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# Perspectives Series: Host/Pathogen Interactions

## Bacterial Toxins That Target Rho Proteins

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Not only “big” GTP-binding proteins (heterotrimeric G-proteins, elongation factor 2) but also “small” GTPases are targets for bacterial protein toxins. In this respect, Rho subfamily proteins are of particular importance. These GTPases are ADP-ribosylated by *Clostridium botulinum* C3-like transferases and are monoglycosylated by large clostridial cytotoxins. They appear to be the target of *Escherichia coli* cytotoxic necrotizing factors (CNF1, 2)<sup>1</sup> and Bordetella dermonecrotic toxin (DNT). Moreover, Rho proteins seem to be essential for uptake of bacteria into eukaryotic cells.

### Rho proteins

Rho proteins (Rho stands for Ras homologous proteins) belong to the superfamily of Ras proteins (1). They are inactive in the GDP-bound form and are activated by GDP/GTP exchange. The active state is terminated by GTP hydrolysis catalyzed by intrinsic GTPase activity. At least three groups of regulatory proteins control Rho proteins. Guanine nucleotide dissociation stimulators facilitate nucleotide exchange. Conversely, guanine nucleotide dissociation inhibitors stabilize the inactive GDP-bound form and extract Rho proteins from the membrane. Finally, GTPase-activating proteins stimulate the low basal GTPase activity, thereby inactivating the protein. In mammalian cells, several Rho subfamily proteins (RhoA, B, C, Rac1 and 2, Cdc42 (G25K), RhoG, RhoD, and RhoE) have been identified. Rho, Rac, and Cdc42, which are best studied, play crucial roles in regulation of the actin cytoskeleton. Rho subtype proteins are involved in formation of stress fibers and focal adhesion complexes. Rac proteins induce lamellipodia formation and membrane ruffling (also induced by Rho in some cell types). Cdc42 was shown to induce formation of filopodia or microspikes. At least in some cell types (e.g., Swiss 3T3 cells), these GTPases act on the actin cytoskeleton in a cascadelike manner. Activated Cdc42 is able to activate Rac which then causes activation of Rho. Rho proteins are not only involved in regulation of the actin cytoskeleton but are molec-

ular switches in various signal transduction processes, e.g., Rho subfamily proteins participate in control of cell-cell contact, integrin signaling, endocytosis, transcriptional activation, proliferation, apoptosis, and cell transformation. Accordingly, numerous potential Rho-interacting effectors have been described, some of which are kinases (e.g., Rho kinase, p60 PAK kinase, PKN kinase, lipid kinases), and some of which are adaptor proteins without kinase activity (e.g., WASP, p67 phox). Several of these processes appear to be important for bacteria-host interactions, signal transduction of cells of the immune system, and host defense mechanisms. This is particularly evident in the case of Rac and its interaction with p67phox, which is an essential cofactor for superoxide anion formation by granulocyte or macrophage NADPH oxidase, but is similarly obvious for a role of Rho proteins in integrin-mediated cell aggregation of lymphoid cells, lymphocyte-mediated cytotoxicity, and for control of cell polarity of T cells towards antigen-presenting target cells by Cdc42. A final example where Rho proteins are of conceivable importance for bacteria-host interaction may be their function as switches in protein kinase cascade, resulting in activation of JNK/SAPK (c-Jun NH<sub>2</sub>-terminal kinases/stress-activated kinases) and p38 kinase. These kinases are stimulated by stress and inflammatory cytokines and may cause growth arrest, apoptosis, or activation of immune cells. Thus, teleologically, it seems to make sense that Rho proteins are targets of bacterial toxins.

### *C. botulinum* C3-like exoenzymes

Rho proteins are targets of ADP-ribosyltransferase C3 (2), which is produced by *C. botulinum* type C and D strains. Several isoforms of C3 exist and C3-like transferases which share 30–70% identity at the amino acid level are produced by certain strains of *Clostridium limosum*, *Bacillus cereus*, and *Staphylococcus aureus*. All these exoenzymes have molecular masses of ~ 25 kD, are very basic proteins (IP > 9), and modify Rho (Rho A, B, and C) but not Rac or Cdc42 at the same site at asparagine 41 in the effector region of the GTPase. This causes inactivation of Rho, rounding-up of cells, redistribution of the actin cytoskeleton, and inhibition of other processes controlled by Rho. ADP-ribosylation inhibits interaction of Rho with its effector(s) or induces sequestration of Rho-activating proteins.

C3-like exoenzymes contain no cell binding and membrane translocation unit. Therefore, cell accessibility is poor and, generally, high concentrations (> 10 µg/ml) are needed to induce unspecific uptake of C3 in cultured cells, a finding that might question the role of C3 as a bacterial toxin. However, some cell types appear to be more sensitive towards C3-like transferases (e.g., keratinocytes) and, after microinjection, C3 is a potent cytotoxin, which is widely used as a cell biological tool to selectively inactivate Rho proteins.

