–DHFR fusion protein was active in vitro and was delivered by C2IIa into the cytosol of cells as detected by rounding of intoxicated cells and subsequent in vitro ADP-ribosylation of the actin of those intoxicated cells (Haug et al. 2003b). When radiolabeled NAD as cosubstrate and C2I was added to lysates from intoxicated cells, no radioactively labeled actin was detectable (Haug et al. 2003b). This demonstrates that the complete actin was already ADP-ribosylated in intact cells by C2 toxin. When methotrexate, a substrate of DHFR, was

present during incubation of cells with C2I–DHFR, intoxication of cells was clearly reduced. Binding of methotrexate (MTX) to DHFR keeps the DHFR domain of the C2I–DHFR fusion protein in a folded conformation, which is not able to unfold during C2IIa-mediated translocation of the protein across endosomal membranes (Haug et al. 2003b). The principle of the DHFR/MTX translocation system is depicted in Fig. 3C. This finding favors a model which includes an at least partial unfolding of C2I during its translocation into the cytosol. Similar results were obtained with anthrax toxin (Wesche et al. 1998).

The host cell chaperone Hsp90 is essential for uptake of C2 toxin

The model that C2I translocates in an unfolded conformation from early endosomes into the cytosol implies that the protein has to become refolded in the cytosol to recover its enzymatic activity. In principle, there are three possibilities how this refolding can be achieved: (a) C2I harbors intrinsic chaperone activity; (b) the C2IIa pore refolds C2I prior to its release into the cytosol; and (c) host cell chaperones are involved in refolding of C2I. To address this question, the specific pharmacological inhibitors for the heat shock protein (Hsp)90, geldanamycin and radicicol were used to block Hsp90 in eukaryotic target cells prior to incubation of the cells with C2 toxin (Haug et al. 2003a). Each of the inhibitors dramatically delayed intoxication of cells by C2 toxin. Further analysis revealed that the Hsp90 inhibitors had no effect on the early steps of C2 toxin uptake into cells such as binding of the toxin to its receptor or interaction of the two toxin components (Haug et al. 2003a). The Hsp90 inhibitors prevented the translocation of C2I from early acidic endosomes into the cytosol. When cells were pretreated with either geldanamycin or radicicol, C2I was trapped in endosomal compartments (Haug et al. 2003a) (see Fig. 4). The role of Hsp90 in cellular uptake is summarized in Fig. 4. Inhibition of Hsp90 also inhibited intoxication of cells by an artificial fusion construct, which was constructed based on the C2 toxin as a protein transport system (Haug et al. 2003a) and intoxication by iota-like toxins (Haug et al. 2004). This was the first time that an involvement of host cell chaperones in the cellular uptake mechanism was reported for binary bacterial toxins. Recently, a similar role of Hsp90 was reported by Ratts and co-workers for the uptake of diphtheria toxin into eukaryotic cells (Ratts et al. 2003), a finding that suggests that the dependence of toxin translocation on host cell chaperones might be a more general principle for bacterial toxins. The precise role of Hsp90 in translocation of bacterial toxins across endosomal membranes has to be elucidated in further studies.

Cellular uptake mechanism of iota-like toxins

Besides the C2 toxin, the iota-like toxins belong to the family of binary actin-ADP-ribosylating toxins. These toxins are very similar to C2 toxin but differ in their substrate specificity. C2 toxin ADP-ribosylates only β/γ-nonmuscle G-actin and γ-smooth muscle actin isoforms but no α-actin isoforms (muscle actin) while the iota-like toxins ADP-ribosylate all actin isoforms, including α-actin isoforms (Mauss et al. 1990). The binary iota toxin, the prototype of the iota-like toxins, is produced by *C. perfringens* type E strains and consists of the enzymatically active Ia component, the ADP-ribosyltransferase, and the binding/translocation component Ib (Stiles and Wilkens 1986). When Ia or Ib are combined,

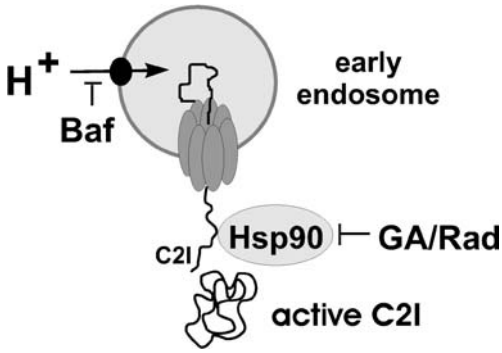

Fig. 4 Model for the translocation of C2 toxin from early endosomes into the cytosol. The translocation of C2I from early endosomes into the cytosol of target cells depends on acidification of the endosomal lumen and on membrane insertion and pore formation of the C2IIa component. Moreover, the host cell chaperone Hsp90 is essential for translocation of C2I across endosomal membranes. Inhibition of Hsp90 by the specific inhibitors radicicol (*Rad*) or geldanamycin (*GA*) prevented delivery of C2I into the cytosol. C2I was trapped in endosomal vesicles

the resulting toxin causes diarrhea in calves and lambs and is lethal to mice and dermonecrotic in guinea pigs (Songer 1996).

The crystal structure of Ia was solved recently (Tsuge et al. 2003). As reported for VIP2 and C2I, Ia consists of two domains, which have similar folding but only little sequence similarity (Tsuge et al. 2003). The catalytic domain is located at the C terminus of Ia. Structure–function analysis of the enzyme component Ia revealed that residues R295, E378, and E380 are the conserved EXE motif, which is found in ADP-ribosyltransferases (Perelle et al. 1993; Tsuge et al. 2003). The 'STS sequence' and the conserved arginine residue, which is characteristic of ADP-ribosyltransferases, was identified upstream of the EXE motif (Tsuge et al. 2003). The N-terminal domain of Ia (residues 1–210) interacts with Ib. However, the Ib docking region on Ia (residues 129–257) is not located within the very N terminus, as found for C2I (residues 1–87) or for *C. difficile* transferase (CDT) (residues 1–240), but it is located more centrally within the N-terminal domain (Marvaud et al. 2002).

The binding/translocation component Ib shares high sequence homology to C2II within domains 1–3 but not within domain 4, which represents the receptor binding domain in both proteins (Blöcker et al. 2000). Like C2II, Ib requires proteolytic activation within the N-terminal domains (Barth et al. 2000a, 2000). Cleavage of Ib occurs at position Ala-211 (Perelle et al. 1993). Like C2IIa, activated Iba forms voltage-dependent ion-permeable channels in black lipid membranes (Knapp et al. 2002). Iba forms SDS-stable heptamers on membranes of intact cells and mediates Ia docking (Stiles et al. 2002). Iba heptamers, which have been formed in solution, behave differently from Iba heptamers, which have been formed on the cell surface. Iba oligomers, which are formed in solution, are less stable on SDS–PAGE and do not facilitate cytotoxicity (Stiles et al. 2002; Blöcker et al. 2001). This is a difference to the observations made for C2II (Blöcker et al. 2001; Barth et al. 2000). Moreover, Iba heptamers, which have been formed in solution, do not facilitate K^+ release from cells, suggesting that they are not able to form pores in cell membranes, and are efficiently digested by protease treatment after binding to the surface of cells (Stiles et al. 2002). This is also different from C2IIa, which forms heptamers in solution that represent the biologically active species of C2II with respect to cell binding, pore for-

mation and transport of the enzyme component (Barth et al. 2000). Ib, like C2II, lacks a furin cleavage site and is not activated over time by a cellular protease after binding to cells (Perelle et al. 1993). As for C2II, the receptor binding site of Ib is located in the C-terminal domain. Deletion of 10 amino acid residues from the C terminus abrogates Ib binding to Vero cells (Marvaud et al. 2001). Oligomerization of Iba is essential for cytotoxicity of iota toxin. This was demonstrated in studies with an antibody, which efficiently prevented Ib oligomerization on the cell surface and did inhibit cytotoxic effects of the toxin (Stiles et al. 2002).

C2 toxin and iota toxin bind to different cellular receptors. While all of the mammalian cell types tested so far are sensitive to C2 toxin, there are several cell types that are resistant to iota toxin. C2IIa binds to carbohydrate structures (Eckhardt et al. 2000) but the receptor for Iba is most likely a protein. The latter was postulated because pretreatment of cells with proteases diminished subsequent binding of Iba (Stiles et al. 2000). Moreover, the cellular distribution of the receptors for C2 toxin and for iota toxin on cells that have been shown to be sensitive to both toxins are significantly different(Blöcker et al. 2001). The receptor for Ib is localized primarily on the basolateral surface of polarized human colon carcinoma (CaCo-2) cells while C2 toxin binds to both the basolateral and the apical side (Blöcker et al. 2001).

Iota toxin is taken up into cells in principle by the same mechanism as that described for C2 toxin. However, there are some slight variations. Like C2 toxin, endocytosed iota toxin translocates from early endosomes into the cytosol as the specific inhibitor of vesicular H^+-ATPase bafilomycin A1 protects cultured cells from intoxication by iota toxin (Blöcker et al. 2001). However, when Ia is translocated directly across the plasma membrane of intact cells via Iba by an acidic pulse, a more acidic pH of the medium is required than for C2 toxin (Blöcker et al. 2001). Cellular uptake of iota toxin and CDT from *C. difficile*, another iota-like toxin, depend on host cell Hsp90 (Haug et al. 2004). When cellular Hsp90 was inactivated by pretreatment with radicicol, intoxication of cells was reduced. This observation confirms that despite differences, C2 toxin and the iota-like toxins share the same general uptake mechanism into the cytosol of eukaryotic cells.

Binary toxins as delivery systems for foreign proteins

One central goal for studies with intact eukaryotic cells in pharmacology and cell biology is the delivery of foreign peptides, such as enzymes, into the cytosol. Several methods of bringing proteins into living cells, such as transfection and microinjection techniques, have been established but the use of such methods does not allow a rapid, synchronous and complete delivery of peptides into cells. Therefore, performance of biochemical assays with many cells at the same time point is very limited. Moreover, such techniques may exhibit effects of the living cells themselves. In comparison with these methods, the delivery of peptides into the cytosol of cells by catalytically inactive bacterial toxins as a transport device has the advantage that existing cellular routes for protein trafficking are used and that all of the cells in a culture are affected simultaneously. The binary toxins described in this review have all the properties that are needed for a protein delivery system. We used the C2 toxin and constructed a fusion toxin, which consists of the N-terminal domain of C2I (amino acid residues 1–225 of C2I, called C2IN) and the C3 exoenzyme of *C. limosum* as a reporter enzyme (Barth et al. 1998a, 2002b) (Fig. 5A). The N domain of

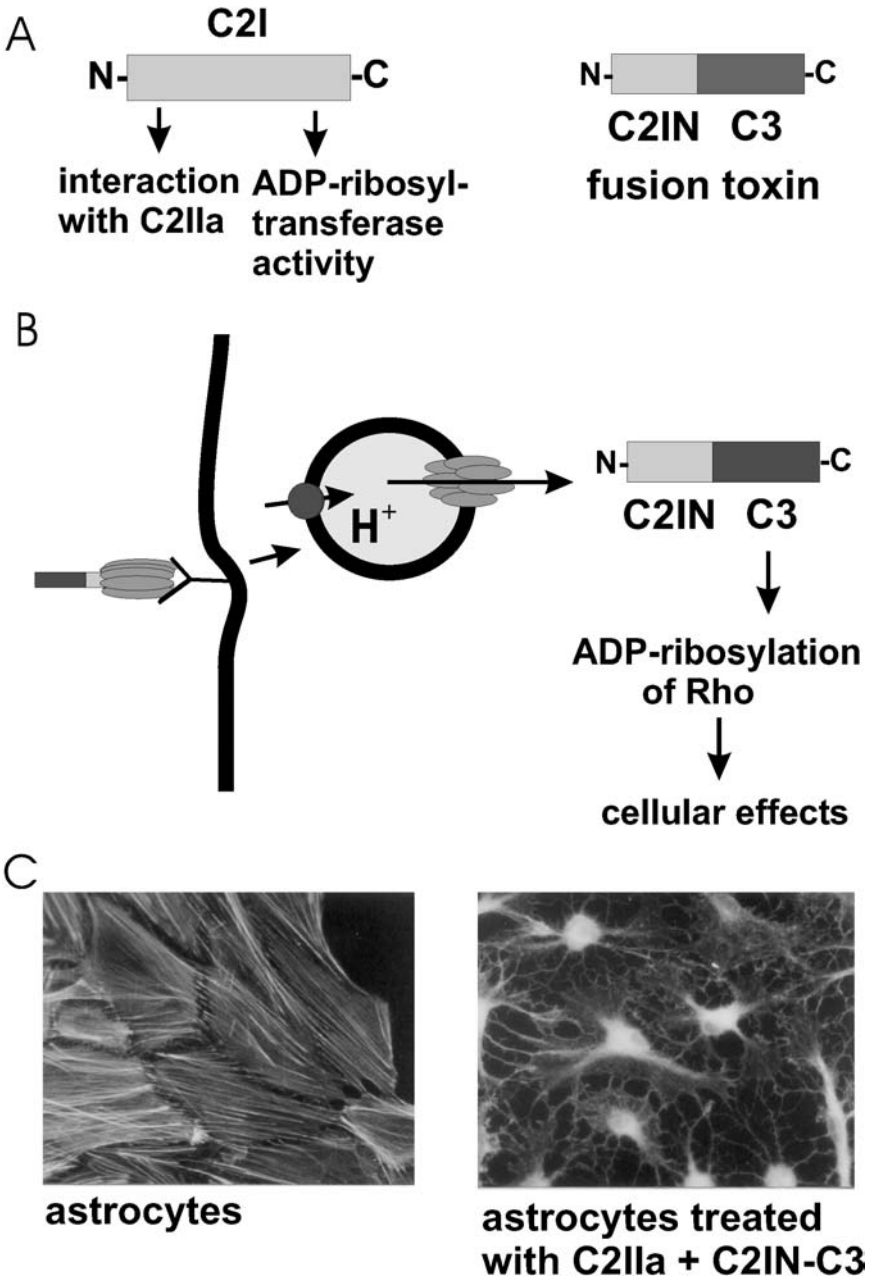

Fig. 5A–C The binary C2 toxin as a cellular protein delivery system for passenger proteins. **A** The C2IN–C3 fusion toxin is composed of the N-terminal domain of C2I (C2IN, amino acid residues 1–225) and the C3 transferase from *C. limosum.* C3 transferase exclusively ADP-ribosylates the GTPase Rho but is not taken up into eukaryotic cells. C2IN acts as an adaptor for C2IIa-mediated delivery of the fusion toxin into target cells. Amino acid residues 1–225 of C2I interact with C2IIa and are sufficient for translocation of fusion proteins into the cytosol. **B** The translocation of the fusion toxin via C2IIa from acidic endosomes is depicted schematically. **C** Cytotoxic effect of C2IN–C3 fusion toxin on cultured rat astrocytes. Astrocytes were incubated for 3 h at 37°C with C2IN–C3 (100 ng/ml) together with C2IIa (200 ng/ml). As a control, cells were incubated without toxin. The actin filaments, which were stained with rhodamine–phalloidin, are shown

