Review

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Rho/Ras-GTPase-dependent and -independent activity of clostridial glucosylating toxins

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Clostridial glucosylating toxins are the main virulence factors of clostridia responsible for gangrene and/or colitis. These toxins have been well characterized to inactivate Rho/Ras-GTPases through glucosylation. However, the signalling pathways downstream of Rho/Ras-GTPases leading to the intracellular effects of these toxins are only partially known. Rac-dependent modification of focal adhesion complexes and phosphoinositide metabolism seem to be key processes involved in actin filament depolymerization and disorganization of intercellular junctions. In addition, clostridial glucosylating toxins induce Rho/Ras-independent intracellular effects such as activation of mitogen-activated protein kinase pathways, which are used by some of these toxins to trigger an inflammatory response.

Introduction

Clostridial glucosylating toxins are 250–300 kDa proteins encompassing *Clostridium difficile* toxins A and B (TcdA and TcdB), *Clostridium sordellii* lethal toxin (TcsL) and haemorrhagic toxin (TcsH), as well as *Clostridium novyi* alpha-toxin (TcnA). TcdB and TcsL are highly related (76 % amino acid sequence identity) and are more distantly related to TcdA and TcnA (48–60 % identity) (Jank & Aktories, 2008).

C. difficile is the aetiological agent of pseudomembranous colitis and of about 30 % of cases of postantibiotic diarrhoea, which are the most frequent nosocomial intestinal diseases (Kelly & LaMont, 2008). TcdA, which experimentally induces necrotic and haemorrhagic intestinal lesions, has been considered as the main virulence factor (Genth *et al.*, 2008; Voth & Ballard, 2005). Since *C. difficile* strains producing both TcdA and TcdB or only TcdB cause enteric disease in humans, TcdB might also be an important enterotoxin (Genth *et al.*, 2008; Voth & Ballard, 2005). Indeed, using genetically modified *C. difficile* strains and the hamster disease model, TcdB was found to be the essential virulence factor by Lyras *et al.* (2009). However, a recent study with isogenic *tdcA* or *tdcB* mutants of *C. difficile* showed that both toxins, TcdA and TcdB, are critical to induce the pathological effects of *C. difficile* (Kuehne *et al.*, 2010). Both TcdB and TcdA participate in the alteration of the intestinal barrier and in the recruitment of inflammatory cells, which are abundant in the lesions. *C. sordellii* is also an agent of haemorrhagic enteritis and enterotoxaemia in cattle (Al-Mashat & Taylor, 1983a, b; Clark, 2003; Lewis & Naylor, 1996, 1998; Popoff, 1984; Richards & Hunt, 1982; Songer, 2005). In addition, both *C. sordellii* and *C. novyi* can invade deeper tissues and are involved in gangrene. Sporadic cases of *C. sordellii*

infection are commonly encountered in women after post-partum wounding, endometritis or postabortion disease. Although rare, these cases are all dramatic. A fulminant toxic shock syndrome (irreversible hypotension, apyrexia, haemoconcentration with hyperproteinaemia, and leukocytosis) accompanies the local infection of the uterus or perineum. Pleural effusions and serosanguinous ascites have been reported in almost all cases (Bitti *et al.*, 1997; Centers for Disease Control and Prevention, 2005; Ho *et al.*, 2009; McGregor *et al.*, 1989; Rørbye *et al.*, 2000; Sinave *et al.*, 2002; Soper, 1986). In recent years, several cases of fatal toxic shock syndrome due to *C. sordellii* have been reported following medical abortion (Cohen *et al.*, 2007; Couzin, 2006; Fischer *et al.*, 2005; Greene, 2005; Miech, 2005; Murray & Woollorton, 2005; Soper, 2007; Winikoff, 2006). Moreover, *C. sordellii* is responsible for myonecrosis associated with a toxic shock syndrome and a high rate of mortality (about 50 %) in injection drug users (Aldape *et al.*, 2006; Kimura *et al.*, 2004).

Clostridial glucosylating toxins are single-chain proteins containing at least four functional domains. The C-terminal third exhibits multiple repeated sequences (31 short repeats and 7 long repeats in TcdA), which are involved in the recognition of a cell-surface receptor. A trisaccharide (Gal- α 1-3Gal- β 1-4GlcNAc) has been found to be the motif recognized by TcdA. The central part contains a hydrophobic segment and probably mediates the translocation of the toxin across the membrane. The enzymic site, which is characterized by the Dx₂D motif surrounded by a hydrophobic region, and the substrate-recognition domain are localized within the 543 N-terminal residues corresponding to the natural cleavage site in TcdB (Hofmann *et al.*, 1997, 1998; Rupnik *et al.*, 2005). Clostridial glucosylating toxins enter cells by

receptor-mediated endocytosis and translocate from early endosomes upon an acidification step. The N-terminal domain is delivered into the cytosol by an autoproteolytic process stimulated by inositol hexakisphosphate. A cysteine protease domain has been identified close to the cutting site in TcdB (amino acids 544–955), which is conserved in all clostridial glucosylating toxins (Egerer *et al.*, 2007, 2009; Reineke *et al.*, 2007).

Clostridial glucosylating toxins catalyse the glucosylation of Rho- and/or Ras-GTPases from UDP-glucose, except TcnA, which uses UDP-*N*-acetylglucosamine as cosubstrate. TcdA and TcdB glucosylate Rho at Thr-37, Rac and Cdc42 at Thr-35, whereas TcsL glucosylates Ras and Rap at Thr-35 as well as Ral at Thr-46, which is equivalent to Thr-35 in the other GTPases (Table 1). The large glucosylating clostridial toxins cleave the cosubstrate and transfer the glucose moiety to the acceptor amino acid of the Rho proteins (Just *et al.*, 1995a, b; Popoff *et al.*, 1996). The conserved Thr, which is glucosylated, is located in switch I. Glucosylation of Thr35 completely prevents the recognition of the downstream effector, blocking the G-protein in the inactive form (Herrmann *et al.*, 1998). The crystal structure of Ras modified by TcsL shows that glucosylation prevents the formation of the GTP conformation of the effector loop of Ras, which is required for the interaction with the effector Raf (Vetter *et al.*, 2000). Similar results were found with RhoA glucosylated by TcdB (Sehr *et al.*, 1998). In addition, glucosylation of GTPase slightly reduces the intrinsic GTPase activity, completely inhibits GAP-stimulated GTP hydrolysis (Herrmann *et al.*, 1998), and leads to accumulation of the GTP-bound form of Rho to

the membrane, where it is tightly bound (Genth *et al.*, 1999).

Clostridial glucosylating toxins alter the actin cytoskeleton and intercellular junctions

By inactivating Rho proteins, clostridial glucosylating toxins induce cell rounding, which is accompanied by a loss of actin stress fibres, reorganization of the cortical actin, disruption of the intercellular junctions and subsequently increase in cell barrier permeability. These effects are also reported as cytopathic effects, in contrast to the cytotoxic effects, which result in toxin-induced cell death (Just & Gerhard, 2004; Rupnik & Just, 2006). Differences in actin cytoskeleton modification are observed between the clostridial glucosylating toxins. TcdB induces loss of actin stress fibres and reorganization of focal adhesions accompanied by cytoplasmic retraction and cell rounding, whereas long protrusions radiating around the cell yield a particular 'actinomorph' morphology (Ottlinger & Lin, 1988). TcdA and TcnA cause similar cell alterations, whereas TcsL produces cell rounding with loss of actin stress fibres but without branched membrane protrusions. These different effects might result from the different Rho proteins targeted by the toxins: TcdA, TcdB and TcnA inactivate the three main Rho-GTPases (Rho, Rac and Cdc42) whereas TcsL only modifies Rac (Table 1). However, Rac1 rather than RhoA or Cdc42 has been shown to be the critical target for the cytopathic effects induced by TcdB (Halabi-Cabezón *et al.*, 2008). Although Rac inactivation seems to be the main mechanism responsible for the cytopathic effects, the additional Rho-GTPases modified by TcdA, TcdB and TcnA are probably involved in different actin cytoskeleton alterations from those induced by TcsL.

TcdA and TcdB alter the barrier function of polarized intestinal cells such as Caco-2 and T84 cells by increasing paracellular permeability (Hecht *et al.*, 1988, 1992; Johal *et al.*, 2004). Concomitant with the permeability increase, TcdA and TcdB disrupt apical and basal actin filaments with a subsequent disorganization of the ultrastructure and component distribution (i.e. ZO-1, ZO-2, occludin, claudin) within tight junctions (TJs). TcdB decreases actin-ZO-1 association and disperses ZO-1 and occludin from lipid-rich membrane microdomains, without changing the occludin phosphorylation status (Chen *et al.*, 2002; Nusrat *et al.*, 2001). Since Rho plays an important role in TJ assembly, the effects of TcdB and TcdA on TJs presumably result from Rho glucosylation (Jou *et al.*, 1998; Popoff & Geny, 2009). In contrast, TcsL-82, which inactivates Rac, and TcsL9048, which inactivates both Rac and Cdc42 (Table 1), induce a specific depolymerization of basolateral actin filaments, an increased cell barrier permeability, and a drastic perturbation of adherens junctions (AJs), whereas apical actin and TJs are preserved. Thereby, TcsLs preferentially depolymerize actin filaments in the basolateral cell compartment and alter AJs. TcsLs

Table 1. Specific substrates of *C. sordellii* and *C. difficile* glucosylating toxins

Substrates according to Boehm *et al.* (2006), Dreger *et al.* (2009), Genth & Just (2010), Just *et al.* (1996), Popoff *et al.* (1996) and unpublished data: +, Preferred substrate; +/-, minor substrate.