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1. Abbreviations used in this paper: CNF, cytotoxic necrotizing factor; DNT, dermonecrotic toxin; LT, *C. sordellii* lethal toxin.

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### *Clostridium difficile* toxins

Rho proteins are the targets for *C. difficile* toxins A and B. *C. difficile* is recognized as a frequent cause of antibiotic-induced diarrhea (in ~ 25% of cases) and is the major causative agent of antibiotic-associated pseudomembranous colitis (3). *C. difficile* produces toxins A and B with 308,000 and 270,000  $M_r$ , respectively, which are ~ 45% identical at the amino acid level. Toxin A designated as enterotoxin because it induces the typical symptoms of enterocolitis in animal models. In contrast, toxin B exhibits no enterotoxic effects under similar conditions. It is, however, 100–1,000-fold more cytotoxic than toxin A in inducing rounding-up of cells and destruction of the actin cytoskeleton. Therefore, toxin B was designated cytotoxin. Recently, however, it was reported that toxin B is more effective in damaging human colonic epithelium than toxin A. After parenteral application, both toxins are lethal at similar doses.

The single-chain *C. difficile* toxins A and B comprise at least three structural parts. At the COOH terminus, groups of repetitive peptides are located which are important for binding to carbohydrates of the eukaryotic cell surface. The middle part of the protein is characterized by a short hydrophobic region, perhaps involved in membrane translocation. The biological activity has been located at the NH<sub>2</sub> terminus of the toxin, as was verified recently by deletion analysis of toxin B showing that an NH<sub>2</sub>-terminal fragment of only 546 amino acids (holotoxin 2,366 amino acids) possesses enzyme activity and is able to induce the typical cytotoxic effects after microinjection.

*C. difficile* toxins affect the actin cytoskeleton by inactivation of Rho proteins (4). The toxins monoglucosylate Rho proteins using UDP-glucose as cosubstrate. Targets are all Rho subfamily proteins (e.g., Rho, Rac, and Cdc42), other low molecular mass GTP-binding proteins, including Ras, Rab, Arf, or Ran subfamilies, or heterotrimeric G proteins, are not modified. Modification of Rho occurs at threonine 37 (Thr-35 of Rac or Cdc42). This threonine residue is highly conserved in all low molecular mass GTPases. Thr-37 binds the nucleotide through coordination of the magnesium cation and is located in the effector region of Rho where coupling with the effector protein takes place. Most likely, glucosylation blocks this interaction. Microinjection of recombinant RhoA previously glucosylated by toxin B into monolayer cells induces cytopathic effects typically occurring after treatment of cultured cells with *C. difficile* toxins, thus verifying the cytotoxic actions of the toxins by glucosylation of Rho.

So far, the exact pathogenetic pathway of *C. difficile*-associated diarrhea and colitis is still obscure. However, *C. difficile* toxins were shown to increase intestinal permeability and to stimulate chloride secretion. Toxin A induces an increase in the permeability of the tight junctions. The essential role of Rho proteins in barrier function of tight junctions was shown with C3 exoenzyme (actually, a membrane-penetrating C3-chimera toxin was used). C3 induces depolymerization of the actin cytoskeleton in the vicinity of tight junctions and causes redistribution of the peripheral tight junction protein ZO-1. Concomitantly, a dramatic increase in the permeability of the epithelium is observed (5). On the other hand, it was suggested that leukocytes are involved in the inflammatory effects caused by the toxins. Toxin A appears to be able to induce production of cytokines (e.g., interleukin 8). However, activation of granulocytes does not fit very well into the pathogenetic

model of Rho inactivation by glucosylation (which rather inhibits granulocyte functions). Also whether mere toxin binding to carbohydrates of the eukaryotic cell membrane (receptor) causes lectinlike signaling relevant for pathogenicity is still an open question.

### *Other large clostridial cytotoxins*

Other members of the family of large clostridial toxins, which share significant structural similarities with *C. difficile* toxins are *Clostridium sordellii* hemorrhagic and lethal toxins and the *Clostridium novyi*  $\alpha$ -toxin. The toxins cause gas gangrene syndromes in humans, cattle, and sheep and may be important for induction of diarrhea and enterotoxaemia, at least in domestic animals. These cytotoxins from *C. sordellii* and *C. novyi* are also glycosyltransferases; however, they exhibit exciting differences in substrate and cosubstrate specificities as compared with *C. difficile* toxin (6). *C. novyi*  $\alpha$ -toxin uses UDP-GlcNAc as cosubstrate but not UDP-glucose. Rho proteins are *N*-acetylglucosaminylated at the same site as they are glucosylated by *C. difficile* toxins and the protein targets are identical with modification of all members of the Rho subfamily. The lethal toxin (LT) from *C. sordellii* that shares 90% similarity with *C. difficile* toxin B uses UDP-glucose as cosubstrate. However, LT glucosylates Rac but not Rho and its ability to modify Cdc42 varies between toxins from various strains. Intriguing is the glucosylation of Ras by the *C. sordellii* toxin. In intact cells, LT inhibits growth factor (e.g., EGF)-induced stimulation of the Ras signaling pathway (activation of MAP kinase cascade). In addition to Ras, Rap and Ral proteins are also substrates. Modification of Ral also depends on the origin of LT. In contrast to LT, the hemorrhagic toxin of *C. sordellii* shares cosubstrate and substrate specificities with *C. difficile* toxins.