Table 2 The C2IN–C3 fusion toxin in studies on the role of Rho in cell biology and pharmacology

Activation of anion channels	Nilius et al. 1999
Secretion of van Willebrand factor	Vischer et al. 2000
Ephrin-A5-induced neuronal growth cone collapse	Wahl et al. 2000
Uptake and degradation of lipoproteins by macrophages	Sakr et al. 2001
Interleukin-1 receptor induced signaling	Dreikhausen et al. 2001
Aquaporin 2 translocation	Klussmann et al. 2001
Cerebellar granule neuronal survival	Linseman et al. 2001
Cyclooxygenase-2-gene expression	Hahn et al. 2002
Monocyte transendothelial migration	Strey et al. 2002
Volume-regulated anion channels in vascular endothelial cells	Carton et al. 2002
Angiotensin II-induced vasoconstriction	Galle et al. 2003
Mechanism of formation of branched dendrites	Leemhuis et al. 2004

C2I is enzymatically inactive but serves as an adaptor for binding to the C2IIa binding/translocation component and is able to translocate into the cytosol (Barth et al. 1998a). The C3 exoenzyme (~23 kDa) ADP-ribosylates and thereby inactivates the Rho-GTPase. Interestingly, C3 exoenzyme is not able to enter cells, because this protein does not have a binding/translocation domain. Therefore, studies with C3 in intact cells require methods like microinjection. As C3 transferase is the only known specific inhibitor for Rho, it is widely used in studies investigating the role of Rho in cellular signal transduction pathways (for a review see Aktories et al. 2000). The constructed C2IN–C3 fusion toxin specifically modified Rho in vitro and was very efficiently (concentrations of 100 ng of toxin per ml medium together with 200 ng/ml of C2IIa) delivered into various cell types by the C2IIa transport component (Barth et al. 1998a). A complete intoxication of cultured cells was obtained after a 3-h incubation time as indicated by morphological analysis and by in vitro ADP-ribosylation of Rho from lysates of intoxicated cells (Barth et al. 1998a, 1999). The effect of this fusion toxin on cell morphology was transient because the cells recovered their normal morphology when the toxin was degraded in the cytosol of cells. Moreover, it was shown that ADP-ribosylated Rho was degraded and de novo synthesis of Rho was sufficient to induce a complete rebuilding of the actin cytoskeleton (Barth et al. 1999). This makes it possible to use this toxin to study the cellular consequences of a transient but complete inactivation of Rho in intact cells. The mode of action of the C2–C3 fusion toxin is depicted in Fig. 5B and its effect on the morphology of cultured rat astrocytes is shown in Fig. 5C. This fusion toxin was used as a tool in various cell biological and pharmacological studies which are summarized in Table 2.

Conclusion

For many bacterial toxins, pore formation in lipid membranes has been reported and it is a widely accepted hypothesis that pore formation is an essential event in the translocation of bacterial toxins across cellular membranes. However, it has not been shown so far that the active domain of a toxin directly translocates through the pore formed by the binding/translocation domain. Binary toxins are an ideal model with which to study translocation of bacterial toxins across cell membranes. One important reason is that the single components of these toxins can be added to cells sequentially and the individual steps involved in translocation—such as membrane insertion, pore formation and translocation—can be observed independently. The interaction of the binding/translocation domain and the en-

zyme domain can be studied more easily than in one-chain AB toxins, because both are individual proteins, which have specific properties. Thus, the binary toxins are important tools for the study of mechanisms of the uptake of protein toxins into cells.

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The enteric toxins of *Clostridium perfringens*

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Abstract The Gram-positive pathogen *Clostridium perfringens* is a major cause of human and veterinary enteric disease largely because this bacterium can produce several toxins when present inside the gastrointestinal tract. The enteric toxins of *C. perfringens* share two common features: (1) they are all single polypeptides of modest (~25–35 kDa) size, although lacking in sequence homology, and (2) they generally act by forming pores or channels in plasma membranes of host cells. These enteric toxins include *C. perfringens* enterotoxin (CPE), which is responsible for the symptoms of a common human food poisoning and acts by forming pores after interacting with intestinal tight junction proteins. Two other *C. perfringens* enteric toxins, ϵ-toxin (a bioterrorism select agent) and β-toxin, cause veterinary enterotoxemias when absorbed from the intestines; β- and ϵ-toxins then apparently act by forming oligomeric pores in intestinal or extra-intestinal target tissues. The action of a newly discovered *C. perfringens* enteric toxin, β2 toxin, has not yet been defined but precedent suggests it might also be a pore-former. Experience with other clostridial toxins certainly warrants continued research on these *C. perfringens* enteric toxins to develop their potential as therapeutic agents and tools for cellular biology.

Introduction

The spore-forming bacterium *Clostridium perfringens* produces an intimidating arsenal consisting of at least 15 different toxins. Individual *C. perfringens* isolates produce only portions of this toxin repertoire, forming the basis for a commonly used toxin typing scheme (McClane et al. 2000) that assigns *C. perfringens* isolates to one of five types (Table 1, A–E) based upon their production of α-, β-, ϵ-, and ι-toxins. Each *C. perfringens* toxinotype is associated with particular human or veterinary diseases (Table 2), indicating

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Table 1 Toxin typing classification scheme for *Clostridium perfringens* isolates

	Toxins produced:			
Toxinotype	*Alpha*	*Beta*	*Epsilon*	*Iota*
A	+	−	−	−
B	+	+	+	−
C	+	+	−	−
D	+	−	+	−
E	+	−	−	+

Table 2 Diseases associated with *C. perfringens* toxinotypes

Toxinotype	Major toxins	Diseases
A	α	Human myonecrosis (gas gangrene), necrotic enteritis in fowls, enterotoxemias of cattle and lambs, mild necrotizing enteritis of piglets
	α, CPE	Human food poisoning, human nonfood-borne GI disease, veterinary diarrhea
	α, CPE, β2	Human nonfood-borne GI disease
	α, β2	Porcine enteritis
B	α, β, ϵ	Dysentery in newborn lambs, chronic enteritis in older lambs, hemorrhagic enteritis in neonatal calves and foals, hemorrhagic enterotoxemias in sheep, biodefense concerns
C	α, β	Human necrotizing enteritis (Pigbel, Darmbrand), enteritis in hemorrhagic/necrotic enterotoxemias in neonatal pigs, lambs, calves goats, and foals, acute enterotoxemia (struck) in adult sheep
	α, β, β2	Porcine enteritis
D	α, ϵ	Enterotoxemia in lambs (pulpy kidney disease) and calves, enterocolitis in neonatal and adult goats and cattle, biodefense concerns
E	α, ι	Canine, bovine, porcine enteritis

that variations in toxin production profoundly influence the virulence properties of *C. perfringens* isolates. These isolate-to-isolate differences in toxin production also help explain the pathogenic versatility of *C. perfringens*, which causes both enteric and histotoxic infections and has a disease spectrum ranging from low incidence/high mortality (clostridial myonecrosis) to high incidence/low mortality (*C. perfringens* type A food poisoning).

This review discusses four *C. perfringens* toxins that are produced in the gastrointestinal (GI) tract and have been implicated in human and/or veterinary enteric disease. These enteric toxins include two *C. perfringens* typing toxins (β- and ϵ-toxins), as well as two nontyping toxins, [*C. perfringens* β2 toxin (CPB2) and *C. perfringens* enterotoxin (CPE)] that are also considered important for enteric disease. Another *C. perfringens* typing toxin, ι-toxin, affects the GI tract during veterinary type E infections; however, ι-toxin is discussed elsewhere in this volume.

The *Clostridium perfringens* enterotoxin

When identified over 30 years ago, the 35-kDa CPE protein was quickly linked to *C. perfringens* type A food poisoning, the second and third most commonly identified foodborne disease in the UK and USA, respectively (Adak et al. 2002; Olsen et al. 2000). The pathogenic importance of CPE for food poisoning received initial support from human

volunteer feeding studies showing that ingestion of purified CPE is sufficient to reproduce the diarrhea and cramping symptoms of the natural *C. perfringens* type A food poisoning (Skjelkvale and Uemura 1977). More recently, molecular Koch's postulates analyses using an isogenic *cpe* mutant of SM101 (a transformable derivative of food poisoning isolate NCTC 8798) confirmed that CPE expression is required for CPE-positive, type A food poisoning isolates to cause GI effects in animal models (Sarker et al. 1999). Additionally, CPE-positive type A isolates became implicated during the mid-1980s as the cause of up to 5–15% of all human nonfood-borne GI disease cases, e.g., antibiotic-associated diarrhea (AAD; Borriello et al. 1984). Molecular Koch's postulate analyses have also supported the importance of CPE expression for the enteric virulence of the nonfood-borne human GI disease type A isolate F4969 (Sarker et al. 1999). Finally, CPE-positive type A isolates have recently been linked to some veterinary diarrheas (Songer 1996).

Most *C. perfringens* isolates carrying the enterotoxin gene (*cpe*) classify as type A isolates. However, even among type A isolates, the presence of the *cpe* gene is uncommon, i.e., <5% of global *C. perfringens* type A isolates are *cpe*-positive (Czeczulin et al. 1993). The *cpe* gene can be present on either the chromosome or on a large plasmid (Collie and McClane 1998; Cornillot et al. 1995). A strong correlation exists between a type A isolate's *cpe* genotype (i.e., whether the *cpe* gene is chromosomal or plasmid-borne) and which CPE-associated GI illness that isolate can cause. Specifically, *C. perfringens* type A food poisoning isolates usually carry a chromosomal *cpe* gene, whereas CPE-positive *C. perfringens* type A isolates causing AAD or sporadic diarrhea (SD) nearly always possess a plasmid-borne *cpe* gene (Collie and McClane 1998; Cornillot et al. 1995). The reasons for these specific *cpe* genotype:GI disease relationships are just now emerging. Chromosomal *cpe* isolates appear to be strongly associated with food poisoning because (1) these are the predominant *cpe*-positive isolates present in foods (Wen 2004), and (2) the higher heat resistance of spores/vegetative cells of these isolates (versus the spores/cells of plasmid *cpe* isolates; Sarker et al. 1999) favors their survival in temperature-abused foods. The strong linkage between plasmid *cpe* isolates and AAD/SD could be explained, at least in part, by recent studies demonstrating the transfer of the *cpe* plasmid between *C. perfringens* isolates (Brynestad et al. 2001). Transmissibility of the *cpe* plasmid could be important since cases of CPE-associated AAD/SD are thought to result from ingestion of small numbers of CPE-positive isolates (McClane et al. 2000), in contrast to the massive numbers of CPE-positive vegetative cells ingested during *C. perfringens* type A food poisoning. Therefore, establishment of AAD/SD might be assisted if the few infecting *cpe*-positive isolates can transfer their *cpe* plasmid to the predominantly *cpe*-negative *C. perfringens* isolates present in the normal intestinal flora, thus converting these commensal isolates to enteropathogens.

Early studies determined that CPE biosynthesis is temporally associated with sporulation (Duncan 1973). Promoter mapping of the *cpe* gene identified three functional promoters, each exhibiting homology to a consensus binding sequence for the sporulation-specific sigma factors SigK or SigE (Melville et al. 1994; Zhao and Melville 1998). During sporulation, CPE is strongly expressed by many CPE-positive type A isolates. This single protein can account for up to 20% of the total protein present in a sporulating cell (Czeczulin et al. 1993). After synthesis, CPE is not secreted but instead accumulates in the cytoplasm of sporulating cells, where it may localize in paracrystalline inclusion bodies (Labbe 1989). CPE is eventually released into the intestinal lumen at the completion of sporulation, when the mother cell lyses to release its mature spore.

The intestinal action of CPE

Purified CPE rapidly damages the rabbit ileum. CPE-induced histopathological effects, including villus shortening and epithelial desquamation (McDonel 1986), are apparently responsible for initiating diarrhea since (1) only those CPE doses capable of causing intestinal damage are able to produce intestinal fluid and electrolyte secretion in the rabbit ileum (McDonel 1986), and (2) fluid and electrolyte secretion consistently follows development of intestinal damage in the CPE-treated rabbit ileum (McDonel 1986; Sherman et al. 1994). Although less sensitive than the ileum, the rabbit duodenum and jejunum also respond to CPE (McDonel 1986). It is interesting to note that the rabbit colon binds, but is insensitive to, CPE (McDonel 1986).

The molecular action of CPE

Once released into the intestinal lumen upon the completion of sporulation, CPE binds to enterocytes. At physiologic CPE concentrations, this binding clearly requires a proteinaceous receptor (Wnek and McClane 1986). Certain claudins have recently been implicated as protein receptors for CPE (Fujita et al. 2000; Katahira et al. 1997a, b). The claudins are a large family of >20 tetra-membrane-spanning proteins that play important roles in tight junction structure and function (Anderson and Van Itallie 1999). Claudin-3 and -4 were initially identified as functional CPE receptors by screening the CPE sensitivity of mouse L-929 fibroblasts (which are naturally CPE-insensitive due to their inability to bind CPE) transfected with cDNA from the CPE-sensitive Vero cell culture line (Katahira et al. 1997a, b). Fujita et al. used similar L-929 cell transfectant approaches to demonstrate that claudins-6, -7, -8, and -14 can also serve as functional CPE receptors (Fujita et al. 2000). However, L-929 cell transfectants expressing claudins-1, -2, -5, or -10 still remained insensitive to CPE (Fujita et al. 2000). Preliminary studies suggest that the receptor domain of claudin-3 lies within the second extracellular loop of the protein (Fujita et al. 2000). The involvement of claudins in tight junction function and regulation is an area of active research and studies continue to evaluate the involvement of certain claudins in CPE's intestinal action.