Substrate	TcsL-82	TcsL-9048	TcdB	TcdA
RhoA	–	–	+	+
RhoB	–	–	+	+
RhoC	–	–	+	+
RhoD	–	–	–	–
RhoG	+	+	+	+
RhoE	–	–	–	–
Cdc42	–	+	+	+
Rac1	+	+	+	+
H-Ras	+	+	–	–
Rap	+	+	–	–
Ral	+	+/-	–	–
TC10	+	+	+	+
TCL	+	+	+	+
R-Ras1	+/-	+/-	–	+/-
R-Ras2 (TC21)	+/-	+/-	–	+/-
R-Ras3 (M-Ras)	+	+/-	–	+/-

cause a dramatic redistribution of E-cadherin and α - and β -catenins from the plasma membrane to the cytoplasm, with a reduced and discontinuous distribution of these molecules at the junctional ring and a diffuse localization into the cytosol. Simultaneously, E-cadherin is reduced at the cell surface and is increased in the detergent-soluble fraction. Thereby, TcsLs remove the whole E-cadherin–catenin complex from the membrane and increase paracellular permeability of cell monolayers (Boehm *et al.*, 2006; Richard *et al.*, 1999).

TcsL is a highly potent lethal toxin but its mechanism of lethality is still elusive. Intraperitoneal injection of TcsL in mice induces an increased vascular permeability, mainly at the level of lung vessels, leading to a massive extravasation of blood fluid in the thoracic cavity, dehydration, increase in haematocrit, hypoxia, and finally cardio-respiratory distress. Alterations of lung endothelial cells are apparent by electron microscopy, showing modifications of AJs, which appear fainter and more discontinuous than in controls. VE-cadherin immunostaining is markedly altered, with an interrupted and locally diffuse staining reflecting a redistribution of VE-cadherin from the junctional ring to the cytoplasm (Geny *et al.*, 2007). Thereby, TcsL, which inactivates Rac among the Rho-GTPases, seems to preferentially target endothelial cells in the cardio-respiratory area, to alter the distribution of VE-cadherin, and subsequently to increase the endothelial barrier permeability, leading to local oedema and haemodynamic perturbations probably responsible for lethality.

Which cell signalling pathways downstream of Rac controlling actin polymerization and intercellular junctions are altered by clostridial glucosylating toxins?

Several Rac effectors controlling the actin cytoskeleton and intercellular junctions have been identified (Fig. 1) (Burridge & Wennerberg, 2004; Van Aelst & D'Souza-Schorey, 1997). Multiple signalling pathways downstream of Rac have been characterized. The main role of Rac is to coordinate the formation of lamellipodia, but it is also involved in the control of other actin structures. Rac activates actin polymerization by stimulating PI4P5-kinase (PI4P5K) and through the WAVE/IRSp53 and PAK (P-21-activated kinase) pathways. In addition, Rac and Cdc42 directly act on the activity and assembly of the E-cadherin–catenin complex, forming AJs through a common effector IQGAP (IQ motif containing GTPase-activating protein) (Fukata & Kaibuchi, 2001). IQGAP1 has been shown to interact with β -catenin and the cytoplasmic domain of E-cadherin. A model has been proposed in which Rac and Cdc42 regulate E-cadherin junctions through IQGAP1. Active Rac and Cdc42 in the GTP-bound form interact with IQGAP1 and thus prevent the association of IQGAP1 with β -catenin, which results in the stabilization of E-cadherin–catenin complex. In the GDP-bound form, non-active Rac and Cdc42 do not interact with IQGAP1,

which associates with β -catenin, thereby displacing α -catenin from its binding to β -catenin. This leads to a dissociation of α -catenin linked to actin filaments from the E-cadherin–catenin complex, conferring a weak adhesive activity (Bashour *et al.*, 1997; Fukata & Kaibuchi, 2001; Fukata *et al.*, 1999; Kaibuchi *et al.*, 1999a, b). Further work with knockdown Rac and/or IQGAP1 supports the idea that Rac positively regulates cell–cell adhesion through IQGAP1 by promoting the accumulation of actin filaments, E-cadherin and β -catenin at MDCK cell–cell contact sites (Noritake *et al.*, 2004).

The various pathways regulated by Rac have been investigated in response to TcsL treatment. In polarized epithelial cells, TcsL induces a redistribution of the E-cadherin–catenin complexes from the membrane (detergent-insoluble fractions) to the cytoplasm (soluble fractions). In contrast to 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which induces cell–cell dissociation via inhibition of Rac, resulting in increased association of IQGAP1 with β -catenin and dissociation of α -catenin from E-cadherin (Fukata & Kaibuchi, 2001), TcsL does not change the ratio of catenin–E-cadherin–IQGAP1 complexes, indicating that this toxin does not modify AJ integrity through the Rac/IQGAP pathway (Boehm *et al.*, 2006). Therefore, TcsL-mediated dissociation of E-cadherin from AJs does not seem to involve the Rac–IQGAP1 pathway directly, but rather Rac-dependent cortical actin depolymerization. Indeed, disruption of actin filaments induced by iota toxin or cytochalasin D, which inhibit actin polymerization by directly interacting with actin molecules, also leads to similar AJ alterations including removal of the E-cadherin–catenin complex from the cell surface (Boehm *et al.*, 2006). But how does TcsL-dependent Rac inactivation disorganize cortical actin filaments and E-cadherin junctions? A Rac pathway involves modifications of PAK–LIM-kinase and cofilin phosphorylation, thereby inhibiting actin depolymerization (Arber *et al.*, 1998; Chen & Macara, 2006; Yang *et al.*, 1998). Since Tcs-82 does not change the expression or phosphorylation of cofilin, this pathway is probably not involved in the toxin process (Geny *et al.*, 2010). However, we have found that TcsL-82 induces a rapid dephosphorylation of paxillin and thus decreases the number of focal adhesions (FAs). This effect is Rac dependent, since paxillin dephosphorylation is prevented in TcsL-82-treated cells overexpressing constitutively active Rac1 (Rac^{G12V}), and cells overexpressing activated Rac1 exhibit a higher number of FAs, a level which is not modified upon intoxication. Paxillin dephosphorylation does not appear to result from inactivation of the Rac major tyrosine kinase, Src or FAK (FA kinase), but rather from an earlier event. Rac inactivation might activate some phosphatase and thereby dephosphorylate proteins from FA complexes such as paxillin (Geny *et al.*, 2010). Several studies have reported the involvement of Rac1 in the control of AJ regulation, probably through reorganization of actin cytoskeleton (Braga *et al.*, 1997, 1999; Fukata & Kaibuchi,

Fig. 1. Main signalling pathways downstream of Rac that are involved in the control of the actin cytoskeleton and intercellular junctions. AJ, adherens junction; LIMK, LIM-kinase; MLCK, myosin light chain kinase; MLC-P, myosin light chain phosphate; myosin HC, myosin heavy chain; PAK, P-21 activated kinase; PI4P5K, phosphatidylinositol 4-phosphate 5-kinase; PIP2, phosphatidylinositol 4, 5-bisphosphate.

2001; Popoff & Geny, 2009; Takaishi *et al.*, 1997). However, the molecular mechanism(s) leading to the loss of cell adherence and cell-cell interaction upon Rac1 inactivation is (are) not yet fully deciphered. We observed an interaction between FA and AJ complexes as paxillin, talin and β -catenin are co-immunoprecipitated in resting cells. Upon cell intoxication with TcsL-82, and thus Rac1 inactivation, interactions between β -catenin, paxillin and talin are markedly decreased, indicating that FA and AJ complexes and their interactions are loosened. Moreover, protein interactions inside FA complexes also appear to be loosened, as the level of talin, which co-immunoprecipitates with paxillin, is decreased noticeably after cell intoxication (Geny *et al.*, 2010). These findings are in agreement with the critical role of Rac1 in FA complex assembly (Guo *et al.*, 2006) and the recent work of the group of Birukov, which demonstrates such an interaction by showing that FA and AJ proteins can be co-immunoprecipitated and that interactions between FA and AJ complexes via paxillin and β -catenin association involve Rac1 as a regulator (Birukova *et al.*, 2007, 2008).