### *Toxins activating Rho proteins*

Heterotrimeric G-proteins are bidirectionally affected by bacterial toxins. They are activated by cholera toxin and are functionally inactivated by pertussis toxin. Similarly, Rho GTPases appear to be bidirectionally affected by toxins. Some recent reports suggest that Rho proteins are activated by DNT and CNFs. DNT isoforms with very similar biological and immunological properties are produced by several *Bordetella* spp. (7). DNT, a heat-labile toxin of 154 kD, causes dermonecrotic lesions when injected intradermally and is lethal (e.g., for mice) after intravenous application. DNT is believed to be a causative agent of porcine atrophic rhinitis. The toxin stimulates DNA synthesis, but inhibits division leading to binucleated cells. DNT stimulates actin filament assembly and formation of focal adhesions, suggesting a role of Rho proteins in its action.

Similar cytopathic effects are caused by both cytotoxic necrotizing factors CNF1 and CNF2 from *E. coli* (8). These toxins are proteins of ~ 115 kD of 85% identity, which share some similarity with DNT at their COOH terminus. At the NH<sub>2</sub> terminus, CNFs exhibit ~ 27% identity (80% conserved residues) with the amino acid sequence of *Pasteurella multocida* toxin. CNF is suggested to be important for *E. coli* pathogenicity and is found in up to 20% of *E. coli* strains isolated from diarrhea and up to 50% of *E. coli* strains isolated from extraintestinal infections. The toxins cause tissue damage and death of the animal host. CNFs induce actin polymerization and increase the F-actin content of cells but inhibit cytokinesis and cause formation of multinucleated cells. Moreover, the

toxins induce dramatic formation of membrane ruffles. Because CNF prevents the cytopathic effects induced by ADP-ribosylation or glucosylation of Rho and changes the migration of Rho on SDS-PAGE (as also shown for DNT), it is suggested that CNFs and DNT attack Rho by an unidentified posttranslational modification, thereby activating the GTPase.

#### Role of Rho proteins in bacterial invasion

Many pathogenic bacteria are able to invade eucaryotic cells by processes involving the actin cytoskeleton. Also, Rho proteins appear to participate in these processes. For example, the above-mentioned induction of membrane ruffles by CNFs largely increases bacterial uptake by endocytosis including passive entry of bacteria which are per se not able to invade host cells. *Shigella* induces formation of filopodia-like structures that, finally, engulf the bacterium in an endocytotic process (9). Concomitantly, Rho proteins are recruited into the bacterial entry sites. Inactivation of Rho by C3-induced ADP-ribosylation blocks *Shigella*-induced membrane ruffling and inhibits bacterial entry. Also, invasive *Salmonella* induce membrane ruffling, which is accompanied by macropinocytosis and increased internalization of bacteria. In contrast to the actions of CNFs or *Shigella*, membrane ruffling induced by *Salmonella* appears to be independent of Rho and Rac but a recent report indicates a specific role of Cdc42 in this process (10).

#### Conclusions

Rho subfamily proteins are involved in the regulation of the actin cytoskeleton and are molecular switches in the control of multiple signaling processes of eukaryotic cells, and therefore, these small GTPases may have evolved as powerful targets to optimize bacteria-host interaction. Rho proteins are inactivated by ADP-ribosylation by C3-like exoenzymes or by monoglycosylation catalyzed by large clostridial cytotoxins. They are targets for bacterial modifications by mechanisms (CNFs, DNT) not yet identified resulting in activation of the GTPase. Because a precise control of these molecular switches is essential, activation of Rho proteins by toxins also has dramatic effects for the eukaryotic organism. Additionally, bacteria may manipulate Rho signal pathways by acting not only on

the GTPases themselves but upstream or downstream of the GTPases, thereby using Rho-dependent processes as tools, e.g., for invading the host cells. Thus, Rho proteins play an important role in the interaction of bacteria with their host. Delineation of this interaction is basic for understanding of bacterial pathogenesis. On the other hand, the bacterial toxins are potent and selective tools in cell biology with which the cellular functions of their eukaryotic targets can be studied. Moreover, in respect to the essential roles of Ras and Rho proteins in cell transformation, metastasis, and invasion by tumor cells, Rho/Ras-modifying toxins may have important implications as novel antitumor agents.

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