After binding, CPE forms a series of protein complexes in plasma membranes of host cells. One such CPE complex, the "small complex," forms concurrently with the binding of enterotoxin to all CPE-sensitive cells studied to date. This small complex, which likely corresponds to CPE bound to its receptor(s) (Wieckowski et al. 1994), is SDS-sensitive, forms at both 4°C and 37°C (McClane and Wnek 1990), and has a molecular weight of ~90 kDa (Wieckowski et al. 1994). The composition of this CPE complex remains to be determined, but coimmunoprecipitation results suggest it contains, in addition to CPE, an ~50-kDa host plasma membrane protein (Wieckowski et al. 1994). Linkage of an ~50-kDa host protein to small complex could either indicate that CPE has a nonclaudin cellular receptor in naturally-sensitive cells, or that the ~50-kDa protein is a claudin aggregate (anomalous migration of claudin aggregates on SDS-PAGE gels has been demonstrated in vitro; Mitic et al. 2003). Discriminating between these possibilities awaits further research.

Small complex formation, although important, is not sufficient to obtain CPE-induced cytotoxicity. While CPE forms small complex at 4°C, no cytotoxic effect occurs at that low temperature (McClane and Wnek 1990). However, if cells containing CPE se-

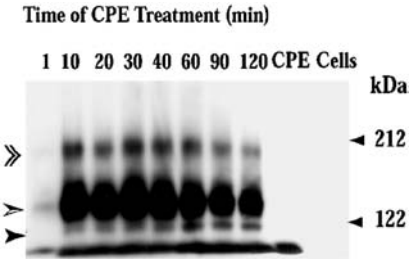

Fig. 1 Kinetics of large CPE complex formation in CaCo-2 cells. When CaCo-2 cells are incubated with CPE at 37°C and extracted with SDS, three CPE-containing protein complexes are visualized when those SDS-extracts are run on low acrylamide percentage (4%) SDS-PAGE gels and then Western immunoblotted with anti-CPE antiserum. These three CPE complexes have approximate molecular weights of 135 (*closed arrow*), 155 (*open arrow*), and 200 kDa (*double arrow*). Time of CPE-treatment is shown above the gel (in minutes); "CPE alone" and "cells alone" controls are shown in the right-most lanes of the gel. (Reproduced from Singh et al. 2000, with permission from the American Society for Biochemistry and Molecular Biology, Inc.).

questered only in small complex are shifted from 4°C to 37°C, there is immediate formation of large CPE-containing complexes in plasma membranes (Wnek and McClane 1989). Concurrently with the development of large, SDS-resistant CPE complexes, those cells exhibit plasma membrane permeability alterations (McClane and Wnek 1990). These results suggest that the small complex is a precursor for formation of the large CPE complexes and that formation of the large complexes initiates cytotoxicity.

The large, CPE-containing complexes have recently been resolved into at least three species, with molecular weights of ~135, ~155, and ~200 kDa (Fig. 1; Singh et al. 2000). The ~155-kDa complex is the first large complex formed when monolayers of CaCo-2 cells (a human enterocyte-like cell line) are treated with CPE at 37°C (Singh et al. 2001). Kinetic studies demonstrated that development of the ~155-kDa complex coincides with onset of membrane permeability changes in CPE-treated CaCo-2 cell monolayers (Singh et al. 2000), suggesting this large complex is responsible for initiating the cytotoxic response. Longer treatment of CPE-sensitive cells results in the formation of an ~200-kDa CPE complex, which contains the tight junction protein occludin (Singh et al. 2000). Formation of the ~200-kDa complex removes occludin from tight junctions and internalizes this tight junction protein into the cytoplasm (Singh et al. 2001). The redistribution of occludin, and probably claudin, induces structural damage to tight junctions, thereby leading to paracellular permeability changes that may contribute to CPE-induced diarrhea (Singh et al. 2001). Finally, the nature and function of the ~135-kDa large CPE complex remains unclear, but this CPE complex could be a precursor for, or intermediate form of, the ~155- or ~200-kDa complexes.

Considerable evidence suggests that formation of the ~155-kDa complex may correspond to the formation/insertion of a pore permeable to water, ions, and other small molecules. Early studies using radiolabeled markers demonstrated that cytoplasmic molecules up to ~3000 Da are released from CPE-treated Vero cells (McClane and Wnek 1990). Evidence supporting CPE's insertion into membranes was obtained (Wieckowski et al. 1998) when membrane-bound CPE localized in large complexes (mainly the ~155-kDa complex) was shown to develop resistance to external Pronase digestion. Whole-cell patch clamping of CaCo-2 cells demonstrated increased cationic permeability of those cells after

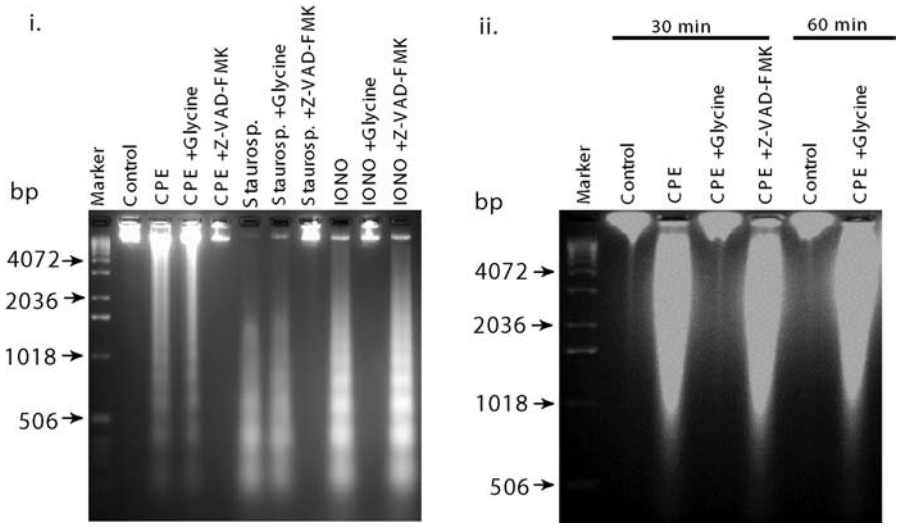

Fig. 2 CPE-induced DNA cleavage in CaCo-2 cells after treatment with 1 (*i*) or 10 (*ii*) μg/ml of CPE. Confluent CaCo-2 cells were pretreated with HBSS that did or did not contain either the broad-spectrum caspase inhibitor Z-VAD-FMK or the oncosis inhibitor glycine. Those cultures were then treated for 30 or 60 min in HBSS with or without 1 or 10 μg/ml of CPE, along with the same inhibitor used (if any) during pretreatment. After CPE treatment, DNA was extracted from each sample, run on a 2% agarose gel, and stained with ethidium bromide. CaCo-2 cells treated for 8 h at 37°C with either staurosporine or ionomycin served as positive controls for apoptosis and oncosis, respectively. Control depicts CaCo-2 cells treated only with HBSS (no CPE). *Arrows* at the *left side* of the gel indicate the migration of DNA size markers in basepairs (bp). (Reproduced from Chakrabarti et al. 2003)

CPE treatment (Hardy et al. 1999). The enterotoxin was also shown to form pores, similar to those observed during CaCo-2 cell patch clamping, in artificial phospholipid bilayer systems lacking any proteins (Hardy et al. 2001; Sugimoto et al. 1988). While artificial membrane studies support the ability of CPE to interact directly with lipid bilayers, it might be noted that these studies used either CPE sonicated into membranes or exceptionally high enterotoxin concentrations, i.e., under pathophysiologic conditions, CPE binding to protein receptors remains essential.

CPE-induced cellular death pathways

CPE has been recognized since the 1970s as a potent cytotoxin that kills sensitive mammalian cells by inducing membrane permeability alterations (McClane et al. 2000). Our laboratory recently identified the cellular death pathways involved in CPE-induced cytotoxicity (Chakrabarti et al. 2003). We found that, in CaCo-2 cells, a 1 μg/ml CPE dose induces features typical of a classical caspase 3-mediated apoptosis (Hockenbery 1995; Majno and Joris 1995; Steller 1995), including morphologic alterations, DNA cleavage into ladder-like fragments (Fig. 2i), mitochondrial membrane depolarization, cytochrome C and caspase 3/7 activation (Chakrabarti et al. 2003). In contrast, a 10 μg/ml CPE dose induced morphologic alterations and random DNA cleavage in CaCo-2 cells (Fig. 2ii); those effects could be transiently inhibited by the oncosis inhibitor glycine, but not by any

caspase inhibitors, suggesting that high CPE doses kill CaCo-2 cells via the oncosis death pathway.

Some earlier in vivo studies using CPE-treated rabbit ileal loops observed inflammatory cell (mostly PMNs) infiltration (McDonel et al. 1978), while other studies using the same in vivo model failed to detect any CPE-induced inflammation (Sarker et al. 1999; Sherman et al. 1994). The presence or absence of inflammatory cell infiltration in those rabbit ileal loop experiments appeared to be CPE dose-dependent, with inflammation more prominent in the ileal loops treated with high CPE doses (McDonel and Duncan 1975). Our results demonstrating that high CPE doses induce oncosis could explain the apparent association between high CPE doses and intestinal inflammation since inflammation is more commonly associated with oncosis than with apoptosis. Since inflammation has been shown to contribute to the symptoms of other GI diseases (Boise and Collins 2001; Brennan and Cookson 2000), and high CPE levels have been measured in the feces of some patients with CPE-associated GI diseases (Bartholomew et al. 1985), the proinflammatory consequences of oncosis could contribute to intestinal pathology in some CPE-induced GI disease cases.

Calcium involvement in CPE-induced cell death

Ca^{2+} must be present in treatment buffers to obtain CPE-induced morphologic damage in cultured Vero or HeLa cells (Matsuda and Sugimoto 1979). Vero cell studies ruled out the possibility that extracellular Ca^{2+} is required for CPE binding or for the development of CPE-induced small molecule permeability alterations (McClane et al. 1988). Since increased cytoplasmic calcium levels can trigger cell death pathways (Boehning et al. 2003; Choi 1995) and CPE can affect plasma membrane permeability for small molecules such as ions (McClane and Wnek 1990), we hypothesized that extracellular Ca^{2+} might be required in treatment buffers to obtain morphologic damage in CPE-treated mammalian cells because the enterotoxin causes an influx of extracellular Ca^{2+} that activates cell death pathways (see above, "CPE-induced cellular death pathways").

When that hypothesis was tested (Chakrabarti and McClane, in press), CaCo-2 cultures exhibited a ladder-like pattern of DNA cleavage, caspase 3/7 activation, and mitochondrial membrane depolarization only when treated with a 1 μg/ml CPE dose dissolved in HBSS containing Ca^{2+}. Similarly, smeared DNA cleavage only developed when CaCo-2 cultures were treated with a 10 μg/ml CPE dose dissolved in HBSS containing Ca^{2+}, i.e., both CPE-induced apoptosis and oncosis require the presence of extracellular calcium. CPE treatment caused a dose-dependent increase in calcium influx that selected whether the oncosis or apoptosis death pathways became activated in CaCo-2 cells. Finally, CPE-mediated apoptosis and oncosis were both shown to involve calmodulin and calpain, two calcium-activated cell proteins.

Model for CPE's cellular and molecular action

An updated model for CPE action (Fig. 3) begins with binding of CPE to the apical membrane of enterocytes via protein receptors, which may include certain claudins. This binding localizes CPE in a small complex of ~90 kDa. In steps that remain to be defined, the small CPE-containing complex then interacts with other proteins to sequester CPE in an

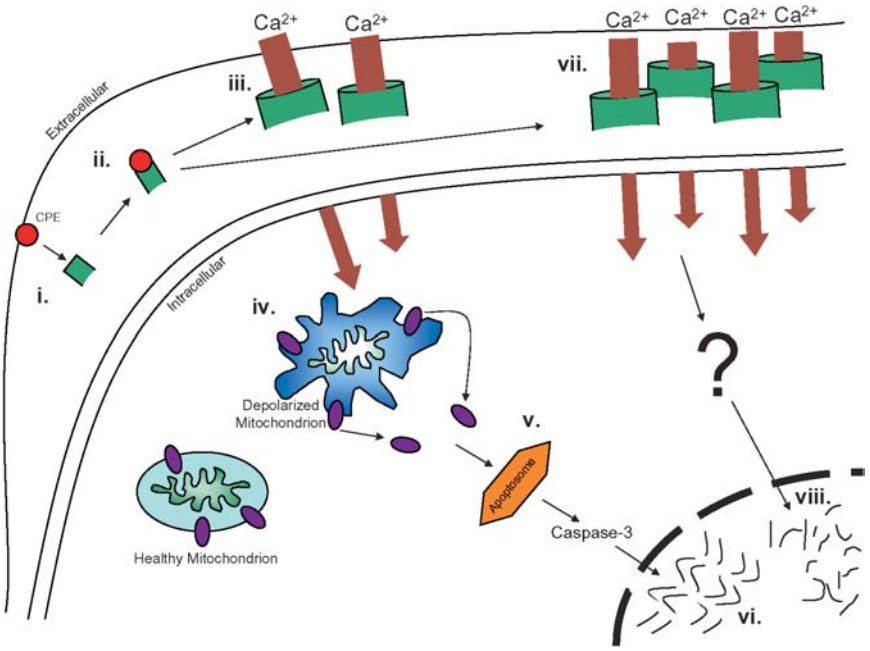

Fig. 3 A current model for the mechanism of action of CPE. (*i*) CPE first binds to protein receptors on the surface of intestinal epithelium, causing (*ii*) formation of the SDS-sensitive small complex. At physiological temperatures, CPE then localizes in (*iii*) an SDS-resistant, ~155-kDa complex that initiates membrane permeability alterations that permit calcium influx into the cell. Low doses of CPE, where modest amounts of calcium influx activate calmodulin and calpain, lead to (*iv*) depolarization of mitochondrial membranes and release of cytochrome C into the cytosol, which results in (*v*) formation of the apoptosome. Apoptotic cell death pathway involves caspase activation that eventually leads to (*vi*) DNA cleavage into regular 200 bp fragments. In contrast, high doses of the enterotoxin typically produce (*vii*) a massive calcium influx that activates the oncosis cell death pathway producing (*viii*) random DNA cleavage through mechanisms involving a vigorous calmodulin and calpain activation

~155-kDa complex. Formation of this large CPE complex, which apparently has pore-like properties, initiates small molecule membrane permeability alterations. Those permeability alterations, via an influx of extracellular Ca^{2+}, rapidly increase cellular Ca^{2+} levels. With very high CPE doses, where many pores form, the increase in cellular Ca^{2+} levels is so rapid and massive that oncosis develops due to strong calmodulin and calpain activation. With lower CPE doses, there is a slower and lesser (but still potent) increase in cellular Ca^{2+} levels that causes a lesser activation of calmodulin and calpain. Activation of those (and other?) cytosolic proteins leads to mitochondrial membrane depolarization inside the CPE-treated cell, which results in substantial cytochrome C release that triggers a rapid apoptotic response involving caspase 3/7 activation.