In addition, we have shown that TcsL-82 induces a rapid decrease (within 1–2 h) in the cellular content of PI4P (phosphatidylinositol 4-phosphate), PI4,5P₂ (phosphatidylinositol 4,5-bisphosphate) and PI3,4,5P₃ (phosphatidylinositol 3,4,5-triphosphate). Polyphosphoinositides regulate numerous cellular processes, including FA formation and modulation of the actin cytoskeleton, two processes modified by TcsL-82 intoxication. Indeed, polyphosphoinositides play a role in membrane anchorage of several proteins or protein complexes, in particular proteins containing a PH (pleckstrin homology) or a PX (phox

homology) domain (Ferguson *et al.*, 1995; Lemmon *et al.*, 2002; Simonsen & Stenmark, 2001) as well as complex organization in lipid raft microdomains. Lipid rafts are enriched not only in cholesterol and sphingolipids but also in polyphosphoinositides such as PI4,5P₂ and PI3,4,5P₃ (Bodin *et al.*, 2001; Caroni, 2001; Doughman *et al.*, 2003; Golub & Caroni, 2005; Logan & Mandato, 2006; van Rheenen *et al.*, 2005). FA and AJ molecules also belong to highly organized membrane microdomains (Gaus *et al.*, 2006), and lipid raft content in AJ and FA proteins is modified upon TcsL-82 intoxication. Indeed, the level of talin, a protein that belongs to the protein complex forming FA, decreases in detergent-insoluble membranes of TcsL-82-intoxicated cells compared to that present in control cells. Several AJ proteins, including E-cadherin, β -catenin and p120 catenin, as well as PI4,5P₂, are also decreased in detergent-insoluble membrane structures of TcsL-82-intoxicated cells compared to control resting cells (Geny *et al.*, 2010). These changes in lipid raft protein content are probably related to modifications in polyphosphoinositides of these membrane structures induced by TcsL-82. Moreover, the TcsL-82-dependent decrease in PI4,5P₂, and possibly also in PI3,4,5P₃, is likely to be responsible for actin depolymerization induced by the toxin. Indeed several actin-associated and/or actin-regulating proteins, including gelsolin (Janmey & Stossel, 1987), profilin (Lassing & Lindberg, 1985), α -actinin (Fukami *et al.*, 1994) and vinculin (Burrige & Mangeat, 1984; Menkel *et al.*, 1994), have been reported to interact with PI4,5P₂. For example, association of PI4,5P₂ with vinculin induces a conformational change in vinculin allowing it to interact with talin, which binds actin (Gilmore & Burrige, 1996). It has been proposed that PI4,5P₂ enhances actin polymerization by uncapping actin

filaments, thereby increasing the number of free barbed ends, which are the actin polymerizing filament ends (Hartwig *et al.*, 1995).

Rac1 appears to play a central role in controlling polyphosphoinositide cell content since this small GTPase has been shown to associate with and regulate the PI4P5K, which generates PI4,5P₂ (Carpenter *et al.*, 1999; Hartwig *et al.*, 1995; Tolias *et al.*, 1995, 2000). PI4P5K was reported to be present in FAs (Di Paolo *et al.*, 2002; Ling *et al.*, 2002). The molecular mode of action of TcsL-82 in decreasing polyphosphoinositides has not yet been elucidated. The main effect of glucosylation of small GTPases by clostridial glucosylating toxins has been reported to be modified interaction with different effectors (Herrmann *et al.*, 1998; Sehr *et al.*, 1998; Vetter *et al.*, 2000). However, no significant difference was found in the association between PI4P5K and non-glucosylated or glucosylated Rac1 loaded either with GDP or with GTP γ S (Geny *et al.*, 2010), but this does not rule out the possibility that TcsL-82 could inactivate PI4P5K via Rac1 at a step other than Rac-PI4P5K interaction. Also, it cannot be excluded that the observed decrease in cellular phosphoinositide content could result from the activation of lipid phosphatases upon TcsL intoxication and Rac inactivation.

Rac seems to be the main target of clostridial glucosylating toxins, and mediates the toxin effects on actin cytoskeleton reorganization. Thereby, TcsL, like TcsL-82, only glucosylates Rac among the Rho-GTPases, and TcdB, which inactivates Rho, Rac and Cdc42 alters the actin cytoskeleton mainly via Rac inactivation rather than RhoA inactivation (Halabi-Cabezón *et al.*, 2008). Therefore, it is likely that clostridial glucosylating toxins trigger a common pathway downstream of Rac including a decrease in phosphoinositides, to induce the actin cytoskeleton disorganization and the subsequent pathological effects, such as the alteration of epithelial and endothelial barrier integrity.

It is noteworthy that TcsL-dependent Rac inactivation through glucosylation at Thr35 does not impair all the Rac downstream signalling pathways (Fig. 2). Rac interacts with more than 31 potential downstream effectors, and it is now known that these effectors use distinct residues within the main docking domains, including switch-I and switch-II regions, and in some cases within the C-terminal area (Bustelo *et al.*, 2007). Indeed, mutation of specific residues results in impaired recognition of only a subset of effectors and prevents the activation of only the corresponding signalling pathways. For instance, Rac amino acids 123–135, known as the insert region, are involved in binding to IQGAP but not to PAK. Tyr40 but not Phe37 from the switch-I is critical for Rac binding to PAK, whereas Phe37 is important for the Rac-dependent change in the actin cytoskeleton (Bishop & Hall, 2000; Bustelo *et al.*, 2007). This could account for the fact that TcsL, which modifies Thr35, impairs a limited number of effectors. However, Rac glucosylation at Thr-35 is reported to block the switch-I domain in its inactive conformation, thus impairing Rac

Fig. 2. TcsL-dependent signalling pathways subsequent to Rac inactivation and leading to alteration of actin cytoskeleton and E-cadherin intercellular junctions. FA, focal adhesion; AJ, adherens junction.

interaction with all the downstream effectors (Genth & Just, 2010; Genth *et al.*, 2008). Since Rho-GTPases including Rac are distributed in various subcellular compartments (Michaelson *et al.*, 2001; Spiering & Hodgson, 2011), it is conceivable that TcsL, as well as the other clostridial glucosylating toxins, preferentially inactivates certain subcellular pools of Rac, which coordinate a restricted number of effectors.

TcdA has also been reported to alter intestinal cell TJs through dephosphorylation of paxillin and FAK (Kim *et al.*, 2009). However, the TcdA-dependent mechanism of paxillin and FAK dephosphorylation seems to be different from that of TcsL. TcdA was reported to directly interact with the catalytic domain of Src, inhibiting its kinase activity towards paxillin and FAK. This process is independent of Rho-GTPase glucosylation, since the glucosyltransferase inhibitor UDP-2',3'-dialdehyde did not prevent dephosphorylation of paxillin and FAK by TcdA, and also independent of tyrosine phosphatases based on experiments with sodium orthovanadate, a tyrosine phosphatase inhibitor, which was not found to impair TcdA-induced dephosphorylation activity (Kim *et al.*, 2009). Further work is required to better understand the clostridial glucosylating toxin activity downstream of Rho-GTPase and to identify possible alternative mechanisms to Rho-

GTPase inhibition in alteration of the actin cytoskeleton and intercellular junctions. In contrast, the recently described TcdA activity consisting of α -tubulin deacetylation leading to microtubule depolymerization, loss of TJs, and proinflammatory cytokine production was found to be Rho-GTPase-dependent (Nam *et al.*, 2010).

Cellular effects induced by clostridial glucosylating toxins independently of Rho/Ras-GTPase inactivation

Clostridial glucosylating toxins have been found to activate the mitogen-activated protein (MAP) kinase (MAPK) pathways, leading to an inflammatory response. TcdA, which glucosylates, and thus inactivates, small GTPases from the Rho family, Rho, Rac and Cdc42, was also reported to activate MAPKs in a monocytic cell line. Activation of ERK1/2 and SAPK/JNK by TcdA was shown to be transient (less than 30 min) whereas that of p38 MAPK, starting within minutes of cellular intoxication, is sustained, lasting for at least 1 h (Warny *et al.*, 2000). The latter MAPK activation was demonstrated to be involved in the inflammatory response accompanying intoxication by TcdA (Kim *et al.*, 2005b; Warny *et al.*, 2000), a response that was not observed during TcsL-82 intoxication in mice (Geny *et al.*, 2007). TcdA-induced activation of p38 MAPK was also shown to lead to p53 activation and finally to apoptosis mostly independently of Rho inactivation in human untransformed NMC460 colonocytes (Kim *et al.*, 2005a). In human mast cells, TcdA and TcdB activate p38 MAPK and ERK1/2, leading to a p38 MAPK-dependent release of interleukin-8 (IL-8) and prostaglandins D₂ and E₂. This process was found to result from toxin entry into cells, but not from a receptor-mediated effect. Although cytoskeleton rearrangement participates in mast cell degranulation, increased secretion of IL-8 and prostaglandins was found to be dependent on the TcdB-mediated p38 MAPK activation and not derived from its actin depolymerization effect (Meyer *et al.*, 2007). This does not rule out the possibility that a Rho-GTPase inactivation-dependent pathway distinct from that of actin depolymerization might be involved. However, ERK1/2-p38 MAPK activation and subsequent IL-8 production induced by TcdA was found to be independent of its glucosyltransferase activity based on the different kinetics of the two activities (Kim *et al.*, 2005b; Warny *et al.*, 2000). MAPK activation possibly results from an initial induction of reactive oxygen species (ROS) by TcdA (Kim *et al.*, 2005b). TcdA also increases IL-8 secretion via activation of the NF- κ B pathway (Kim *et al.*, 2006), which is probably mediated by ROS generation from mitochondria independently of Rho inactivation (He *et al.*, 2000, 2002). In addition, a possible role of RhoB activation (Gerhard *et al.*, 2005) subsequent to TcdA-dependent Rho inactivation in NF- κ B activation has been hypothesized (Kim *et al.*, 2006).

