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Glucosylation of Rho/Ras Proteins by Lethal Toxin – Implications of Actin Re-Organization and Apoptosis in *C. Sordellii*-Associated Disease

Harald Genth*, Florian Schulz and Ingo Just

Institute of Toxicology, Hannover Medical School, D-30623 Hannover, Germany

Abstract: *Clostridium sordellii* causes disease in livestock and life-threatening illnesses in humans. Pathogenic *C. sordellii* strains produce up to seven virulence factors, including lethal toxin (TcsL), hemorrhagic toxin, a hemolysin, a DNAse, a collagenase, and a lysolecithinase cell. TcsL exhibits an A-B toxin-like structure and enters its target cells by receptor-mediated endocytosis. Inside the, TcsL mono-glucosylates low molecular weight GTP-binding proteins of the Ras and Rho families. This article reviews recent progress for (i) re-enforcing (H/K/N)Ras glucosylation and subsequent inhibition of the phosphoinositide 3-kinase (PI3K) / Akt survival signalling pathway as the cause of TcsL-induced apoptotic cell death, and (ii) showing the critical nature of Rac1 glucosylation in the loss of epithelial and endothelial barrier function. Finally, the detection of TcsL-induced glucosylation of Rac1 and (H/K/N)Ras using glucosylation-sensitive antibodies is presented as a new method to track TcsL activity.

Keywords: Myonecrosis, Phagocytosis, Mono-glucosylation, (H/K/N)Ras, Rac.

C. SORDELLII-ASSOCIATED DISEASES IN HUMANS AND LIVESTOCK

Clostridium sordellii is an emerging pathogen in humans and livestock. The severity of *C. sordellii*-associated disease is caused by the organism's ability to grow rapidly and release a variety of soluble virulence factors, which damage host cells. Pathogenic *C. sordellii* produce up to seven virulence factors, including lethal toxin (TcsL), hemorrhagic toxin (TcsH), a hemolysin, a DNAse, a collagenase and a lysolecithinase [1]. Only two of these, TcsL and (to some extent) TcsH, have been extensively studied.

Infection with *C. sordellii* is a rare event in humans, which occurs after trauma, childbirth, and routine gynecological procedures, or intravenous drug abuse [2, 3]. Typical clinical symptoms of infection are nausea, dizziness, lethargy, hypotension, and tachycardia. *C. sordellii* bacteremia occurs in patients with predisposing factors such as malignancy or immuno-suppression and is associated with a high lethality of about 70 %. Patients with *C. sordellii* infections should receive combined surgical and drug treatment. The antibiotics of choice are penicillin, metronidazole, clindamycin, or the new broad-spectrum glycylcycline tigecycline [4].

In livestock, *Clostridium sordellii* has been associated with sudden death in sheep [5] and haemorrhagic enteritis, enterotoxemia, and myonecrosis (gas gangrene) in cattle, sheep, and other ruminants [6, 7].

*Address correspondence to this author at the Institute of Toxicology, Hannover Medical School, D-30623 Hannover, Germany;
Tel: +49 511 532 9168; Fax: +49 511 532 2879;
E-mail: genth.harald@mh-hannover.de

THE BIOLOGICAL ACTIVITY OF *C. SORDELLII* TOXINS IN ANIMAL MODELS

C. sordellii toxins TcsL and TcsH are regarded as the major virulence factors of *C. sordellii*-associated diseases, as both toxins exhibit a remarkably low LD_{50}, ranging from 5 to 50 ng/kg in mice [8]. When intraperitoneally injected into mice, TcsL causes massive extravasation of blood fluid in the thoracic cage, resulting from an increase in lung vascular permeability. Mice exhibit dehydration, an increase in hematocrit, hypoxia, and finally, cardiorespiratory failure [9]. Intra-muscular injection of a sublethal dose of TcsL into mice causes pronounced localized symptoms such as edema, inflammation, myofibril disassembly, and degeneration of skeletal muscle fibres within 24 h. The damage persists for 6 to 9 days and (slowly) regenerates over a period of 60 days [10]. Intradermal injection of TcsL into animals results in local necrosis, progressive edema due to local and systemic vascular permeability, and death [11, 12]. The symptoms observed upon TcsL injection in mice resemble those occurring in *C. sordellii* infections in humans [3].