The resultant morphologic damage from CPE-induced oncosis or apoptosis exposes the basolateral membranes on the CPE-treated cell and adjacent cells present in the intestinal epithelium. This exposure allows unbound CPE access to additional CPE receptors on the basolateral membranes, from which the receptor-bound CPE can interact with occludin. Those interactions cause formation of additional ~155-kDa complex and initial formation of the ~200-kDa CPE complex, containing occludin. Formation of the ~200-kDa complex triggers internalization of tight junction proteins such as occludin, thereby damaging tight

Fig. 4 Functional map of CPE. Removal of the first 44 amino acids (shown in *red*) from native CPE activates toxicity by promoting 155-kDa complex formation; this effect corresponds to proteolytic processing during in vivo disease. *Blue*, deletion and point mutational analyses have identified a region between Asp-45 and Glu-53 that is essential for cytotoxicity by promoting formation of the ~155 kDa large complex. Depicted in this region are amino acid changes that result in attenuation of the enterotoxin's toxicity. *Black*, receptor binding activity of CPE maps to the extreme 30 amino acids at the C-terminus of the toxin

junction structure and function. That tight junction disruption produces paracellular permeability alterations which contribute to CPE-induced diarrhea during GI disease. Studies are now underway to further test this model.

CPE structure–function relationships

Understanding of CPE functional domains has been evolving over the past 15 years. Initial studies, utilizing truncated CPE fragments and synthetic peptides, mapped receptor binding activity to the 30 C-terminal CPE amino acids (Fig. 4; Hanna and McClane 1991; Hanna et al. 1989, 1991, 1992). The importance of the toxin's extreme C-terminus for binding was then confirmed by further deletion analysis, which demonstrated removing the five C-terminal amino acids from recombinant CPE (rCPE) eliminates receptor binding activity (Kokai-Kun and McClane 1997). Fine-mapping of this C-terminal binding region and its interaction with CPE cellular receptors is the subject of ongoing studies.

Early studies had shown that limited trypsin or chymotrypsin treatments cleave CPE at its N-terminus, removing (respectively) 25 or 36 amino acids (Granum and Richardson 1991; Granum et al. 1981; Hanna et al. 1992; Richardson and Granum 1983). Those protease treatments increase CPE activity ~2–3 fold by promoting increased formation of large CPE complexes (particularly the ~155-kDa complex). Since trypsin and chymotrypsin are both intestinal proteases, it seems likely that CPE is subjected to similar proteolytic activation during in vivo disease (Granum and Richardson 1991; Granum et al. 1981; Hanna et al. 1992).

The observation that C-terminal CPE fragments can bind, yet do not kill mammalian cells, suggested that sequences in the N-terminal half of CPE must be important for cytotoxicity (Hanna and McClane 1991; Hanna et al. 1989). Further rCPE deletion mutagenesis studies identified a region in the N-terminal half of CPE that appears to be essential for cytotoxicity (Kokai-Kun and McClane 1997). Specifically, rCPE fragments lacking the N-terminal 36 or 44 amino acids were found to display the same "activated" phenotype produced by limited proteolysis (as described in the preceding paragraph). However, deleting the first 52 N-terminal residues produced an rCPE fragment that binds but is completely noncytotoxic (Fig. 4; Kokai-Kun and McClane 1997). These important findings mapped a CPE region between Asp-45 and Gly-53 as being essential for CPE activity. Random point mutagenesis performed on rCPE using the *Escherichia coli* mutator strain, XL-1

Red, confirmed the importance of this putative Asp-45 to Gly-53 cytotoxicity region when a G49D mutant was produced that displays similar binding properties as rCPE, yet does not form any large complexes or elicit a cytotoxic response (Fig. 4; Kokai-Kun et al. 1999).

In order to more precisely map the N-terminal CPE "cytotoxicity" domain, alanine-scanning mutagenesis has recently been performed (J.G. Smedley, III and B.A. McClane, in press). When alanine substitutions were introduced by site-directed mutagenesis into each residue of the Asp-45 to Glu-54 region of CPE, two rCPE variants (D48A and I51A) were identified that are nontoxic or strongly attenuated for cytotoxicity, respectively (Fig. 4). Both variants exhibited wild-type levels of binding and small complex formation, but had a phenotype deficient in large complex formation and in the ability to elicit ^{86}Rb-release from cells. When more conservative mutations were introduced at these same two amino acid positions in CPE, a glutamate or asparagine substitution at Asp-48 also produced nontoxic CPE variants. However, substituting either leucine or valine at residue Ile-51 resulted in variants with full cytotoxic activity. These findings indicate that the side chain length and charge of aspartic acid is required at position 48, and that residue volume/hydrophobic interactions are important at amino acid 51 in this N-terminal cytotoxicity domain of the toxin. Further deciphering of the precise role of this region in the CPE's mechanism of action is continuing.

CPE: foe or friend?

CPE's binding to certain claudins becomes interesting given recent observations that claudins are often upregulated in tumors (Long et al. 2001; Michl et al. 2001). This has inspired recent research exploring potential therapeutic uses for the enterotoxin in cancer treatment. An initial study showed that claudin-4 is overexpressed in many pancreatic cancer tissues and cell lines. (Michl et al. 2001). In vivo experiments then demonstrated that injection of CPE into a pancreatic tumor xenograft grown on the back of mice stopped the growth (and caused necrosis) of that tumor, without harming the mouse. Another study illustrating CPE's potential antitumor use noted that metastatic prostate adenocarcinoma cells obtained from a patient suffering from prostate cancer overexpress both claudin-3 and -4 (Long et al. 2001). Those cells were shown to be highly sensitive to CPE-mediated lysis. Collectively, these results support the need for increased understanding of CPE-claudin interactions and for continued study of the enterotoxin's molecular mechanism of action.

The *Clostridium perfringens* ϵ-toxin

Epsilon toxin, a 33-kDa protein produced by *C. perfringens* type B and type D strains (Table 1), is the third most lethal of all clostridial toxins, ranking behind only tetanus and botulinum neurotoxins. Type B and D strains producing ϵ-toxin cause fatal enterotoxemias, mainly in lambs and goats (Table 2; Songer 1996), and this toxin is considered a major potential bioterrorism agent.

Epsilon toxin is synthesized and secreted as a relatively inactive prototoxin, which is then converted to an active mature toxin by removing 13 residues from the N-terminus and 22 residues from the C-terminus. This proteolytic activation can be catalyzed by intes-

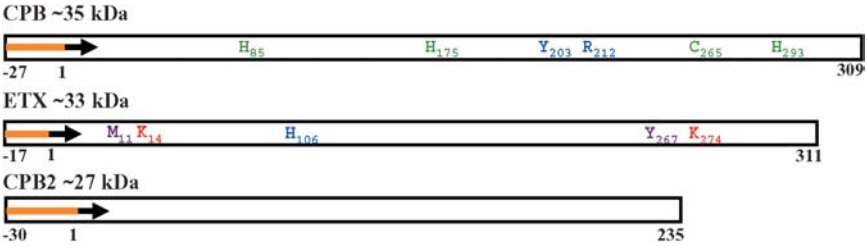

Fig. 5 The primary amino acid structures of CPB, ETX, and CPB2. Secretory peptides are shown in *orange* (these peptides are removed from the mature toxin and thus not included in the molecular weights). ETX or CPB amino acids modified in various studies using site-directed mutagenesis are shown within each peptide; mutagenesis of *green* residues did not decrease toxicity, whereas mutagenesis of *blue* residues caused a decrease in toxicity. The *purple* and *red* amino acids depict trypsin and chymotrypsin cleavage sites, respectively

tinal proteases, such as trypsin and α-chymotrypsin (Hunter et al. 1992; Miyata et al. 2001). Lambda toxin, a protease produced by some type B and D isolates, can also remove 11 N-terminal and 29 C-terminal amino acids from the ϵ-prototoxin to make active toxin (Jin et al. 1996; Minami et al. 1997). Activated ϵ-toxin possesses lethal, dermonecrotic, and edematic activity (Hunter et al. 1992).

The ϵ-toxin structural gene, *etx*, is carried on a poorly-studied large plasmid (Katayama et al. 1996). An insertion sequence, IS*1151,* which has sequence similarity with IS*231* from *Bacillus thuringiensis*, is located 96 bp upstream of the *etx* gene (Daube et al. 1996; Matsushita et al. 1994). Sequences downstream of *etx* shows similarity to the transposon Tn*4001* of *Staphylococcus aureus* (Hunter et al. 1992).

Epsilon toxin appears to be composed primarily of β sheets (Hunter et al. 1992) and has 20–27% sequence identity with the mosquitocidal toxin Mtx2 and Mtx3 of *Bacillus sphaericus* and with a parasporal crystal protein c53 of *B. thuringiensis* (Liu et al. 1996; Thanabalu and Porter 1996). When the nucleotide sequences of the *etx* open reading frame from a type B isolate (NCTC 8533) and a type D isolate (NCTC 8346) were compared, nucleotide changes were found at positions 762 and 962 (Havard et al. 1992); S. Sayeed et al., unpublished data). While the nucleotide difference at position 762 results only in a silent mutation, the second difference (position 962) codes for a serine in the type B strain and for a tyrosine in the ϵ-toxin variant of the type D strain. Despite these changes, both toxin variants are biologically active and cross-react immunologically (Hunter et al. 1992). Sequence differences in putative –10 and –35 regions upstream of the *etx* ORF have also been identified between the two types, but any significance of those observations for *etx* transcription is unclear.

Mutational analysis of ϵ-toxin lethality

Several ϵ-toxin amino acid residues have been identified that are clearly important for toxicity. Chemical modification results suggest that one tryptophan (Kumar et al. 2002; Sakurai and Nagahama 1985), one histidine (Sakurai and Fujii 1987), one tyrosine (Sakurai and Fujii 1987), three or four aspartic or glutamic acid (Sakurai and Fujii 1987), and eight lysine (Sakurai and Nagahama 1986) residues are essential for the lethal activity of the toxin (Fig. 5). Oysten et al. constructed a nontoxic mutant by replacing proline with

a histidine at residue 106 of the native ϵ-toxin (Oysten et al. 1998). Immunization of mice with this nontoxic ϵ-toxin mutant induced a specific antibody response that protected the mice against 1,000 LD_{50} doses of the wild-type ϵ-toxin.

ϵ-Toxin mechanism of action

During type B- and D-mediated disease, ϵ-toxin is produced within the gut of the infected animal. The toxin is then absorbed across the mucosal barrier, either by inducing direct damage, as occurs in goats (Fernandez Miyakawa and Uzal 2003), or as a result of eating a high fiber diet which can also damage intestinal permeability (Songer 1996). Once ϵ-toxin has crossed the mucosal barrier, it is rapidly carried through the bloodstream to target organs. A recent study using whole-body autoradiograms taken immediately after death showed that ϵ-toxin injected systemically into mice localizes primarily in the nasal cavity, brain, spinal cord, and kidneys (Tamai et al. 2003). Those observations are consistent with cumulative findings identifying ϵ-toxin's primary biological action as production of edema and hemorrhage in the brain, heart, lung, liver, and the kidneys (Petit et al. 2003). An interesting clinical phenomenon of ϵ-toxin intoxication is the appearance of neurological symptoms including opisthotonous and convulsions, which are thought to result from the toxin's ability to cross the blood-brain barrier, followed by damage to brain vasculature and direct effects on cells of the hippocampus (resulting in increased glutamate release) (Miyamoto et al. 1998, 2000). These potent effects have led to the inclusion of ϵ-toxin as a Class B Select Agent by the U.S. Centers for Disease Control.

In the late 1970s it was hypothesized that ϵ-toxin requires a cell-specific receptor for binding to mammalian cells (Buxton 1978). Those early studies focused on interactions between ϵ-toxin and tissue derived from rat or mouse brains and kidneys. Within the past 10 years ϵ-toxin susceptible cell culture lines (i.e., Madine-Darby canine kidney (MDCK) cells and human leiomyoblastoma (G-402) cells) have become available for in vitro research (Petit et al. 2003). Because MDCK cells are much more sensitive to ϵ-toxin than are G-402 cells, most studies have used MDCK cells as a model for in vitro cytotoxicity.

The limited number of sensitive cell lines is consistent with ϵ-toxin requiring a specific receptor. It was initially proposed that a sialoglycoprotein residing in a specific membrane environment is required for ϵ-toxin binding (Nagahama and Sakurai 1992). This conclusion was drawn from experiments demonstrating that rat brain tissue treated with Pronase, neuramidase, or lipase lost sensitivity to ϵ-toxin, whereas trypsin- or phospholipase C-treated rat brain tissue remained sensitive to ϵ-toxin (Nagahama and Sakurai 1992). The involvement of proteins in ϵ-toxin binding received further support from recent studies culturing cells normally sensitive to ϵ-toxin and selecting for resistance to the toxin (Beal et al. 2003). Two-dimensional gel electrophoresis studies of those resistant cells uncovered several proteins shifted in molecular weight and/or pI which may be putative receptors for ϵ-toxin.