We have also observed that TcsL-82 activates the three MAPK pathways ERK1/2, p38 MAPK and SAPK/JNK and

subsequently the phosphorylation of transcriptional factors such as c-Jun and ATF2. This effect is transient. In HeLa cells, TcsL-82-dependent activation of MAPK pathways occurs within about 1 h, reaches a maximum after 2 h and then decreases (Geny & Popoff, 2009). The kinetics of TcsL-82-dependent activation of MAPK pathways is similar to that of *in vivo* enzymic activity towards Rac/Ras-GTPases. This raises the question whether MAPK activation is dependent on Rac/Ras-GTPase glucosylation by TcsL-82. A derivative cell line (DonQ) from the cell line Don has a low content of UDP-glucose and thus is resistant to TcsL-82 intracellular activity. Although glucosylation of Rho/Ras-GTPases and cell rounding are prevented in DonQ cells in contrast to the parental Don cells, TcsL-82 induces MAPK activation in both cell lines (Geny & Popoff, 2009), indicating that glucosylation of Rac/Ras-GTPases is not required for the toxin-dependent MAPK activation. Similar findings have been obtained with TcdA: activation of MAPK by TcdA was reported to be independent of Rho-GTPase. The early p38 MAPK activation by TcdA precedes Rho glucosylation, suggesting that the two effects are not related. It has been proposed that a toxin interaction with an as yet unknown cell-surface receptor and/or intracellular entry of the toxin might trigger p38 MAPK activation (Kim *et al.*, 2005b; Warny *et al.*, 2000). We have tested whether the C-terminal part of TcsL-82, which interacts with a cell-surface receptor, might induce MAPK activation. No effect on MAPK activation has been found with a recombinant TcsL C-terminal protein. The whole toxin is required to induce this effect. In cells pretreated with bafilomycin A1, which blocks the translocation of the N-terminal part of the toxin from acidified endosome to the cytosol, TcsL-82 is unable to trigger the MAPK activation (Geny & Popoff, 2009). Therefore, the N-terminal part of TcsL-82, which contains the enzymic site, has to be translocated into the cytosol to induce the MAPK activation, but independently of Rac/Ras-GTPase glucosylation.

Interestingly, a specific JNK inhibitor, JNK inhibitor II, prevents the TcsL-82-dependent cytoskeleton modifications and actin depolymerization but in a transient manner, whereas inhibitors of ERK1/2 and p38 MAPK are without effect (Geny & Popoff, 2009). JNK inhibitor II delays the TcsL-82-dependent glucosylation of Rac and Ras proteins. As mentioned in the previous paragraph, TcsL-82 induces stress fibre depolymerization and cell rounding, most probably as a consequence of Rac inactivation. Retardation of Rac glucosylation by JNK inhibitor II accounts for the delayed effects of TcsL-82 on actin depolymerization and cell morphology modification. JNK inhibitor II does not directly impair Rac glucosylation by TcsL-82, since the kinetics of *in vitro* Rac glucosylation by TcsL-82 is not modified in its presence, but rather acts indirectly. It is presumed that JNK activation by TcsL-82 induces a facilitation of Rac and Ras glucosylation, possibly mediated by another, as yet unknown, cofactor. In contrast, changes in cell morphology and actin depoly-

merization resulting from TcdB intoxication are not prevented by JNK inhibitor II, although this intoxication is also accompanied by an activation of the SAPK/JNK pathway (Geny & Popoff, 2009). This further argues that TcsL-82-dependent glucosylation of Rac/Ras-GTPases and activation of the SAPK/JNK pathway proceed by two independent mechanisms. Cell stress, such as cytoskeleton alteration, might induce MAPK activation. Indeed, cytochalasin D, which caps actin and thus induces its depolymerization, activates the JNK pathway. But the specific JNK inhibitor II does not prevent cytochalasin D-dependent actin depolymerization (Geny & Popoff, 2009). Therefore, the SAPK/JNK pathway seems to be connected to Rac/Ras-GTPases and subsequently to the control of actin polymerization, but not directly to the actin cytoskeleton, and this interplay between the SAPK/JNK and Rac/Ras-GTPase pathways seems to be specifically targeted by TcsL and not by the other toxins active on Rho-GTPases.

Another consequence of TcdA and TcdB intoxication independent of Rho-GTPase inactivation is apoptosis. Contradictory mechanisms of apoptosis induced by TcdA have been reported, including p38 MAPK-dependent p53 activation leading to cytochrome *c* release and caspase-3 activation in a Rho-GTPase independent manner (Kim *et al.*, 2005a), and a caspase-3/caspase-8 activation via Rho-GTPase glucosylation and independently of p53 (Brito *et al.*, 2002; Nottrott *et al.*, 2007). The TcdB apoptotic effect has been found to interact with mitochondria, leading to membrane permeability alteration, mitochondrial swelling and cytochrome *c* release independently of Rho-inhibiting activity (Matarrese *et al.*, 2007).

Alteration of intercellular junctions by clostridial glucosylating toxins as a consequence of actin depolymerization following Rho-GTPase inactivation is well documented (Just & Gerhard, 2004; Popoff & Stiles, 2006). However, an additional mechanism not requiring Rho-GTPase has been reported. Indeed, TcdA seems to disturb TJs and increase cell monolayer permeability through redistribution of ZO-1 from detergent-resistant membrane fractions to soluble fractions via a protein kinase C (PKC) pathway (Chen *et al.*, 2002).

From clostridial glucosylating toxin-dependent cellular effects to pathology

A major difference between *C. difficile* and *C. sordellii* infections is that these two pathogens modulate the host inflammatory response differently. *C. difficile* can grow in the intestinal lumen and secrete toxins which alter the intestinal mucosa and induce a strong inflammatory response, whereas *C. sordellii*, which is also an agent of haemorrhagic enteritis and enterotoxaemia, mainly observed in animals, can in addition, most often as a result of a wound, enter and multiply in deeper tissues, like muscle and connective tissues, without inducing or triggering only a modest inflammatory response of the

host. The absence of or a weak inflammatory response is observed in natural cases of *C. sordellii* infections as well as in experimental animals (Aldape *et al.*, 2006; Geny *et al.*, 2007). Thus *C. sordellii* avoids the host innate defence, proliferates and locally produces toxins, which diffuse and induce a toxic shock syndrome. *C. sordellii* and *C. difficile* toxins, although they share some common mechanisms of action, exert different cellular effects leading to alteration of epithelial/endothelial barrier integrity accompanied by a strong inflammatory response or by killing inflammatory cells. The different set of Rho/Ras-GTPases inactivated by *C. sordellii* and *C. difficile* toxins accounts in part for these different effects. Inactivation of Rho-GTPases mainly induces disorganization of the actin cytoskeleton and subsequent modification of cell morphology and loss of cell-cell contacts, whereas inhibition of Ras-GTPases causes cell killing by cell cycle arrest and/or apoptosis through several signalling pathways including the PI3/AKT, RalGEF/Ral and Raf/ERK pathways (reviewed by Genth & Just, 2010). A subtle balance between these two main effects, called cytopathic and cytotoxic, is controlled by each toxin according to the cell type. TcdB and TcdA, via inactivation of Rho, Rac and Cdc42, profoundly disorganize the actin cytoskeleton and tight and basolateral intercellular junctions, leading to severe dysfunctions and lesions of the intestinal mucosa (diarrhoea, necrosis). TcsL, by inactivating only Rac from the Rho-GTPases, more specifically alters basolateral intercellular junctions, leading to alteration of endothelial barriers and oedema, notably in the cardio-respiratory system, and by inactivating Ras induces lymphocyte and phagocytic cell killing (Fig. 3). Moreover, Rac inactivation in lymphocytes and phagocytic cells probably impairs their migration and phagocytic function via actin filament depolymerization.