CLOSTRIDIAL GLUCOSYLATING TOXINS

TcsL and TcsH are structurally and immunologically related to Toxin A (TcdA) and Toxin B (TcdB) from *Clostridium difficile*, the causative agents of the *C. difficile* associated diarrhea (CDAD) [13-15]. Anti-TcsL antibodies cross-react with TcdB, the crucial finding that led to the discovery of the *C. difficile* toxins [16-18]. TcdA/TcdB and TcsH/TcsL have formerly been classified as "Large Clostridial Cytotoxins" [19]. Nowadays, they are referred to as "Clostridial Glucosylating Toxins", due to their inherent glucosyltransferase activity [14]. The "Clostridial Glucosylating Toxins" are single-chained protein toxins with an AB toxin-like structure. The C-terminal delivery domain (B domain) harbors domains required for receptor binding,

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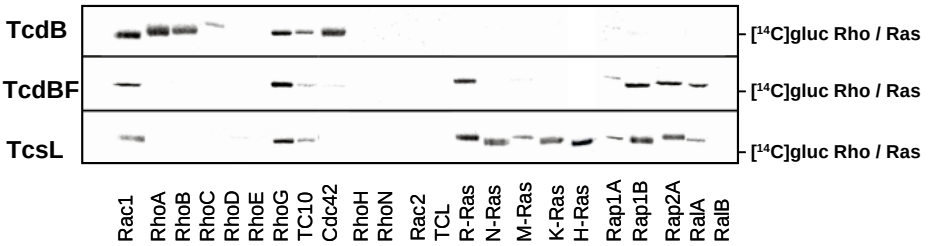

Fig. (1). Differences in the protein substrate specificity of clostridial glucosylating toxins. Rho/Ras proteins purified as GST fusion proteins from *E. coli* were incubated with either TcdB, TcdBF, or TcsL as indicated in the presence of UDP-[^{14}C]glucose for 30 min. Proteins were resolved on SDS-PAGE and [^{14}C]glucosylated Rho/Ras proteins were visualized by autoradiography. TcdB represents a broad spectrum inhibitor of Rho proteins, while TcsL is a broad-spectrum inhibitor of Ras proteins. TcdBF and TcsL differ in their protein substrate with respect to the glucosylation of (H/K/M/N)Ras that are glucosylated by TcsL, but not TcdBF.

membrane translocation, and auto-catalytic processing, allowing the N-terminally located glucosyltransferase domain (A domain) to enter the target cell cytosol by receptor-mediated endocytosis [20, 21]. The structure of the glucosyltransferase domain of TcsL has been solved, leading to its classification as an A family glycosyltransferase [22].

The closest relative of TcsL among the clostridial glucosylating toxins is Toxin B from the "variant" *C. difficile* serotype F strain 1470 (TcdBF), which has been characterized as a functional hybrid between TcdB and TcsL: TcdBF shares the delivery domain with TcdB but the glucosyltransferase domain with TcsL [23, 24].

GLUCOSYLATION OF RHO/RAS PROTEINS BY TCSL

Low molecular weight GTP-binding proteins act as molecular switches, as they cycle between an active GTP-bound conformation and an inactive GDP-bound conformation. Rho and Ras proteins in the GTP-bound form specifically bind to their effector proteins such as kinases, lipases or scaffold proteins, triggering downstream signaling [25].