Epsilon toxin action appears to be more complicated than simple interactions between this toxin and protein receptors. MDCK cells depleted of cholesterol lose their ϵ-toxin sensitivity (Miyata et al. 2002). When combined with the discovery of detergent-resistant membrane domains (DRMs) within cell membranes, this finding led researchers to explore whether or not ϵ-toxin interacts with DRMs. Using Triton X-100 cold-extracted membranes separated on sucrose gradients, ϵ-toxin was localized to fractions containing glycosphingolipid-enriched DRMs (Miyata et al. 2002).

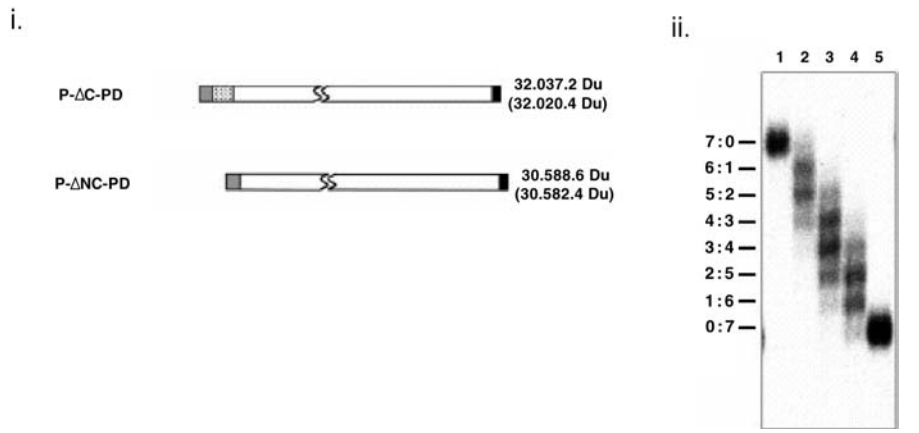

Fig. 6 Demonstration of *C. perfringens* ϵ-toxin heptamerization in rat synaptosomal membranes. Two recombinant ϵ-toxin species were engineered that corresponded to either the mature toxin with no cleavage peptides present (P-ΔNC-PD) or a protoxin with only the N-terminal cleavage peptide remaining (P-ΔC-PD) *i*. Each ϵ-toxin species was radiolabeled for detection and the molecular weight of each ϵ-toxin species (to the *left* of each construct) was then determined from the predicted sequence (*top number*) and experimentally by MALDI-TOF (*in parenthesis*). The two constructs were incubated in different ratios (*left hand margin* of *ii*) totaling 12 ng of protein, with 2.2 mg of synaptosomal membranes in TBS. Samples were treated with SDS-sample buffer, resolved on a 5% SDS-PAGE gel, and developed using autoradiography (*ii*). Note the presence of seven different molecular weight species which is indicative of heptamer formed with varying ratios of the two different constructs. (Reproduced from Miyata et al. 2001)

Previous work has shown that ϵ-toxin forms SDS-resistant large molecular weight species in MDCK cells or mouse brain homogenates, but not in nonsensitive cells or heat-treated brain homogenates (Nagahama and Sakurai 1992). Later studies further defined ϵ-toxin interactions with MDCK cells and artificial lipid bilayers by showing that: (1) ϵ-toxin is a pore-forming toxin (Petit et al. 2001), (2) functional ϵ-toxin pore formation, but not binding, requires removal of the 23 C-terminal amino acids from ϵ-toxin monomers (Fig. 6; Miyata et al. 2001), (3) the functional pore corresponds to an ϵ-toxin heptamer within MDCK cells and rat synaptosomal membranes (Miyata et al. 2002), and (4) ϵ-toxin is not internalized after pore formation (Petit et al. 2003). The ϵ-toxin pores formed in lipid-bilayers have been shown to allow molecules up to 1 kDa to diffuse freely (Petit et al. 2001).

Pore-formation in ϵ-toxin-sensitive cells or artificial lipid-bilayers leads to increased cellular anion/cation permeability and also causes decreased transepithelial resistance (Petit et al. 2001, 2003). These effects produce net water movement across endothelial/epithelial layers, possibly explaining the edema associated with ϵ-toxin-induced disease. Similar to the *Helicobacter pylori* VacA toxin, the decrease in transepithelial resistance is not accompanied by loss of tight junction contacts (Petit et al. 2003). Immunofluorescence studies have shown that MDCK cells treated with ϵ-toxin had no redistribution of their junction-associated proteins, including actin filaments, ZO-1, E-cadherin, and α catenin (Petit et al. 2003).

Together, these studies indicate that once ϵ-toxin is activated by proteolytic cleavage in the gut, the toxin moves across the intestinal epithelium sometimes by directly inducing damage. It is then transported through the circulatory system to target organs including the

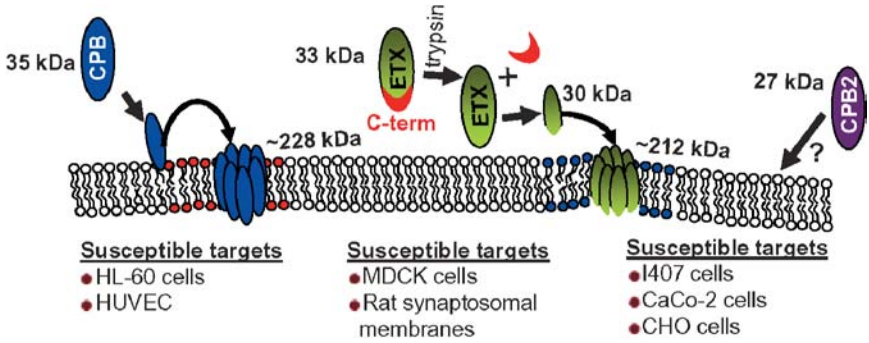

Fig. 7 Proposed mechanisms of action of CPB, ETX, and CPB2. CPB is believed to bind within lipid rafts (*red* lipids), where it apparently interacts with other CPB monomers to form a heptameric pore. A similar mechanism has been proposed for ETX; however, a processing step involving trypsin/chymotrypsin cleavage of the C-terminal end is necessary for pore formation. The mechanism of action of the CPB2 toxin remains to be determined. Cell lines shown to be susceptible to each toxin are shown *below* the respective mechanism of action cartoon

brain, kidneys, and lungs. Upon reaching target organs, the ϵ-toxin binds to cells containing the appropriate receptors (probably glycosylated-proteins located within DRMs; Fig. 7). After binding, the ϵ-toxin forms a nonselective pore by oligomerizing into a heptamer (Fig. 6). This pore results in loss of transepithelial resistance, and ultimately in net water movement that creates edema within intoxicated tissues. Further studies are needed to confirm that these effects are responsible for all neurotoxicity and nephrotoxicity associated with ϵ-toxin.

The *Clostridium perfringens* β-toxin

β-Toxin is a lethal toxin produced by *C. perfringens* type B and type C strains (Table 1), which causes a necrotic enteritis characterized by hemorrhagic mucosal ulceration or superficial mucosal necrosis of the small intestine (Sakurai 1995; Sakurai and Fujii 1987) in humans (type C only) or animals (type B and C). β-Toxin is secreted in late log growth phase, is thermo-labile, and highly-sensitive to proteases. The molecular weight of the β-toxin polypeptide is ~35 kDa (Fig. 5; Hunter et al. 1992).

The gene (*cpb*) encoding β-toxin is carried on a plasmid (Katayama et al. 1996; Sakurai and Duncan 1978) that has received little attention thus far except for studies demonstrating the presence of IS*1151* sequences (Daube et al. 1996). The β-toxin gene has been cloned and sequenced (Hunter et al. 1993), which revealed an open reading frame encoding a 336 amino acid polypeptide that includes a 27-amino acid signal sequence removed from the mature β-toxin (Fig. 5). A possible Shine-Delgarno sequence is located 7 bp upstream of the start codon of the *cpb* gene and the intervening region is composed entirely of thymidine residues. Due to its high A+T content, potential promoter sequence prediction for *cpb* is difficult. Also, unlike the genes encoding most of these toxins, the *cpb* gene does not contain any stem-loop structures followed by a poly (T) tail, suggesting that rho-independent termination of *cpb* transcription does not occur (Hunter et al. 1993). Comparison of *cpb* sequences between *C. perfringens* type C and B isolates detected 1–6 base dif-

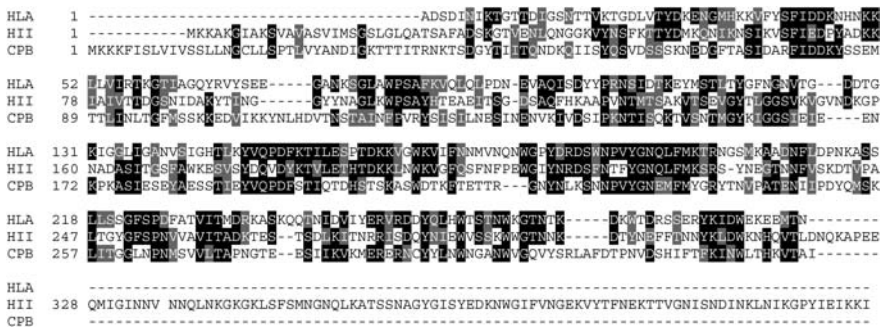

Fig. 8 Amino acid sequence alignment between CPB and other members of the leukocidin and γ-toxin families. CPB shows approximately 17–29% amino acid similarity with the leukocidin toxin (HLA) and γ-toxin (HII) of *Staphylococcus aureus*. This sequence comparison suggest a similar mode of action between CPB and these pore-forming toxin families. Identical residues are shown in *black* and similar residues are shown in *gray*. The alignment was performed using ClustalW Multiple Sequence Alignment program (http://www.ebi.ac.uk) and illustrated using Boxshade (http://www.ch.embnet.org/software/BOX_form.html)

ference within the open reading frame (Steinthorsdottir et al. 1995; D.J. Fisher et al., unpublished data). Some of those changes result in amino acid substitutions, but the significance of those changes for toxicity is unclear.

The deduced amino acid sequence of β-toxin significantly resembles several pore-forming toxins, with 28% similarity to *Staphylococcus aureus* α-toxin (Hunter et al. 1993), 22%, 28%, and 18% similarity, respectively, to the A, B, and C components of γ-toxin (Cooney et al. 1993), 17% and 28% similarity, respectively, to the S and F components of leukocidin, and 25% similarity to cytotoxin K of *Bacillus cereus* ATCC 14579 (Fig. 8). These similarities are dispersed throughout the β-toxin protein, with the weakest similarity at the N-terminal region. While a common feature of the toxins sharing sequence homology with β-toxin is their hemolytic activity, β-toxin is not hemolytic for either rabbit or sheep erythrocytes (Steinthorsdottir et al. 1998)

Mutational analysis of β-toxin lethality

Chemical modification experiments identified certain residues likely to be essential for β-toxin lethal activity (Fig. 5). One study created 11 single mutations in the β-toxin protein and found that an arginine at position 212 is very important, i.e., replacement of that Arg-212 residue with glutamic acid resulted in an 11.5-fold reduction in toxicity (Steinthorsdottir et al. 1998). However, replacing the same Arg-212 residue with glutamine reduced lethal activity only by about 5.5-fold. Another study determined that the thiol group on β-toxin's single cysteine residue is sensitive to modification but is not strictly required for lethal activity, i.e., replacing this cysteine residue (at position 265) with either alanine or serine has very little effect on lethal activity (Nagahama et al. 1999). However, changes at residues 266, 268, and 275 in β-toxin's C-terminal region containing the cysteine residue caused a complete loss of activity. This result suggests sites adjacent to the cysteine residue play a critical role in mediating toxicity. Nagahama et al. have proposed

that this C-terminal region is either required for β-toxin binding to its receptor or for formation of β-toxin oligomers (Nagahama et al. 1999).

β-Toxin mechanism of action

Until recently, detailed studies of β-toxin's action were delayed by the lack of commonly used in vitro models sensitive to this toxin. In 1997, Gilbert et al. first reported weak β-toxin cytotoxicity on intestinal I407 cells, but that has not since been confirmed or reproduced by other groups (Gibert et al. 1997). Steinthorsdottir et al. (2000) later showed that β-toxin forms heat-stable multimers on the membrane of HUVEC (human umbilical vein endothelial cell) cells, causing leakage of inositol and arachidonic acid from these cells (Fig. 7). However, it was not clear whether this release is a direct result of toxin-containing transmembrane channels or is an indirect cellular effect of β-toxin. Direct evidence of β-toxin pore formation came from later work done by Shatursky et al. (2000), who demonstrated that this toxin forms cation-selective pores in bilayer lipid membranes (BLM) composed of approximately 50% phosphatidylcholine and cholesterol. The size of these toxin pores was determined to be approximately 12 Å in diameter and they were shown to be selective for monovalent cations like Na^+ and K^+. This study also reported the insertion rate of β-toxin into membranes is stimulated by the presence of divalent cations. Nagahama et al. (2003) recently demonstrated the HL 60 (Human leukemia cell) cell line is also susceptible to β-toxin; in this cell culture model, β-toxin induces K^+ efflux and Ca^{2+}, Na^+ and Cl^- influxes, which then produce cell swelling and cell lysis.

Collectively, available information strongly suggests that β-toxin is a pore-forming toxin that oligomerizes to form channels in susceptible membranes (Fig. 7). The resultant membrane permeability changes result in cell death. The role of β-toxin in infections is still poorly understood. For example, while type C infections result in intestinal necrosis, purified β-toxin does not by itself induce intestinal damage in animal models. However, a β-toxoid vaccine protects humans against necrotizing enteritis from type C isolates of *C. perfringens* (Lawrence et al. 1990), which implies that β-toxin does have some intestinal activity at least in combination with other *C. perfringens* toxins. Tweten (2001) has proposed that, once absorbed from the intestines into the circulation by unknown mechanisms, β-toxin might exert neurological effects on the host by directly affecting the distribution of cations across the membranes of susceptible nervous system cells.

The *Clostridium perfringens* β2 toxin

The nucleotide sequence encoding the β2 toxin (CPB2) was first reported in 1997 (Gibert et al. 1997). Despite its name, the CPB2 toxin has no significant amino acid similarity with the β-toxin (15% nucleotide similarity), nor with any other known or hypothetical proteins (Gibert et al. 1997).