Most of the intracellularly active toxins unleash their cytotoxic programme by interacting with a unique intracellular mechanism of action most often consisting in an enzymic modification of a unique or a set of related targets. But some toxins and type III secretion virulence factors from invasive bacteria, which differ from the toxins by their mode of entry into cells based on a direct injection into the cytosol through a bacterial secretion machinery, are multifunctional proteins. They contain several enzymic domains able to modify more than one substrate (Sansone, 2004). This strategy ensures a greater efficiency of the overall toxic activity towards a wide set of cell types. The Rho/Ras-GTPase-independent effects of *C. sordellii* and *C. difficile* toxins contribute again to the different effects of these toxins. Indeed TcdA attacks TJ integrity by Rho-GTPase inactivation and alternative pathways (Chen *et al.*, 2002; Kim *et al.*, 2009). TcsL-82-dependent SAP/JNK activation facilitates Rac and Ras glucosylation, thus reinforcing the effects of Rac/Ras inhibition in target cells, namely epithelial/endothelial barrier dysfunction as well as phagocytic and immune cell alteration (migration and phagocytosis impairment, apoptosis). Inhibition of the inflammatory response possibly also results from TcsL

Fig. 3. Model of *C. sordellii* and *C. difficile*-associated diseases based on the different toxin effects. *C. difficile* and *C. sordellii* can colonize the intestine and secrete toxins, which through inactivation of Rho-GTPases alter the intestinal epithelial barrier (actin cytoskeleton and intercellular junction disorganization, increased permeability resulting in diarrhoea, necrotic lesions). In addition, *C. difficile* toxins, mainly TcdA, via activation of p38 MAPK in a Rho-GTPase-independent manner induce a strong inflammatory response, which contributes to the intestinal lesions. In contrast, *C. sordellii* infections, which can occur in the intestinal tract but also in deep tissues, are accompanied by no or only a modest inflammatory response. TcsL targets only Rac among the Rho-GTPases, which is involved in increased endothelial permeability and oedema as well as in migration and phagocytosis impairment in immune cells, and Ras proteins, which lead to apoptosis in immune and phagocytic cells. In addition, the Rac/Ras-independent TcsL effects probably contribute to the anti-inflammatory response through SAP/JNK activation, which facilitates Rac and Ras glucosylation, and possibly through activation of additional signalling cascades subsequent to activation of MAPK pathways.

activation of other signalling cascades subsequent to MAPK activation, since TcsL-82 has been found to activate several transcriptional factors (Geny & Popoff, 2009). In contrast, TcdB-dependent activation of ERK, p38 MAPK or SAP/JNK does not influence Rho-GTPase glucosylation,

and activation of the MAPK and NF- κ B pathways in a Rho/Ras-GTPase-independent manner, mainly p38 MAPK activation by TcdA, results in a strong inflammatory response (Geny & Popoff, 2009; Kim *et al.*, 2005a; Warny *et al.*, 2000). The fact that *C. sordellii* is recognized by the

innate immune system through Toll-like receptors (TLRs) like other pathogens further supports the idea that the absence of an inflammatory response is not related to inadequate immune detection but rather to a specific activity of *C. sordellii* toxins (Aldape *et al.*, 2010). An additional Rac/Ras-independent effect of clostridial glucosylating toxins might include the repression of glucocorticoid receptor and reduced glucocorticoid suppression of proinflammatory cytokine secretion, since this effect correlates with a decreased activation of p38 MAPK. Prevention of p38 MAPK activation occurs at lower toxin concentrations than those used in the studies showing an activation of p38. TcsL was shown to prevent the glucocorticoid suppression of TNF α (tumour necrotizing factor) secretion in *ex vivo* splenocytes and to amplify the effects of the glucocorticoid antagonist RU-486, which has been associated with dramatic *C. sordellii* toxic shock in women (Miech, 2005; Tait *et al.*, 2007). Interruption of the glucocorticoid response by TcsL probably contributes to the toxic shock syndrome induced by this toxin. Therefore, clostridial glucosylating toxins display complex sets of small GTPase-dependent and -independent activities in cells, leading to two main pathological effects: epithelial/endothelial barrier alteration and cell killing, mainly of immune and inflammatory cells. This results in two distinct pathologies, although produced by closely related toxins: *C. difficile* colitis, associated with a strong inflammatory response; and *C. sordellii* infection, characterized by bacterial proliferation in deeper tissues via an inhibition of the inflammatory response and associated with a toxic shock syndrome.

Conclusion

Clostridial glucosylating toxins induce multiple cellular effects, the main characteristics of which are actin cytoskeleton depolymerization and intercellular junction alterations leading to an increased permeability of epithelial and endothelial barriers. The enzymic activity of this toxin family, consisting in inactivation of a subset of

small GTPases through glucosylation, has been extensively analysed in the last decade. However, the signalling pathways downstream of Rho/Ras-GTPases involved in the effects of the toxins are still only partially known. Among the Rho/Ras-GTPase effectors that are involved in specific signalling pathways, only some of them are impaired by the clostridial glucosylating toxins. Rac inactivation and subsequent alteration of FA complexes, including dephosphorylation of paxillin and leading to actin filament depolymerization and disorganization of intercellular junctions, seems to be a key feature of the intracellular activity of these toxins. In addition, alteration of the phosphoinositide metabolism, notably the decrease in PI4,5P₂, probably plays an important role in actin filament depolymerization. The small GTPase pathways altered by these toxins could also differ according to the cell type. However, an increasing number of studies shows that all the intracellular effects of clostridial glucosylating toxins are not Rho/Ras-GTPase-dependent but imply alteration of other signalling pathways (Table 2). Several studies support the idea that activation of MAPK pathways, which can be used by some clostridial glucosylating toxins to induce an inflammatory response in certain cell types, is Rho/Ras-GTPase independent.

The complex sets of cellular signalling pathways altered by *C. difficile* and *C. sordellii* toxins result in two main pathological situations: *C. difficile*, being able to proliferate in the intestinal tract and to attack the intestinal barrier, leads to a strong inflammatory response; *C. sordellii*, which can also colonize the intestinal ecosystem but can prevent the inflammatory response and invade deeper tissues of the host, induces a toxic shock syndrome. *C. difficile* and *C. sordellii* are probably representative of two intermediate evolutionary steps between a commensal micro-organism of the intestine and a highly invasive pathogen. This also suggests that modulation of host inflammation is a critical mechanism used by pathogens. Triggering of gut inflammation seems to be a common strategy of enteropathogenic bacteria to colonize the intestinal ecosystem (Stecher & Hardt, 2008). This strategy is probably used by *C. difficile*

Table 2. Rho/Ras-GTPase-independent activities of clostridial glucosylating toxins

Toxin	Cell type	Activity independent of Rho/Ras GTPase inhibition	Reference(s)
TcdA	Human monocytic THP-1 cells, NCM 460 human colonocytes	Activation of ERK1/2, SAP/JNK and p38 MAPK	Kim <i>et al.</i> (2005a); Warny <i>et al.</i> (2000)
TcsL-82	HeLa cells	Activation of ERK1/2, SAP/JNK and p38 MAPK	Geny & Popoff (2009)
TcdB	HEp-2 cells	Apoptosis via alteration of mitochondrial membrane permeability and release of cytochrome <i>c</i>	Matarrese <i>et al.</i> (2007)
TcdA	T84 colonocytes	Activation of PKC, redistribution of ZO-1 and alteration of tight junctions	Chen <i>et al.</i> (2002)
TcdA	HT-29 colonocytes, primary human colon epithelial cells	Activation of the NF- κ B pathway and IL-8 secretion	Kim <i>et al.</i> (2006)
TcdA	Chinese hamster ovary (CHO) cells	Induction of reactive oxygen species from mitochondria and activation of the NF- κ B pathway and IL-8 secretion	He <i>et al.</i> (2000, 2002)
TcdA	NCM 460 human colonocytes	Paxillin and FAK dephosphorylation	Kim <i>et al.</i> (2009)

in addition to antibiotic-dependent disruption of the physiological microbiota to proliferate in the intestine. *C. sordellii* has developed a further adaptive mechanism based on a differential modulation of host inflammation, permitting its proliferation in deeper tissues, once having crossed the epithelial barrier.