Rho/Ras proteins are mono-glucosylated by TcsL at a pivotal threonine residue within the effector region. Mono-glucosylation of H-Ras at Thr-35 stabilizes the effector loop in the inactive GDP-bound state, preventing Ras activation and subsequent effector coupling [26, 27]. Glucosylation thus renders Rho/Ras proteins functionally inactive. The substrate spectrum of TcsL in general covers the Rho subtype proteins Rac and Cdc42, as well as the Ras family proteins (H/K/N/R/M)Ras, Rap [1,2], and RalA (Fig. **1**). Although initially suggested to exhibit identical substrate specifites, TcsL and TcdBF differ in their substrate spectra [23, 28]: TcsL glucosylates (H/K/N/M)Ras, while TcdBF does not (Fig. **1**). In contrast, TcdB (Fig. **1**), TcdA, and TcsH (data not shown) specifically glucosylate the Rho family proteins Rho(A/B/C), Rac1, RhoG, TC10, and Cdc42, leading to their classification as broad-spectrum inhibitors of Rho proteins [15, 29].

The glucosylation of either Rac1 or (H/K/N)Ras from TcsL-treated cells can be tracked using the glucosylation-sensitive antibodies Rac1(Mab clone 102) or Ras(Mab clone 27H5) [28, 30, 31]. As mono-glucosylation at Thr-35 blocks binding of either antibody, glucosylation is reflected by (apparently) decreasing levels of either Rac1 or (H/K/N)Ras in immuno-blot analysis (Fig. **2**). The levels of Rac1 and Ras are constant, if they are analysed by immuno-blot applying alternative antibodies Rac1(Mab clone 23A8) or K-Ras(F234) that are not sensitive to glucosylation. These observations confirmed that the decrease is due to glucosylation and not degradation (Fig. **2**). Toxin-induced glucosylation of Rho/Ras proteins by TcsL has been classically tracked using sequential [^{14}C]glucosylation [23, 27, 32]. Detection of Rho/Ras glucosylation using glucosylation-sensitive antibodies helps to avoid working with radioactivity, for which reason this non-radioactive method has been appreciated by many researchers in the toxin field [28, 33-35].

CONSEQUENCES OF TcsL-CATALYSED GLUCOSYLATION OF RHO PROTEINS

Rho proteins are master regulators of the actin cytoskeleton. Treatment of cultured cell lines with TcsL

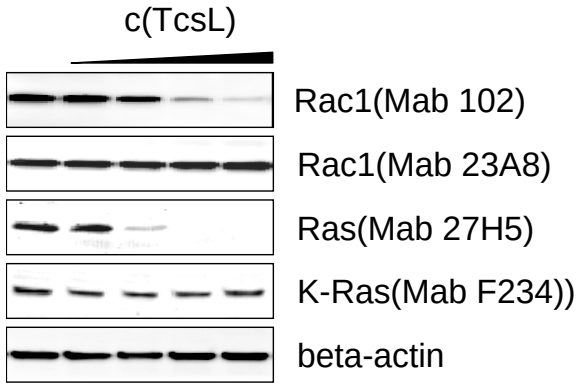

Fig. (2). Detection of the glucosylation of substrate proteins by TcsL by immuno-blot using glucosylation-sensitive antibodies. Rat basophilic leukaemia cells were treated with increasing concentrations of TcsL for 4 h. The cells were lysed and analysed for Rac1 and Ras glucosylation by immuno-blot using the glucosylation-sensitive antibodies Rac1(Mab clone 102) and Ras(Mab clone 27H5) as indicated. Glucosylation is reflected by apparently decreasing levels of either Rac1 or Ras. The decrease was due to glucosylation and not degradation, as the level of Rac1 and Ras were constant, as analysed by immuno-blot applying alternative antibodies Rac1(Mab clone 23A8) or K-Ras(F234) that were not sensitive to glucosylation.

results in the disappearance of actin stress fibres, disorganization of focal complexes, and finally in the complete loss of cell shape i.e. cell rounding ("cytopathic effect"). These cytoskeletal re-arrangements have been attributed to TcsL-induced glucosylation of the Rho-subtype protein Rac [36, 37].