Subsequent publications (Bueschel et al. 2003; Gibert et al. 1997) determined that the CPB2 toxin is produced as a 265 amino acid polypeptide containing an N-terminal secretion signal of 30 amino acids (Fig. 5). After secretion, the CPB2 toxin has a molecular weight of 27.7 kDa and a pI of 5.01 (Bueschel et al. 2003; Gibert et al. 1997). The secreted CPB2 toxin is highly susceptible to proteolytic cleavage, as treatment with trypsin

cleaves CPB2 into two inactive species, with apparent molecular weights of 13 kDa and 15 kDa (Gibert et al. 1997).

As is true of genes encoding many other *C. perfringens* toxins, the *cpb2* gene is carried on plasmids, which range in size from 54 kb to ~100 kb (Gibert et al. 1997; Shimizu et al. 2002). The *cpb2* gene can be found in all *C. perfringens* toxin types (Bueschel et al. 2003; D.J. Fisher et al., unpublished data). In some *C. perfringens* type A human nonfood-borne GI disease isolates, the *cpb2* gene resides on the same plasmid carrying the *cpe* gene (D.J. Fisher et al., unpublished data). Expression of the *cpb2* gene is positively regulated by the VirR/VirS two component bacterial regulatory system and reaches a maximal level during late log phase during in vitro growth (Ohtani et al. 2003).

Mechanism of CPB2 action

Due in part to its relatively recent discovery, detailed studies of the mechanism of action of CPB2 have not been performed and structure-function studies have yet to be published. Type A and C isolates carrying *cpb2* have, however, been linked to GI diseases in various animals, especially adult pigs and piglets (Bueschel et al. 2003; Waters et al. 2003). CPB2 has been identified, by immunohistochemistry, in intestinal lesions of an elephant and horses suffering from natural gastroenteritis (Bacciarini et al. 2001, 2003). Also consistent with this toxin playing a role in intestinal disease, purified CPB2 toxin causes necrotic lesions in a guinea pig ileal loop model (Jolivet-Renaud 1986). Several cell lines are susceptible to purified CPB2 toxin at concentrations ranging from 0.2 to 20 μg/ml, including I407 cells (originally thought to be a human intestinal embryonic cell line, but now listed as a HeLa cell contaminant by the American Type Culture Collection) and Chinese hamster ovary cells (Gibert et al. 1997). Our laboratory has recently determined (D.J. Fisher et al., unpublished data) that CaCo-2 cells (a human colon carcinoma cell line) are also sensitive to CPB2, opening the possibility of this toxin playing a role in some human intestinal diseases. The LD_{50} of CPB2 in a mouse i.v. injection model is 3 μg (Jolivet-Renaud 1986).

When sensitive cells are treated with CPB2, they exhibit cell rounding, membrane bleb formation, and eventually detach from the cell culture matrix (Gibert et al. 1997; D.J. Fisher et al., unpublished data). Purified CPB2 toxin does not disturb the actin cytoskeleton in treated I407 cells (as visualized with phalloidin staining), nor does this toxin modify actin or small G-proteins via ADP ribosylation or UDP-glucosylation (Gibert et al. 1997). Those negative results, along with the physical appearance of toxin-treated cells, suggest that CPB2 resembles many other enterically-active *C. perfringens* toxins by functioning as a pore-forming toxin.

Concluding remarks

The recent emphasis on biodefense research has spawned a renaissance of interest in the *C. perfringens* toxins, particularly the enteric toxins. Epsilon toxin, a class B select agent, is often expressed by type B or D isolates also producing other *C. perfringens* enteric toxins, such as CPE, β-toxin, and CPB2. Coupling their biodefense importance with the current interest in therapeutic development, the *C. perfringens* enteric toxins are likely to receive extensive study in the coming years. Hopefully those studies will elucidate answers

to many important questions, such as: What is the composition of the ~155-kDa CPE complex? What are the physiologic receptors for each of the enterically-active *C. perfringens* toxins? How does CPB2 work? What is the structure of these toxins[1]? How does pore formation explain the physiologic effects of β- and ϵ-toxin?

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[1] Note added during revision: The structure of ϵ-toxin has recently been solved (Cole et al. 2004). Structurally, ϵ-toxin consists of three domains containing mainly β sheets and shares similarities with aerolysin, another pore-forming toxin.

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The *Helicobacter pylori* vacuolating cytotoxin: from cellular vacuolation to immunosuppressive activities

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Abstract *Helicobacter pylori* is a highly successful bacterial pathogen of humans, infecting the stomach of more than half of the world's population. The *H. pylori* infection results in chronic gastritis, eventually followed by peptic ulceration and, more rarely, gastric cancer. *H. pylori* has developed a unique set of virulence factors, actively supporting its survival in the special ecological niche of the human stomach. Vacuolating cytotoxin (VacA) and cytotoxin-associated antigen A (CagA) are two major bacterial virulence factors involved in host cell modulation. VacA, so far mainly regarded as a cytotoxin of the gastric epithelial cell layer, now turns out to be a potent immunomodulatory toxin, targeting the adapted immune system. Thus, in addition to the well-known vacuolating activity, VacA has been reported to induce apoptosis in epithelial cells, to affect B lymphocyte antigen presentation, to inhibit the activation and proliferation of T lymphocytes, and to modulate the T cell-mediated cytokine response.

Introduction

Since the discovery of vacuolating cytotoxin (VacA) 16 years ago (Leunk et al. 1988) and the first description of its purification (Cover and Blaser 1992), numerous studies have been performed to elucidate its mechanism(s) of action and its biological role. Particularly the effect of VacA on epithelial cells has been examined in great detail. The biological significance of VacA has been derived from several observations. First of all, large quantities of purified VacA can induce ulcer-like erosions when administered into the mouse stomach (Marchetti et al. 1995). In addition, VacA increases the risk of gastric ulcer formation in experimentally infected gerbils (Ogura et al. 2000) and also enhances the bacterial colonization rate in a mouse model of infection (Salama et al. 2001). Furthermore, certain *vacA* genotypes causing a high vacuolating activity are correlated with more severe disease in humans (Atherton et al. 1995). The *vacA* gene displays a considerable polymorphism, especially in the signal region (where the genotypes s1 and s2 may be dis-

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criminated) and in a mid region (genotypes m1 and m2). Vacuolating activity is higher in s1/m1 genotypes than in s1/m2 genotypes, and absent in s2/m2 genotypes (Atherton et al. 1995). Consequently, *vacA* s1/m1 genotypes are more frequently associated with peptic ulceration and gastric carcinoma. One further difference is a higher expression level of the s1/m1 as compared to the s2/m2 *vacA* genes (Forsyth et al. 1998).

Structure of the *vacA* gene and its product

The VacA protein is synthesized as a 140-kDa precursor protein (Cover et al. 1994; Schmitt and Haas 1994; Telford et al. 1994), consisting of an N-terminal signal sequence, the toxin domain, and a C-terminal extension of about 50 kDa. The C-terminal part of this extension is sufficient to accomplish secretion to the extracellular medium via an autotransporter mechanism (Fischer et al. 2001). Extracellularly, the mature 95-kDa toxin may be cleaved into an N-terminal 33-kDa (here p33, sometimes also designated as p37) and a C-terminal 55-kDa protein (here p55, sometimes also designated as p58) (Telford et al. 1994; Nguyen et al. 2001), respectively, that remain non-covalently associated (Telford et al. 1994). Purified VacA was shown to assemble into hexameric, heptameric, or dodecameric structures (Lupetti et al. 1996; Cover et al. 1997). Mutant forms of VacA that are unable to form oligomers also lack a vacuolating activity (Vinion-Dubiel et al. 1999), suggesting that oligomerization is an important feature of purified VacA. Under acid or alkaline treatment, these oligomeric forms become activated by dissociation into monomers (Cover et al. 1997; Molinari et al. 1998a; Yahiro et al. 1999). However, culture supernatants and VacA associated with the bacteria do not seem to require activation (Leunk et al. 1988; Pelicic et al. 1999), suggesting that active VacA may be monomeric in vivo, and oligomerization, which requires acid activation for its function, might be an artefact of protein purification.

Effects of VacA on epithelial cells

The vacuolating activity on epithelial cells, originally described by Leunk et al. (1988), is the most apparent effect of VacA. Despite a very extensive characterization, the mechanism of vacuolation is not completely understood. The vacuolating activity is also observed when the VacA protein is produced in epithelial cells, e.g., after transfection of the *vacA* gene cloned in an appropriate plasmid (de Bernard et al. 1997), suggesting an intracellular target for VacA vacuolating activity. On the other hand, VacA is able to form anion-selective membrane channels both in lipid bilayers (Czajkowsky et al. 1999; Szabo et al. 1999; Iwamoto et al. 1999) and in the plasma membrane of epithelial cells (Szabo et al. 1999). However, the channels seem to be structurally different in these two situations (Adrian et al. 2002). These pores induce a membrane depolarization (Szabo et al. 1999; Iwamoto et al. 1999; Schraw et al. 2002) and seem to be necessary for vacuole formation. In fact, inhibition of channel formation by addition of anion-channel blockers to VacA-treated epithelial cells also prevents vacuolation (Szabo et al. 1999; Tombola et al. 1999). Moreover, mutant forms of VacA lacking an N-terminal hydrophobic region or containing site-specific mutations in this region are defective in membrane channel as well as vacuole formation (Vinion-Dubiel et al. 1999; McClain et al. 2003). The pores are formed by oligomerization of membrane-bound monomers (Vinion-Dubiel et al. 1999), for which the N-terminal hydrophobic region is essential (McClain et al. 2001b, 2003). The N-terminal

sequence of s2-encoded VacA proteins contains a hydrophilic extension in comparison to s1-encoded VacA proteins, which is necessary and sufficient to render the proteins non-toxic (Letley and Atherton 2000; Letley et al. 2003; McClain et al. 2001b).

VacA binding to receptor and/or lipid rafts

As reported recently by Ilver et al. (2004), a significant portion of the toxin remains associated with the bacterial surface, where it seems to be organized into distinct domains. The bacteria-associated toxin was shown to be biologically active and may be transferred from the bacteria to the target cell by a contact-dependent mechanism (Ilver et al. 2004). The mechanism of a direct delivery or the involvement of a potential receptor for the direct toxin delivery to eukaryotic cells is still unknown. In contrast to surface-associated VacA, the interaction of secreted VacA with the target cell was reported to be dependent on specific protein receptors, but VacA inserts into artificial lipid membranes in the absence of protein receptors as well (Molinari et al. 1998a; Moll et al. 1995; Pagliaccia et al. 2000). This indicates that the toxin may not bind specifically to a single receptor but rather interacts with multiple components on the cell surface (Fig. 1). The first protein described functioning as a receptor for VacA was the epidermal growth factor (EGF) receptor (Seto et al. 1998). Later, Yahiro et al. (1999) identified the receptor-like protein tyrosine phosphatase (RPTP)-β, a 250-kDa surface glycoprotein, as a VacA-binding receptor on the surface of the gastric adenocarcinoma cell line AZ-521. Interestingly, this interaction was shown to be relevant for the development of VacA-induced gastric ulcers. Fujikawa et al. (2003) reported that wild-type mice, but not mice deficient in RPTP-β, showed mucosal damage and ulcer induction by VacA. RPTP-β is known to be widely expressed in cells of the nervous system, where it regulates the maturation, development, and differentiation of neuronal and glial cells. But Fujikawa et al. demonstrated its expression in gastric tissue as well (Fujikawa et al. 2003). Studies with primary RPTP-$\beta^{+/+}$ and RPTP-$\beta^{-/-}$ gastric epithelial cells indicated that VacA was taken up and induced vacuolation in both cell types to the same extent (Fujikawa et al. 2003). This suggested the existence of other receptors or an internalization mechanism for uptake of VacA besides RPTP-β. Remarkably, binding of VacA to RPTP-β increased significantly the tyrosine phosphorylation of the G protein-coupled receptor kinase interactor (Git)-1, a substrate of RPTP-β, and subsequently induced the detachment of the cells from a reconstituted basement membrane. The authors concluded that erroneous RPTP-β signaling might be involved in the development of gastric ulcers (Fujikawa et al. 2003). Co-immunoprecipitation experiments using RPTP-β-negative, but VacA-sensitive cells led to the identification of another receptor-like protein tyrosine phosphatase, RPTP-α, as a receptor for VacA (Yahiro et al. 2003). In contrast to RPTP-β, RPTP-α is ubiquitously expressed (Yahiro et al. 2003). Besides the interaction of VacA with these specific receptors, VacA was also shown to bind to detergent-resistant microdomains (lipid rafts) of the cell membrane, which are enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol-anchored proteins (GPI-APs) (Fig. 1). As reported by several groups, depletion of membrane cholesterol significantly reduces the entry of VacA into target cells (Patel et al. 2002; Schraw et al. 2002; Kuo and Wang 2003). Contrasting results exist concerning the involvement of GPI-APs in internalization of VacA via lipid rafts. Kuo and Wang (2003) demonstrated that removal of GPI-APs from the cell surface by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) blocked VacA-induced vacuolation, but did not affect binding of VacA to lipid rafts. Similar data were reported by Ricci et al. (2000). In agreement with these findings,