References

- Aldape, M. J., Bryant, A. E. & Stevens, D. L. (2006). *Clostridium sordellii* infection: epidemiology, clinical findings, and current perspectives on diagnosis and treatment. *Clin Infect Dis* **43**, 1436–1446.
- Aldape, M. J., Bryant, A. E., Katahira, E. J., Hajjar, A. M., Finegold, S. M., Ma, Y. & Stevens, D. L. (2010). Innate immune recognition of, and response to, *Clostridium sordellii*. *Anaerobe* **16**, 125–130.
- Al-Mashat, R. R. & Taylor, D. J. (1983a). Production of diarrhoea and enteric lesions in calves by the oral inoculation of pure cultures of *Clostridium sordellii*. *Vet Rec* **112**, 141–146.
- Al-Mashat, R. R. & Taylor, D. J. (1983b). *Clostridium sordellii* in enteritis in an adult sheep. *Vet Rec* **112**, 19.
- Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O. & Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* **393**, 805–809.
- Bashour, A. M., Fullerton, A. T., Hart, M. J. & Bloom, G. S. (1997). IQGAP1, a Rac- and Cdc42-binding protein, directly binds and cross-links microfilaments. *J Cell Biol* **137**, 1555–1566.
- Birukova, A. A., Malyukova, I., Poroyko, V. & Birukov, K. G. (2007). Paxillin-beta-catenin interactions are involved in Rac/Cdc42-mediated endothelial barrier-protective response to oxidized phospholipids. *Am J Physiol Lung Cell Mol Physiol* **293**, L199–L211.
- Birukova, A. A., Alekseeva, E., Cokic, I., Turner, C. E. & Birukov, K. G. (2008). Cross talk between paxillin and Rac is critical for mediation of barrier-protective effects by oxidized phospholipids. *Am J Physiol Lung Cell Mol Physiol* **295**, L593–L602.
- Bishop, A. L. & Hall, A. (2000). Rho GTPases and their effector proteins. *Biochem J* **348**, 241–255.
- Bitti, A., Mastrantonio, P., Spigaglia, P., Urru, G., Spano, A. I., Moretti, G. & Cherchi, G. B. (1997). A fatal postpartum *Clostridium sordellii* associated toxic shock syndrome. *J Clin Pathol* **50**, 259–260.
- Bodin, S., Giuriato, S., Ragab, J., Humbel, B. M., Viala, C., Vieu, C., Chap, H. & Payrastre, B. (2001). Production of phosphatidylinositol 3,4,5-trisphosphate and phosphatidic acid in platelet rafts: evidence for a critical role of cholesterol-enriched domains in human platelet activation. *Biochemistry* **40**, 15290–15299.
- Boehm, C., Gibert, M., Geny, B., Popoff, M. R. & Rodriguez, P. (2006). Modification of epithelial cell barrier permeability and intercellular junctions by *Clostridium sordellii* lethal toxins. *Cell Microbiol* **8**, 1070–1085.
- Braga, V. M., Machesky, L. M., Hall, A. & Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J Cell Biol* **137**, 1421–1431.
- Braga, V. M. M., Del Maschio, A., Machesky, L. & Dejana, E. (1999). Regulation of cadherin function by Rho and Rac: modulation by junction maturation and cellular context. *Mol Biol Cell* **10**, 9–22.
- Brito, G. A. C., Fujii, J., Carneiro-Filho, B. A., Lima, A. A., Obrig, T. & Guerrant, R. L. (2002). Mechanism of *Clostridium difficile* toxin A-induced apoptosis in T84 cells. *J Infect Dis* **186**, 1438–1447.
- Burridge, K. & Mangeat, P. (1984). An interaction between vinculin and talin. *Nature* **308**, 744–746.
- Burridge, K. & Wennerberg, K. (2004). Rho and Rac take center stage. *Cell* **116**, 167–179.
- Bustelo, X. R., Sauzeau, V. & Berenjeno, I. M. (2007). GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *Bioessays* **29**, 356–370.
- Caroni, P. (2001). New EMBO members' review: actin cytoskeleton regulation through modulation of PI(4,5)P(2) rafts. *EMBO J* **20**, 4332–4336.
- Carpenter, C. L., Tolias, K. F., Van Vugt, A. & Hartwig, J. (1999). Lipid kinases are novel effectors of the GTPase Rac1. *Adv Enzyme Regul* **39**, 299–312.
- Centers for Disease Control and Prevention (2005). *Clostridium sordellii* toxic shock syndrome after medical abortion with mifepristone and intravaginal misoprostol – United States and Canada, 2001–2005. *MMWR Morb Mortal Wkly Rep* **54**, 724.
- Chen, X. & Macara, I. G. (2006). Par-3 mediates the inhibition of LIM kinase 2 to regulate cofilin phosphorylation and tight junction assembly. *J Cell Biol* **172**, 671–678.
- Chen, M. L., Pothoulakis, C. & LaMont, J. T. (2002). Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to *Clostridium difficile* toxin A. *J Biol Chem* **277**, 4247–4254.
- Clark, S. (2003). Sudden death in periparturient sheep associated with *Clostridium sordellii*. *Vet Rec* **153**, 340.
- Cohen, A. L., Bhatnagar, J., Reagan, S., Zane, S. B., D'Angeli, M. A., Fischer, M., Killgore, G., Kwan-Gett, T. S., Blossom, D. B. & other authors (2007). Toxic shock associated with *Clostridium sordellii* and *Clostridium perfringens* after medical and spontaneous abortion. *Obstet Gynecol* **110**, 1027–1033.
- Couzin, J. (2006). Infectious disease. RU-486-linked deaths open debate about risky bacteria. *Science* **312**, 986.
- Di Paolo, G., Pellegrini, L., Letinic, K., Cestra, G., Zoncu, R., Voronov, S., Chang, S., Guo, J., Wenk, M. R. & De Camilli, P. (2002). Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 γ by the FERM domain of talin. *Nature* **420**, 85–89.
- Doughman, R. L., Firestone, A. J. & Anderson, R. A. (2003). Phosphatidylinositol phosphate kinases put PI4,5P(2) in its place. *J Membr Biol* **194**, 77–89.
- Dreger, S. C., Schulz, F., Huelsenbeck, J., Gerhard, R., Hofmann, F., Just, I. & Genth, H. (2009). Killing of rat basophilic leukemia cells by lethal toxin from *Clostridium sordellii*: critical role of phosphatidylinositol 3'-OH kinase/Akt signaling. *Biochemistry* **48**, 1785–1792.
- Egerer, M., Giesemann, T., Jank, T., Satchell, K. J. & Aktories, K. (2007). Auto-catalytic cleavage of *Clostridium difficile* toxins A and B depends on cysteine protease activity. *J Biol Chem* **282**, 25314–25321.
- Egerer, M., Giesemann, T., Herrmann, C. & Aktories, K. (2009). Autocatalytic processing of *Clostridium difficile* toxin B. Binding of inositol hexakisphosphate. *J Biol Chem* **284**, 3389–3395.
- Ferguson, K. M., Lemmon, M. A., Schlessinger, J. & Sigler, P. B. (1995). Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* **83**, 1037–1046.
- Fischer, M., Bhatnagar, J., Guarner, J., Reagan, S., Hacker, J. K., Van Meter, S. H., Poukens, V., Whiteman, D. B., Iton, A. & other authors (2005). Fatal toxic shock syndrome associated with *Clostridium sordellii* after medical abortion. *N Engl J Med* **353**, 2352–2360.
- Fukami, K., Endo, T., Imamura, M. & Takenawa, T. (1994). alpha-Actinin and vinculin are PIP2-binding proteins involved in signaling by tyrosine kinase. *J Biol Chem* **269**, 1518–1522.
- Fukata, M. & Kaibuchi, K. (2001). Rho-family GTPases in cadherin-mediated cell-cell adhesion. *Nat Rev Mol Cell Biol* **2**, 887–897.