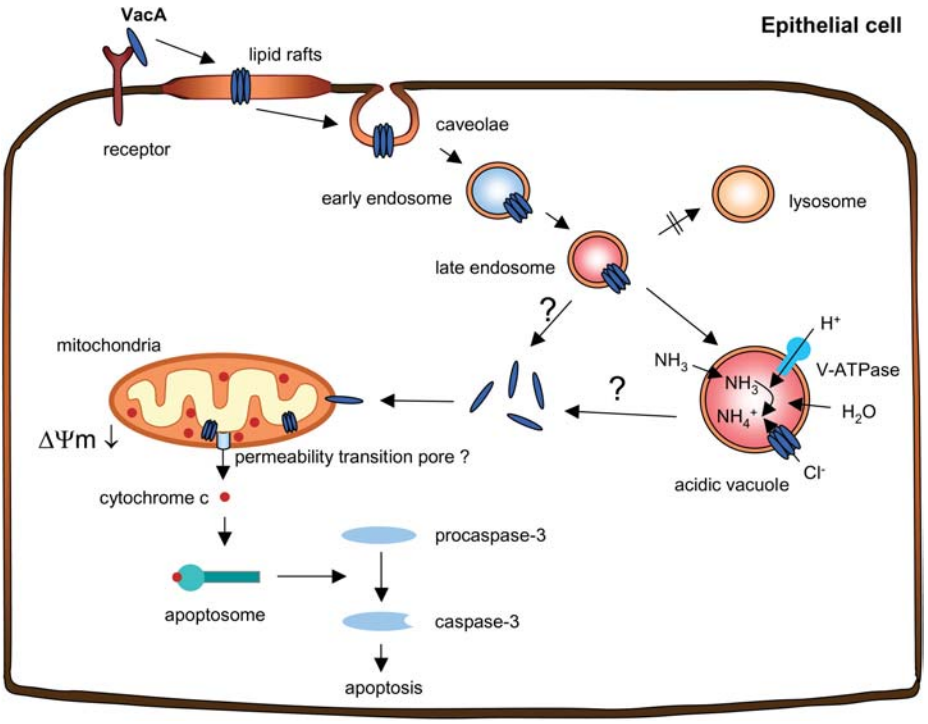

Fig. 1 VacA membrane interaction, intracellular vesicle trafficking, and induction of apoptosis. VacA interacts with a cellular receptor and associates with lipid rafts, where it inserts and oligomerizes to form anion-selective channels (chloride channels). The channels are endocytosed via caveolae and mature from early endosomes via late endosomes into acidic vacuoles. The activation of a vacuolar proton pump (V-ATPase) acidifies the endosomal compartment, which takes up weak bases (NH_3) and H_2O by diffusion. The VacA channel supports the entry of Cl^- ions, finally resulting in swelling and acidic vacuole formation. VacA then enters the cytosol and accumulates in mitochondria by a yet-unknown mechanism. Either direct insertion of VacA into the mitochondrial inner membrane or the activation of endogenous channels results in a reduction of the membrane potential ($\Delta\Psi m$) and cytochrome *c* release, which activates the caspase 3 pathway and finally leads to apoptosis

Gauthier et al. (2004) showed that GPI-APs are required for the formation of active VacA channels in the plasma membrane, but not for binding of the toxin to lipid rafts. This indicated that GPI-APs might be necessary for internalization of VacA, but not for binding to the eukaryotic cell. Kuo and Wang (2003) suggested a common endocytic pathway of GPI-APs and VacA, since they found that internalized VacA co-migrates along with the model lipid raft antigen GPI-AP fasciclin I, which has been constitutively produced in a Chinese hamster ovary (CHO) cell line. In contrast to these results, Schraw et al. (2002) demonstrated VacA cytotoxicity independent of GPI-APs. VacA was taken up into GPI-AP-positive and GPI-AP negative CHO cells to the same extent, but vacuolation was induced in both cell types (Schraw et al. 2002). After insertion into the plasma membrane, VacA channels are internalized. Endocytosis of the VacA channels does not occur via the clathrin-coated pit pathway but rather via an actin-dependent caveolae-like internalization process (Ricci et al. 2000) (Fig. 1). Disruption of the actin cytoskeleton by treatment with cytochalasin D did not inhibit binding of VacA to the lipid rafts, but blocked internalization of VacA and vacuole formation (Gauthier et al. 2004).

Trafficking and formation of acidic vacuoles

VacA-induced vacuoles are hybrid compartments of late endosomal origin with some lysosomal markers (Molinari et al. 1997). The formation of visible vacuoles depends on the presence of weak bases, such as NH_4Cl, that promote osmotic swelling. But even without the addition of weak bases, VacA induces a perinuclear redistribution and clustering of late endosomal compartments, which may be considered as a prerequisite for the development of vacuoles (Li et al. 2004). Vacuole formation depends on the presence and activity of a number of cellular molecules, such as the vacuolar (V-) ATPase (Papini et al. 1993), Rab7 (Papini et al. 1997), Rac1 (Hotchin et al. 2000), or dynamin (Suzuki et al. 2001). The SNARE protein syntaxin 7 also seems to be involved in trafficking or vacuolation, as confirmed by transfection with a dominant-negative syntaxin 7 construct (Suzuki et al. 2003). Another study, however, excluded the involvement of SNARE proteins and suggested that the enlargement of vacuoles might be achieved by fusion of internal vesicles of multivesicular body-like structures (de Bernard et al. 2002). Notably, an involvement of the protein kinase PIKfyve in vacuole formation was demonstrated as well. In this study over-expression of PIKfyve, or microinjection of its substrate, phosphatidylinositol-3,5-bisphosphate, was sufficient to induce vacuole formation (Ikonomov et al. 2002). Apart from these proteins necessary for vacuolation, VacA was shown by a yeast two-hybrid analysis to interact with the intermediate filament protein VIP54 (de Bernard et al. 2000) and with the scaffolding protein RACK1 (Hennig et al. 2001). In both cases, the significance of these interactions is not clear. The generation of late endosome–lysosome hybrid compartments in VacA-intoxicated cells is accompanied by an inhibition of vesicle trafficking, which leads to a defect in procathepsin D maturation, or EGF degradation (Satin et al. 1997).

In order to exert a vacuolating activity, the 33-kDa fragment is not sufficient; at least part of the 55-kDa domain must be present when VacA is produced intracellularly. The minimal intracellular active domain consists of p33 and the N-terminal 100 amino acids of the p55 (de Bernard et al. 1998; Ye et al. 1999). The vacuolating activity also depends on interactions between p33 and p55 (Ye and Blanke 2002; Willhite et al. 2002; Torres et al. 2004) and requires a hydrophobic N-terminal region (Ye and Blanke 2000; McClain et al. 2001a). This hydrophobic region probably contains a transmembrane dimerization sequence, which contains an essential proline residue and a GXXXG motif, which is typical for transmembrane dimerization sequences (Ye and Blanke 2000; McClain et al. 2001a, 2003). Interestingly, an inactivating mutation in the 55-kDa domain can functionally complement another inactivating mutation within this hydrophobic sequence (Ye and Blanke 2002). All mutations that inactivate VacA for vacuolation also lead to a defect in channel formation.

VacA and apoptosis

Several studies correlated *H. pylori* with an increased level of apoptosis in the human gastric mucosa (Moss et al. 1996; Mannick et al. 1996; Rudi et al. 1998). Several *H. pylori* antigens were described to be responsible for apoptosis induction, such as urease (Fan et al. 2000), proteins of the *cag* pathogenicity island (Peek et al. 1999), and lipopolysaccharide (Wagner et al. 1997). Recently, several groups presented conclusive evidence that the VacA toxin is able to induce apoptosis in epithelial cells in the absence of other *H. pylori* factors (Cover et al. 2003; Galmiche et al. 2000; Kuck et al. 2001). Cover et al. (2003)

clearly demonstrated that wild-type bacteria, or their culture supernatants, but not the isogenic *vacA* mutant strains or the corresponding culture supernatant, induced apoptosis. In the same way, purified toxin caused apoptosis in a dose-dependent manner in the presence of ammonium chloride. Furthermore, the induction of programmed cell death was shown to be dependent on the s1m1 form of the VacA protein, since a chimeric s2m1 VacA protein did not initiate apoptosis.

Mitochondria as target for VacA and apoptosis induction

It was reported that intracellularly expressed VacA and the N-terminal p37 VacA fragment, as well as extracellularly applied VacA, are selectively targeted to the mitochondria. VacA is found in the inner mitochondrial membrane, where it induces the release of cytochrome *c*, activating the caspase-3-dependent cell death signaling cascade (Galmiche et al. 2000; Willhite and Blanke 2004) (Fig. 1). Over-expression of the anti-apoptotic Bcl-2 protein blocked apoptosis induction (Galmiche et al. 2000), indicating that VacA-induced apoptosis occurs via a mitochondria-dependent pathway. Later, Willhite et al. (2003) examined the relation between vacuolation, channel forming activity, and induction of apoptosis in more detail. They found that vacuolation and induction of cytochrome *c* release are independent outcomes of VacA intoxication and that both depend on internalization of VacA and formation of anion-selective membrane channels. Interestingly, mutant forms of VacA known to be deficient in channel formation neither caused vacuolation nor cytochrome *c* release, although they localized to the mitochondria as the wild-type toxin does (Willhite and Blanke 2004). Furthermore, the authors investigated the mechanism of VacA-mediated cytochrome *c* release and demonstrated that VacA intoxication of cells leads to a breakdown of the mitochondrial transmembrane potential (Willhite and Blanke 2004), which may be associated with changes in the permeability of the mitochondrial membrane (Fig. 1). Experiments with the well-characterized mutant forms, VacA-P9A and VacA-G14A, which are unable to form membrane channels, and with the specific channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) clearly showed that VacA membrane channel formation is essential for reduction of the mitochondrial membrane potential ($\Delta\psi m$) and subsequent cytochrome *c* release. The authors speculate that VacA might induce changes in the mitochondrial membrane permeability by a mechanism that is independent of cellular caspase activity or the mitochondrial permeability transition.

The simplest model might be that VacA directly permeabilizes the mitochondrial inner membrane by inserting and forming pores. Such pores could result in a reduction in $\Delta\Psi m$, ultimately leading to further changes in the outer membrane and release of cytochrome *c* into the cytosol (Fig. 1). Alternatively, VacA interaction with the mitochondria could activate endogenous channels of the mitochondria, such as the adenine nucleotide translocase within the inner membrane, or the voltage-dependent anion channel (VDAC), an abundant protein located in the outer membrane of mitochondria, which is thought to facilitate cytochrome *c* release (Tsujimoto and Shimizu 2002). Interestingly, the ability of VacA to induce apoptosis seems to be cell type-dependent, since it was found that VacA induces apoptosis in parietal cells (Neu et al. 2002), which might facilitate *H. pylori* colonization of the gastric mucosa, but no apoptosis could be detected in VacA-treated activated T cells (Gebert et al. 2003; Boncristiano et al. 2003).

Novel activities of VacA toward cells of the immune system

The effect of VacA has been studied for years exclusively on epithelial cells. This might be explained by the fact that the epithelial cell layer constitutes the first barrier for VacA in the gastric mucosa. Originally, the vacuolating cytotoxin was described as a secreted molecule (Leunk et al. 1988; Cover and Blaser 1992). The contact-dependent transfer of VacA described by Ilver et al. (2004) might be relevant for delivery of VacA into gastric epithelial cells, but secreted VacA is able to penetrate into deeper tissues, getting possibly into contact with many other cell types, especially cells of the immune system, such as granulocytes, macrophages, dendritic cells, B cells, and T cells. The activity of VacA on these cell types is discussed below.

Opening of tight junctions and cooperation of CagA and VacA

The intact epithelial barrier is usually very tight and the deeper tissue is not accessible for molecules of the gastric surface. It has been observed, however, that colonization of the gastric surface by *H. pylori* results in a partial opening of the tight junctions, which are responsible for the controlled sealing separating the apical side of the gastric surface from the sub-mucosa (Terres et al. 1998; Suzuki et al. 2002). Originally, the transepithelial electrical resistance (TER) of polarized epithelial cell monolayers was found to be lowered by VacA, which was believed to allow the epithelial permeability of Fe^{3+} and Ni^{2+} ions, essential for *H. pylori* survival in vivo (Papini et al. 1998). This effect does not require acid activation and is also observed with m2-type VacA (Pelicic et al. 1999). VacA might also act as a specific urea transporter, as demonstrated by the generation of a transepithelial flux of urea across model epithelia from various polarized and non-polarized epithelial cell lines (Tombola et al. 2001). A more profound effect on the integrity of tight junctions may be caused by cytotoxin-associated antigen (CagA). As demonstrated under in vitro (Odenbreit et al. 2000) and in vivo (Azuma et al. 2003) conditions, CagA might be directly injected into polarized epithelial cells via the type IV secretion system of *H. pylori*. Notably, CagA was reported to disrupt the epithelial barrier function by directly targeting the epithelial tight-junction scaffolding protein ZO-1 and the transmembrane protein junctional adhesion molecule (JAM) (Amieva et al. 2003). It can be assumed that a long-term CagA delivery to polarized epithelia causes a severe disruption of the epithelial barrier function. Thus, it seems that the two major virulence factors of *H. pylori,* VacA and CagA, have a possible synergistic effect within the gastric mucosa, a finding assumed already for a long time because of a frequent association of certain *vacA* and *cagA* genotypes. CagA opens the tight junctions allowing VacA to enter the gastric sub-mucosa and to come into contact with a number of non-epithelial cell types, such as polymorphonuclear leukocytes (PMNs), macrophages, dendritic cells, B cells, and T cells.

Interaction of *H. pylori* with polymorphonuclear granulocytes, monocytes, and macrophages and role of VacA

H. pylori infection induces a specific immune response, as demonstrated by high titers of *H. pylori* antibodies (Blanchard et al. 1999) and the recruitment of PMNs, mononuclear phagocytes, and lymphocytes to the gastric mucosa of infected individuals (Blaser and

Parsonnet 1994; Telford et al. 1997). Despite the presence of such a vigorous immune response, *H. pylori* eradication is not observed, unless specific antibiotic therapy is administered. This finding suggests, that *H. pylori* possesses properties allowing evasion of the host immune response.

Against extracellular bacterial pathogens, activated granulocytes and macrophages play a central role in the inflammatory response. Despite a predominantly extracellular lifestyle in vivo, *H. pylori* has also been described as occasionally entering epithelial cells, especially under in vitro conditions (Kwok et al. 2002), or to be taken up by professional phagocytes (Odenbreit et al. 2001). In epithelial cells, *H. pylori* seems to enter large cytoplasmic vacuoles, where the bacteria are reported to remain viable and motile (Amieva et al. 2002). It has been speculated that such intracellular vacuoles may constitute a reservoir of live *H. pylori*, difficult to attack by antibiotics or phagocytes. Whether or not VacA actually supports survival of *H. pylori* in such vacuoles is still a point of controversy (Petersen et al. 2001; Amieva et al. 2002). Whereas Ramarao et al. (2000) provided evidence that phagocytosis of *H. pylori* is inhibited by the *cag*-PAI type IV secretion system of *H. pylori*, another study found no difference in phagocytosis and intracellular survival between type I strains and isogenic mutants lacking the type IV secretion system (Odenbreit et al. 2001). Delayed phagocytosis, homotypic phagosome fusion, and prolonged survival in macrophages was reported for type I, but not type II, *H. pylori* strains (Allen et al. 2000). A further study found an enhanced survival of *H. pylori* in professional phagocytes, such as the macrophage cell lines THP-1 (human) and RAW 264.7 (murine), which was dependent on the production of VacA (Zheng and Jones 2003). They reported that VacA-producing strains of *H. pylori* reside in a compartment with early endosome properties and avoid fusion with lysosomes (Zheng and Jones 2003). The vacuolating cytotoxin seems to arrest phagosome maturation by recruiting and retaining tryptophane aspartate-containing coat protein (TACO), a mechanism also exploited by *Mycobacterium bovis* to prevent phagosome trafficking and maturation (Ferrari et al. 1999). In another study the presence of *H. pylori*-induced homotypic fusions were confirmed in isolated human monocytes, but neither VacA nor the *cag*-PAI were involved in prolonged intracellular survival (Rittig et al. 2003). Thus, the role of bacterial invasion into epithelial cells and of uptake by phagocytes as a bacterial evasion mechanism has to be clarified more rigorously in future.