- Fukata, M., Nakagawa, M., Kuroda, S. & Kaibuchi, K. (1999). Cell adhesion and Rho small GTPases. *J Cell Sci* **112**, 4491–4500.
- Gaus, K., Le Lay, S., Balasubramanian, N. & Schwartz, M. A. (2006). Integrin-mediated adhesion regulates membrane order. *J Cell Biol* **174**, 725–734.
- Genth, H. & Just, I. (2010). Functional implications of lethal toxin-catalysed glucosylation of (H/K/N)Ras and Rac1 in *Clostridium sordellii*-associated disease. *Eur J Cell Biol* [Epub ahead of print] doi: 10.1016/j.ejcb.2010.10.009.
- Genth, H., Aktories, K. & Just, I. (1999). Monoglucosylation of RhoA at threonine 37 blocks cytosol-membrane cycling. *J Biol Chem* **274**, 29050–29056.
- Genth, H., Dreger, S. C., Huelsenbeck, J. & Just, I. (2008). *Clostridium difficile* toxins: more than mere inhibitors of Rho proteins. *Int J Biochem Cell Biol* **40**, 592–597.
- Geny, B. & Popoff, M. R. (2009). Activation of a c-Jun-NH₂-terminal kinase pathway by the lethal toxin from *Clostridium sordellii*, TcsL-82, occurs independently of the toxin intrinsic enzymatic activity and facilitates small GTPase glucosylation. *Cell Microbiol* **11**, 1102–1113.
- Geny, B., Khun, H., Fitting, C., Zarantonelli, L., Mazuet, C., Cayet, N., Szatanik, M., Prevost, M. C., Cavaillon, J. M. & Huerre, M. (2007). *Clostridium sordellii* lethal toxin kills mice by inducing a major increase in lung vascular permeability. *Am J Pathol* **170**, 1003–1017.
- Geny, B., Grassart, A., Manich, M., Chicanne, G., Payrastra, B., Sauvonnnet, N. & Popoff, M. R. (2010). Rac1 inactivation by lethal toxin from *Clostridium sordellii* modifies focal adhesions upstream of actin depolymerization. *Cell Microbiol* **12**, 217–232.
- Gerhard, R., Tatge, H., Genth, H., Thum, T., Borlak, J., Fritz, G. & Just, I. (2005). *Clostridium difficile* toxin A induces expression of the stress-induced early gene product RhoB. *J Biol Chem* **280**, 1499–1505.
- Gilmore, A. P. & Burridge, K. (1996). Regulation of vinculin binding to talin and actin by phosphatidylinositol-4-5-bisphosphate. *Nature* **381**, 531–535.
- Golub, T. & Caroni, P. (2005). PI(4,5)P₂-dependent microdomain assemblies capture microtubules to promote and control leading edge motility. *J Cell Biol* **169**, 151–165.
- Greene, M. F. (2005). Fatal infections associated with mifepristone-induced abortion. *N Engl J Med* **353**, 2317–2318.
- Guo, F., Debidda, M., Yang, L., Williams, D. A. & Zheng, Y. (2006). Genetic deletion of Rac1 GTPase reveals its critical role in actin stress fiber formation and focal adhesion complex assembly. *J Biol Chem* **281**, 18652–18659.
- Halabi-Cabezón, I., Huelsenbeck, J., May, M., Ladwein, M., Rottner, K., Just, I. & Genth, H. (2008). Prevention of the cytopathic effect induced by *Clostridium difficile* toxin B by active Rac1. *FEBS Lett* **582**, 3751–3756.
- Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A. & Stossel, T. P. (1995). Thrombin receptor ligation and activated Rac uncouple actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* **82**, 643–653.
- He, D., Hagen, S. J., Pothoulakis, C., Chen, M., Medina, N. D., Warny, M. & LaMont, J. T. (2000). *Clostridium difficile* toxin A causes early damage to mitochondria in cultured cells. *Gastroenterology* **119**, 139–150.
- He, D., Sougioultzis, S., Hagen, S., Liu, J., Keates, S., Keates, A. C., Pothoulakis, C. & LaMont, J. T. (2002). *Clostridium difficile* toxin A triggers human colonocyte IL-8 release via mitochondrial oxygen radical generation. *Gastroenterology* **122**, 1048–1057.
- Hecht, G., Pothoulakis, C., LaMont, J. T. & Madara, J. L. (1988). *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. *J Clin Invest* **82**, 1516–1524.
- Hecht, G., Koutsouris, A., Pothoulakis, C., LaMont, J. T. & Madara, J. L. (1992). *Clostridium difficile* toxin B disrupts the barrier function of T84 monolayers. *Gastroenterology* **102**, 416–423.
- Herrmann, C., Ahmadian, M. R., Hofmann, F. & Just, I. (1998). Functional consequences of monoglucosylation of Ha-Ras at effector domain amino acid threonine 35. *J Biol Chem* **273**, 16134–16139.
- Ho, C. S., Bhatnagar, J., Cohen, A. L., Hacker, J. K., Zane, S. B., Reagan, S., Fischer, M., Shieh, W. J., Guarner, J. & Ahmad, S. (2009). Undiagnosed cases of fatal *Clostridium*-associated toxic shock in Californian women of childbearing age. *Am J Obstet Gynecol* **201**, 459.e1–459.e7.
- Hofmann, F., Busch, C., Prepens, U., Just, I. & Aktories, K. (1997). Localization of the glucosyltransferase activity of *Clostridium difficile* toxin B to the N-terminal part of the holotoxin. *J Biol Chem* **272**, 11074–11078.
- Hofmann, F., Busch, C. & Aktories, K. (1998). Chimeric clostridial cytotoxins: identification of the N-terminal region involved in protein substrate recognition. *Infect Immun* **66**, 1076–1081.
- Jank, T. & Aktories, K. (2008). Structure and mode of action of clostridial glucosylating toxins: the ABCD model. *Trends Microbiol* **16**, 222–229.
- Janmey, P. A. & Stossel, T. P. (1987). Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature* **325**, 362–364.
- Johal, S. S., Solomon, K., Dodson, S., Borriello, S. P. & Mahida, Y. R. (2004). Differential effects of varying concentrations of *Clostridium difficile* toxin A on epithelial barrier function and expression of cytokines. *J Infect Dis* **189**, 2110–2119.
- Jou, T. S., Schneeberger, E. E. & Nelson, W. J. (1998). Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. *J Cell Biol* **142**, 101–115.
- Just, I. & Gerhard, R. (2004). Large clostridial cytotoxins. *Rev Physiol Biochem Pharmacol* **152**, 23–47.
- Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. & Aktories, K. (1995a). Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* **375**, 500–503.
- Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streiber, C., Mann, M. & Aktories, K. (1995b). The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. *J Biol Chem* **270**, 13932–13936.
- Just, I., Selzer, J., Hofmann, F., Green, G. A. & Aktories, K. (1996). Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. *J Biol Chem* **271**, 10149–10153.
- Kaibuchi, K., Kuroda, S., Fukata, M. & Nakagawa, M. (1999a). Regulation of cadherin-mediated cell-cell adhesion by the Rho family GTPases. *Curr Opin Cell Biol* **11**, 591–596.
- Kaibuchi, K., Kuroda, S. & Amano, M. (1999b). Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu Rev Biochem* **68**, 459–486.
- Kelly, C. P. & LaMont, J. T. (2008). *Clostridium difficile* – more difficult than ever. *N Engl J Med* **359**, 1932–1940.
- Kim, H., Kokkotou, E., Na, X., Rhee, S. H., Moyer, M. P., Pothoulakis, C. & LaMont, J. T. (2005a). *Clostridium difficile* toxin A-induced colonocyte apoptosis involves p53-dependent p21(WAF1/CIP1) induction via p38 mitogen-activated protein kinase. *Gastroenterology* **129**, 1875–1888.
- Kim, H., Rhee, S. H., Kokkotou, E., Na, X., Savidge, T., Moyer, M. P., Pothoulakis, C. & LaMont, J. T. (2005b). *Clostridium difficile* toxin A regulates inducible cyclooxygenase-2 and prostaglandin E₂ synthesis in colonocytes via reactive oxygen species and activation of p38 MAPK. *J Biol Chem* **280**, 21237–21245.
- Kim, J. M., Lee, J. Y., Yoon, Y. M., Oh, Y. K., Youn, J. & Kim, Y. J. (2006). NF- κ B activation pathway is essential for the chemokine

expression in intestinal epithelial cells stimulated with *Clostridium difficile* toxin A. *Scand J Immunol* **63**, 453–460.

Kim, H., Rhee, S. H., Pothoulakis, C. & LaMont, J. T. (2009). *Clostridium difficile* toxin A binds colonocyte Src causing dephosphorylation of focal adhesion kinase and paxillin. *Exp Cell Res* **315**, 3336–3344.

Kimura, A. C., Higa, J. I., Levin, R. M., Simpson, G., Vargas, Y. & Vugia, D. J. (2004). Outbreak of necrotizing fasciitis due to *Clostridium sordellii* among black-tar heroin users. *Clin Infect Dis* **38**, e87–e91.

Kuehne, S. A., Cartman, S. T., Heap, J. T., Kelly, M. L., Cockayne, A. & Minton, N. P. (2010). The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature* **467**, 711–713.

Lassing, I. & Lindberg, U. (1985). Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature* **314**, 472–474.

Lemmon, M. A., Ferguson, K. M. & Abrams, C. S. (2002). Pleckstrin homology domains and the cytoskeleton. *FEBS Lett* **513**, 71–76.

Lewis, C. J. & Naylor, R. (1996). Sudden death in lambs associated with *Clostridium sordellii* infection. *Vet Rec* **138**, 262.

Lewis, C. J. & Naylor, R. D. (1998). Sudden death in sheep associated with *Clostridium sordellii*. *Vet Rec* **142**, 417–421.

Ling, K., Doughman, R. L., Firestone, A. J., Bunce, M. W. & Anderson, R. A. (2002). Type I γ phosphatidylinositol phosphate kinase targets and regulates focal adhesions. *Nature* **420**, 89–93.

Logan, M. R. & Mandato, C. A. (2006). Regulation of the actin cytoskeleton by PIP2 in cytokinesis. *Biol Cell* **98**, 377–388.

Lyras, D., O'Connor, J. R., Howarth, P. M., Sambol, S. P., Carter, G. P., Phumoonna, T., Poon, R., Adams, V., Vedantam, G. & other authors (2009). Toxin B is essential for virulence of *Clostridium difficile*. *Nature* **458**, 1176–1179.

Matarrese, P., Falzano, L., Fabbri, A., Gambardella, L., Frank, C., Geny, B., Popoff, M. R., Malorni, W. & Fiorentini, C. (2007). *Clostridium difficile* toxin B causes apoptosis in epithelial cells by thrilling mitochondria. Involvement of ATP-sensitive mitochondrial potassium channels. *J Biol Chem* **282**, 9029–9041.

McGregor, J. A., Soper, D. E., Lovell, G. & Todd, J. K. (1989). Maternal deaths associated with *Clostridium sordellii* infection. *Am J Obstet Gynecol* **161**, 987–995.