Interaction of *H. pylori* with B cells and interference of VacA with antigen presentation

As discussed above, the cytotoxin might enter the gastric sub-mucosa by using the intercellular route. At this time it cannot be excluded, however, that VacA also uses a pathway of transcytosis to enter the sub-epithelial layer. In the sub-mucosa, VacA will come into contact with a number of different cell types. Molinari et al. (1998b) presented the first detailed evidence of how VacA might be able to interfere with the immune response. They demonstrated that VacA can inhibit processing of antigenic peptides in B cells and their presentation to human $CD4^+$ T cells. VacA-treated B cells showed a selective inhibition of antigen processing and surface presentation by the invariant chain (Ii)-dependent pathway. This pathway is associated with peptide loading and T cell presentation of newly synthesized major histocompatibility complex (MHC) class II antigen. The antigen presentation, which is dependent on recycling MHC class II was not affected by VacA.

Immunosuppressive activities of VacA

The reason for the successful chronic persistence of *H. pylori* in its special niche is not well understood. As postulated earlier, *H. pylori* may evade host responses through the inhibition of antigen-specific T cell proliferation (Knipp et al. 1994; Fan et al. 1994). Induction of apoptosis in gastric T cells expressing Fas ligand (FasL) by *H. pylori* was also suggested as a mechanism to explain immune evasion of the bacterial pathogen (Wang et al. 2001). Vaccination data from the *H. pylori* mouse model indicated that $CD4^+$ T cells are crucial for control of the *H. pylori* infection by the host (Ermak et al. 1998; Aebischer et al. 2000). A direct correlation was reported between the level of protection against *H. pylori* infection and the density of T cells recruited to the gastric mucosa. In contrast to MHC class I and B cell knockout mice, MHC class II knockout mice were not protected by vaccination, indicating that control of the *H. pylori* infection is dependent on MHC class II-restricted, cell-mediated mechanisms. Furthermore, the adoptive transfer of UreA-specific $CD4^+$ T cells from vaccinated BALB/c mice into naïve syngeneic recipients also demonstrated the importance of $CD4^+$ T cells in controlling the *H. pylori* infection (Lucas et al. 2001). Whether or not VacA has an immunosuppressive activity in vivo in infected individuals is not known. Clinical data by Fan et al. (1994) show significantly lower peripheral blood lymphocyte proliferative responses to *H. pylori* in *H. pylori*-positive as compared to *H. pylori*-negative patients. Furthermore, the observation that *H. pylori* infection of mice infected with vaccinia virus reduces the vaccinia-specific cytotoxic T cell response and prolongs the viral infection would support such an activity (Shirai et al. 1998). Taken together, these data suggest that T cells might be a putative target of manipulation by *H. pylori* during infection.

Until recently, neither a bacterial effector nor any target for immunosuppression by *H. pylori* were known. Now, T cells have been identified as a major target for *H. pylori* VacA (Gebert et al. 2003; Boncristiano et al. 2003). Two factors of *H. pylori*—an hitherto unknown membrane-associated factor and VacA—were shown to efficiently inhibit the proliferation of T cells activated by polyclonal stimulators, such as phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) and Ca^{2+} ionophores (ionomycin, A23187). A productive T cell receptor ligation, together with a costimulatory signal via the CD28 surface receptor triggers an effective interleukin-2 (IL-2) secretion. In parallel, the high-affinity IL-2 receptor α (IL-2Rα) is up-regulated on T cells. VacA was found to efficiently block both IL-2 secretion and IL-2Rα surface location and thus inhibits the existing autocrine loop (Gebert et al. 2003) (Fig. 2). Notably, a low ratio of one to five bacteria per T cell resulted already in a clear effect on IL-2 secretion of T cells (Gebert et al. 2003). Such low amounts of VacA, that may be present during gastric infection in vivo, support the idea that a high-affinity receptor for VacA is present on T cells (Boncristiano et al. 2003). The production of cyclins D3 and E were efficiently down-regulated in purified human peripheral blood lymphocytes (PBLCs) treated with VacA, resulting in a reduced phosphorylation and activation of the retinoblastoma protein (Rb), a key regulator of the cell cycle G1/S phase progression (Gebert et al. 2003). This implies that VacA might have a direct effect on the proliferative response of T cells by modulating the cell cycle.

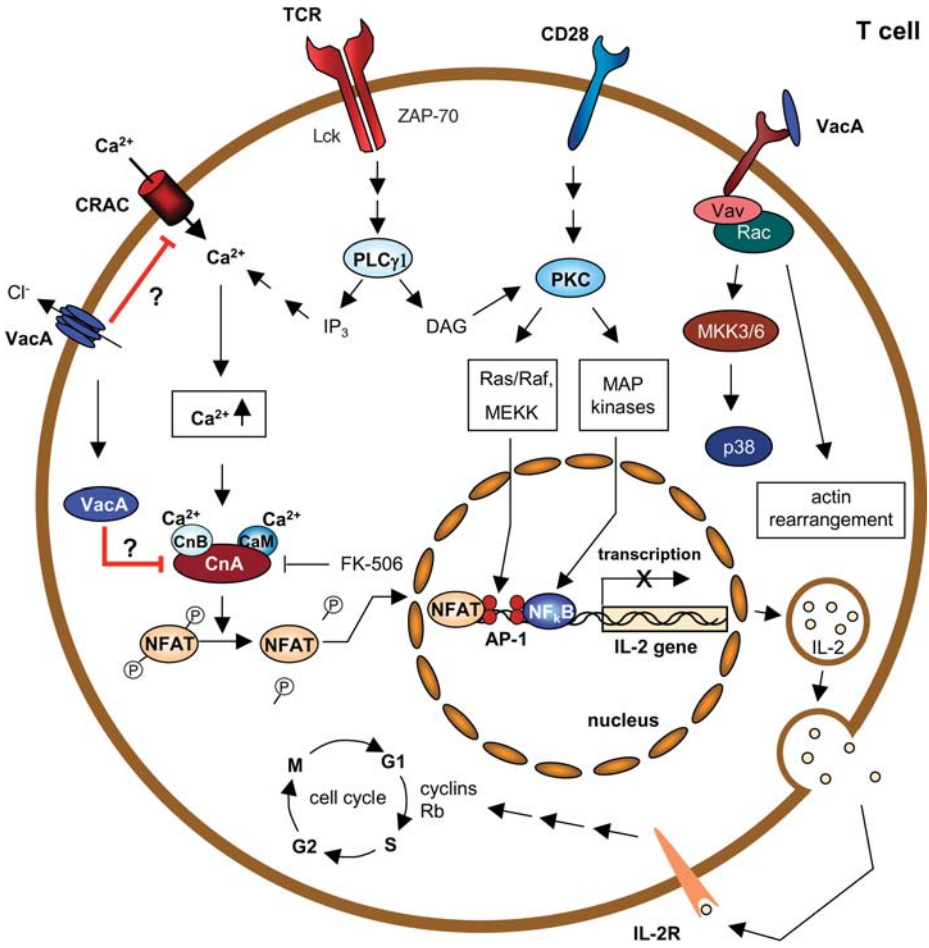

Fig. 2 Model of the interference of VacA with T cell activation. VacA inhibits T cell activation and proliferation by interfering with the T cell receptor (*TCR*) signaling pathway. Upon stimulation of T cells via TCR and CD28, two major pathways are activated [Ca^{2+}-dependent pathway and mitogen-activated protein (*MAP*) kinase pathway]. The anion-selective channel activity of VacA is supposed to depolarize the plasma membrane and to prevent the opening of the Ca^{2+} release-activated Ca^{2+} (*CRAC*) calcium channel (Boncristiano et al. 2003), which is operated by calcium released from intracellular stores. Alternatively, VacA might also block calcineurin activation directly. Inhibition of calcineurin, a prominent target for the immunosuppressive drugs cyclosporin A and FK506, prevents dephosphorylation and translocation of NFAT into the nucleus. As a result, transcription of IL-2 and IL-2Rα genes cannot be initiated (Gebert et al. 2003). The missing IL-2 signaling may arrest the cell cycle via cyclin expression and Rb protein phosphorylation. At low doses, VacA inhibits T cell activation by inducing a cascade of phosphorylation events involving a still-unidentified receptor, Vav, and MKK3/6, resulting in an increase of the active form of p38. Vav induces actin rearrangement through the small GTPase Rac, which leads to inhibition of T cell proliferation (Boncristiano et al. 2003). *AP-1* activator protein 1, *CaM* calmodulin, *CD28* costimulatory molecule, *CnA* calcineurin A subunit, *CnB* calcineurin B subunit, *CRAC* Ca^{2+} release-activated Ca^{2+} channel, *DAG* diacylglycerol, *IL-2* interleukin-2, *IL-2R* interleukin-2-receptor, *IP3* inositol-1,4,5-trisphosphate, *MEKK* MAP/ERK (extracellular regulated kinase) kinase kinase, *MKK* MAP kinase kinase, *NFAT* nuclear factor of activated T cells, *PKC* protein kinase C, *PLCγ1* phospholipase C γ1, *Rb* retinoblastoma protein, *TCR* T cell receptor

Mechanism of T cell inhibition resembles the activity of cyclosporin A and FK506

IL-2 is a major cytokine essential for the proliferation of T cells on antigen stimulation. Transcription of the IL-2 gene is under control of several transcription factors, such as nuclear factor (NF)-κB, activator protein (AP)-1, and nuclear factor of activated T cells (NFAT). In resting T cells, NFAT is phosphorylated at serine–threonine motifs and located in the cytosol. On T cell activation, a massive cytoplasmic Ca^{2+} influx is triggered, first from internal stores and subsequently from external milieu by opening of the Ca^{2+} release-activated Ca^{2+} (CRAC) channels. VacA is able to interfere with the Ca^{2+} signaling pathway, which results in blocking of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (PP2B) (Fig. 2). Ca^{2+}-activated calcineurin dephosphorylates NFAT, which subsequently migrates into the nucleus to initiate transcription of a number of genes, including the IL-2 gene. VacA abolishes the translocation of NFAT into the nucleus by specifically blocking the activation of calcineurin, but leaves NF-κB and AP-1 unaffected (Gebert et al. 2003; Boncristiano et al. 2003). This results in a failure of the activated T cell to produce IL-2 and IL2Rα, but also a number of chemokines—such as single C motif-1β (SCM-1β), SCM-1α (lymphotactin, ATAC), macrophage inflammatory protein (MIP)-1α, and MIP-1β—that might be essential for a concerted immune response (Gebert et al. 2003). Thus, VacA displays a similar effect on T cells as the well-known immunosuppressive drugs cyclosporin A and FK506, which block activation of calcineurin. One explanation of calcineurin inhibition might be due to the VacA anion channel activity (Szabo et al. 1999). In the presence of NPPB, VacA had no effect on the A23187-induced cytosolic Ca^{2+} increase, which suggests that the opening of anion channels might influence the CRAC channel activity and inhibit Ca^{2+} influx (Boncristiano et al. 2003). Other possibilities, for example a direct interaction of VacA with calcineurin, cannot be excluded yet, and further investigations are necessary to clarify the underlying mechanisms.

Immune suppression and proinflammatory activities of VacA

In addition to the immune-suppressive activity of the complete VacA protein, which implies the uptake of VacA into cells, Boncristiano et al. (2003) reported on a second activity of VacA mediated from outside by binding of the COOH-terminal p58 domain of VacA to an unknown high-affinity receptor. This binding was supposed to cause activation of Rac and phosphorylation of stress kinases, such as p38, but not Erk1/2, resulting in actin rearrangement, inhibition of T cell proliferation, and an anergic state of T cells (Boncristiano et al. 2003; Montecucco and de Bernard 2003) (Fig. 2). In the gastric cell line AZ-521, VacA treatment induced p38 as well as Erk1/2 phosphorylation and induction of the p38/ATF-2-mediated pathway (Nakayama et al. 2004). In addition to immune suppression, VacA is also postulated to have proinflammatory effects, e.g., by stimulating expression of the proinflammatory enzyme cyclo-oxygenase (COX)-2, not only in T cells, but also in granulocytes and macrophages (Boncristiano et al. 2003). A further study published by Jüttner et al. (2003) showed transcriptional up-regulation of the COX-2 gene in AGS epithelial cells, but the effect was independent of the *cag*-PAI and of VacA (Jüttner et al. 2003). VacA-independent stimulation of COX-2 was also reported for gastric epithelial cells by Busiello et al. (2004). These authors purified the stimulating activity from bacterial culture supernatant and identified it as the secreted protein gamma-glutamyltranspeptidase. Furthermore, VacA was reported to activate mast cells to produce proinflammatory cytokines tumor necrosis factor (TNF)-α and IL-6 (Supajatura et al. 2002).

Conclusions

Over the last 16 years, significant progress has been made in the understanding of the structure and function of VacA. Besides its vacuolating activity, it was surprising to find completely novel effects of VacA, especially on cells of the immune system. The interference of VacA with antigen presentation and the blocking of T cell activation might be a special form of immunosuppression by *H. pylori,* which might help the bacteria establish a chronic persistent infection. The targeting of VacA to mitochondria and the induction of apoptosis in certain cell types has been well documented meanwhile, but the mechanism of action of VacA has to be further established. In future it will be important to analyze the novel activities observed in vitro for their role in vivo during *H. pylori* chronic infection—in animal models or patients—to find out which of the activities are finally relevant.

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