Menkel, A. R., Kroemker, M., Bubeck, P., Ronsiek, M., Nikolai, G. & Jockusch, B. M. (1994). Characterization of an F-actin-binding domain in the cytoskeletal protein vinculin. *J Cell Biol* **126**, 1231–1240.

Meyer, G. K., Neetz, A., Brandes, G., Tsikas, D., Butterfield, J. H., Just, I. & Gerhard, R. (2007). *Clostridium difficile* toxins A and B directly stimulate human mast cells. *Infect Immun* **75**, 3868–3876.

Michaelson, D., Silletti, J., Murphy, G., D'Eustachio, P., Rush, M. & Philips, M. R. (2001). Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J Cell Biol* **152**, 111–126.

Miech, R. P. (2005). Pathophysiology of mifepristone-induced septic shock due to *Clostridium sordellii*. *Ann Pharmacother* **39**, 1483–1488.

Murray, S. & Woollorton, E. (2005). Septic shock after medical abortions with mifepristone (Mifeprex, RU 486) and misoprostol. *CMAJ* **173**, 485.

Nam, H. J., Kang, J. K., Kim, S. K., Ahn, K. J., Seok, H., Park, S. J., Chang, J. S., Pothoulakis, C., Lamont, J. T. & Kim, H. (2010). *Clostridium difficile* toxin A decreases acetylation of tubulin, leading to microtubule depolymerization through activation of histone deacetylase 6, and this mediates acute inflammation. *J Biol Chem* **285**, 32888–32896.

Noritake, J., Fukata, M., Sato, K., Nakagawa, M., Watanabe, T., Izumi, N., Wang, S., Fukata, Y. & Kaibuchi, K. (2004). Positive role of IQGAP1, an effector of Rac1, in actin-meshwork formation at sites of cell-cell contact. *Mol Biol Cell* **15**, 1065–1076.

Nottrott, S., Schoentaube, J., Genth, H., Just, I. & Gerhard, R. (2007). *Clostridium difficile* toxin A-induced apoptosis is p53-independent but depends on glucosylation of Rho GTPases. *Apoptosis* **12**, 1443–1453.

Nusrat, A., von Eichel-Streiber, C., Turner, J. R., Verkade, P., Madara, J. L. & Parkos, C. A. (2001). *Clostridium difficile* toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins. *Infect Immun* **69**, 1329–1336.

Ottlinger, M. E. & Lin, S. (1988). *Clostridium difficile* toxin B induces reorganization of actin, vinculin, and talin in cultured cells. *Exp Cell Res* **174**, 215–229.

Popoff, M. R. (1984). Bacteriological examination in enterotoxaemia of sheep and lamb. *Vet Rec* **114**, 324.

Popoff, M. R. & Geny, B. (2009). Multifaceted role of Rho, Rac, Cdc42 and Ras in intercellular junctions, lessons from toxins. *Biochim Biophys Acta* **1788**, 797–812.

Popoff, M. R. & Stiles, B. G. (2006). Bacterial toxins and virulence factors targeting the actin cytoskeleton and intercellular junctions. In *The Comprehensive Sourcebook of Bacterial Protein Toxins*, 3rd edn, pp. 154–187. Edited by J. E. Alouf & M. R. Popoff. Amsterdam: Elsevier, Academic Press.

Popoff, M. R., Chaves-Olarte, E., Lemichez, E., von Eichel-Streiber, C., Thelestam, M., Chardin, P., Cussac, D., Antonny, B., Chavrier, P. & other authors (1996). Ras, Rap, and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. *J Biol Chem* **271**, 10217–10224.

Reineke, J., Tenzer, S., Rupnik, M., Koschinski, A., Hasselmayer, O., Schratzenholz, A., Schild, H. & von Eichel-Streiber, C. (2007). Autocatalytic cleavage of *Clostridium difficile* toxin B. *Nature* **446**, 415–419.

Richard, J. F., Petit, L., Gibert, M., Marvaud, J. C., Bouchaud, C. & Popoff, M. R. (1999). Bacterial toxins modifying the actin cytoskeleton. *Int Microbiol* **2**, 185–194.

Richards, S. M. & Hunt, B. W. (1982). *Clostridium sordellii* in lambs. *Vet Rec* **111**, 22.

Rørbye, C., Petersen, I. S. & Nilas, L. (2000). Postpartum *Clostridium sordellii* infection associated with fatal toxic shock syndrome. *Acta Obstet Gynecol Scand* **79**, 1134–1135.

Rupnik, M. & Just, I. (2006). Large clostridial cytotoxins modifying small GTPases. In *The Sourcebook of Bacterial Protein Toxins*, 3rd edn, pp. 409–429. Edited by J. E. Alouf & M. R. Popoff. Amsterdam: Elsevier, Academic Press.

Rupnik, M., Pabst, S., Rupnik, M., von Eichel-Streiber, C., Urlaub, H. & Söling, H. D. (2005). Characterization of the cleavage site and function of resulting cleavage fragments after limited proteolysis of *Clostridium difficile* toxin B (TcdB) by host cells. *Microbiology* **151**, 199–208.

Sansonetti, P. J. (2004). War and peace at mucosal surfaces. *Nat Rev Immunol* **4**, 953–964.

Sehr, P., Joseph, G., Genth, H., Just, I., Pick, E. & Aktories, K. (1998). Glucosylation and ADP ribosylation of rho proteins: effects on nucleotide binding, GTPase activity, and effector coupling. *Biochemistry* **37**, 5296–5304.

Simonsen, A. & Stenmark, H. (2001). PX domains: attracted by phosphoinositides. *Nat Cell Biol* **3**, E179–E182.

Sinave, C., Le Templier, G., Blouin, D., Léveillé, F. & Deland, E. (2002). Toxic shock syndrome due to *Clostridium sordellii*: a dramatic postpartum and postabortion disease. *Clin Infect Dis* **35**, 1441–1443.

- Songer, J. G. (2005).** Clostridial diseases in domestic animals. In *Handbook on Clostridia*, pp. 527–542. Edited by P. Dürre. Boca Raton: CRC Press, Taylor and Francis Group.
- Soper, D. E. (1986).** Clostridial myonecrosis arising from an episiotomy. *Obstet Gynecol* **68** (Suppl.), 26S–28S.
- Soper, D. E. (2007).** Abortion and clostridial toxic shock syndrome. *Obstet Gynecol* **110**, 970–971.
- Spiering, D. & Hodgson, L. (2011).** Dynamics of the Rho-family small GTPases in actin regulation and motility. *Cell Adh Migr* **5**, 170–180.
- Stecher, B. & Hardt, W. D. (2008).** The role of microbiota in infectious disease. *Trends Microbiol* **16**, 107–114.
- Tait, A. S., Dalton, M., Geny, B., D'Agnillo, F., Popoff, M. R. & Sternberg, E. M. (2007).** The large clostridial toxins from *Clostridium sordellii* and *C. difficile* repress glucocorticoid receptor activity. *Infect Immun* **75**, 3935–3940.
- Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H. & Takai, Y. (1997).** Regulation of cell-cell adhesion by Rac and Rho small G proteins in MDCK cells. *J Cell Biol* **139**, 1047–1059.
- Tolias, K. F., Cantley, L. C. & Carpenter, C. L. (1995).** Rho family GTPases bind to phosphoinositide kinases. *J Biol Chem* **270**, 17656–17659.
- Tolias, K. F., Hartwig, J. H., Ishihara, H., Shibasaki, Y., Cantley, L. C. & Carpenter, C. L. (2000).** Type I α phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr Biol* **10**, 153–156.
- Van Aelst, L. & D'Souza-Schorey, C. (1997).** Rho GTPases and signaling networks. *Genes Dev* **11**, 2295–2322.
- van Rheenen, J., Achame, E. M., Janssen, H., Calafat, J. & Jalink, K. (2005).** PIP₂ signaling in lipid domains: a critical re-evaluation. *EMBO J* **24**, 1664–1673.
- Vetter, I. R., Hofmann, F., Wohlgemuth, S., Herrmann, C. & Just, I. (2000).** Structural consequences of mono-glucosylation of Ha-Ras by *Clostridium sordellii* lethal toxin. *J Mol Biol* **301**, 1091–1095.
- Voth, D. E. & Ballard, J. D. (2005).** *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin Microbiol Rev* **18**, 247–263.
- Warny, M., Keates, A. C., Keates, S., Castagliuolo, I., Zacks, J. K., Aboudola, S., Qamar, A., Pothoulakis, C., LaMont, J. T. & Kelly, C. P. (2000).** p38 MAP kinase activation by *Clostridium difficile* toxin A mediates monocyte necrosis, IL-8 production, and enteritis. *J Clin Invest* **105**, 1147–1156.
- Winikoff, B. (2006).** *Clostridium sordellii* infection in medical abortion. *Clin Infect Dis* **43**, 1447–1448.
- Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. & Mizuno, K. (1998).** Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* **393**, 809–812.