In contrast to the cytopathic effect induced by the related TcdA and TcdB, TcsL-induced cell rounding is accompanied by cell clustering and rapid detachment from the matrix (Fig. **3**). TcsL-induced cell clustering is therefore exploited as a "diagnostic" marker to distinguish between TcdA/TcdB- and TcsL-induced cytopathic effects [38]. The difference in cytopathic features may be based on the additional inactivation of Ras proteins by TcsL [24].

One of the main primary host target tissues of TcsL in livestock is the intestinal barrier formed of epithelial cells linked by intercellular junctions, including apical tight junctions and basolateral adherens junctions [37]. TcsL is suggested to increase the epithelial permeability and drastically perturb adherens junctions. This dysfunction of cell barrier permeability is thought to contribute to increased fluid secretion and diarrhoea observed in *C. sordellii*-associated enteritis in animals [37].

In a mouse model, TcsL is reported to increase vascular permeability in the lung, resulting in massive extravasation of blood fluid in the thoracic cavity [9]. TcsL is suggested to cause perturbation of adherens junctions, with the junction protein VE-cadherin being redistributed from membranes to cytosol [9]. As endothelial (as well as epithelial) barrier function depends on Rac1, its glucosylation by TcsL is the molecular basis for the loss of junctions and barrier function [39].

CONSEQUENCES OF THE TcsL-CATALYSED GLUCOSYLATION OF RAS PROTEINS

Ras proteins regulate cell proliferation and cell survival through a network of signal transduction pathways, including PI3K/Akt, RalGEF/Ral, and Raf/ERK (Fig. **4**) [40]. These pathways predominantly lead to activation or inhibition of transcription factors (e.g. NFkappaB, Elk-1, AFX) that regulate expression of both pro- and anti-apoptotic proteins. Ras glucosylation by TcsL inhibits Raf/ERK [27, 32, 41], RalGEF/Ral [42], as well as PI3K/Akt signaling [28, 43]. Inhibition of (H/K/N)Ras-dependent survival signaling pathways is most likely the basis for TcsL-induced apoptotic cell death, as TcdBF that does not glucosylate (H/K/N)Ras fails to induce apoptosis under identical settings [28].

TcsL induces apoptosis in cultured epithelial, endothelial, or myeloid cells, characterized by the activation of caspases-3/8/9, chromatin condensation and nucleus fragmentation, cytochrome C release, phosphatidylserine exposure, and the reduction of cell viability [28, 43, 44]. In a non-synchronized population of cells, a sub-population of about 30 % of total cells are sensitive to TcsL-induced apoptosis [45]. This property has been reported for other apoptosis-inducing agents and is likely based on the fact that onset of the execution phase of apoptosis is markedly asynchronous across a population of cells [46]. If cells are synchronized using the thymidine double block technique, the complete population of cells is sensitive to TcsL-induced apoptosis [28].

THE CRITICAL ROLE OF PI3K/AKT SIGNALING IN TcsL-INDUCED APOPTOSIS

Many cell-surface receptors mediate the production of second messengers that activate PI3K. PI3K generates phosphorylated phosphatidylinositides (PI-3,4-P2 and PI-3,4,5-P3) in the cell membrane that bind to the amino-terminal pleckstrin homology (PH) domain of the serine/threonine kinase Akt. Activated Akt promotes cell survival through suppression of apoptosis by (i) phosphorylation of the Bad component of the Bad/Bcl-$_{XL}$ complex, (ii) degradation of IKK-alpha that ultimately leads to NF-kB activation and cell survival [47], and (iii) suppression of the pro-apoptotic GTP-binding protein RhoB [48, 49]. As Ras is up-stream of PI3K/Akt signalling, its glucosylation by TcsL results in Akt dephosphorylation (indicative of inhibited PI3K/Akt signaling) and de-suppression of apoptosis [28, 31, 43]. The critical role of PI3K/Akt signalling in TcsL-induced apoptosis has been evaluated using tauroursodeoxycholic acid (TUDCA) as a pharmacological tool. TUDCA is the taurine-conjugate of the endogenous hydrophilic bile acid ursodeoxycholic acid

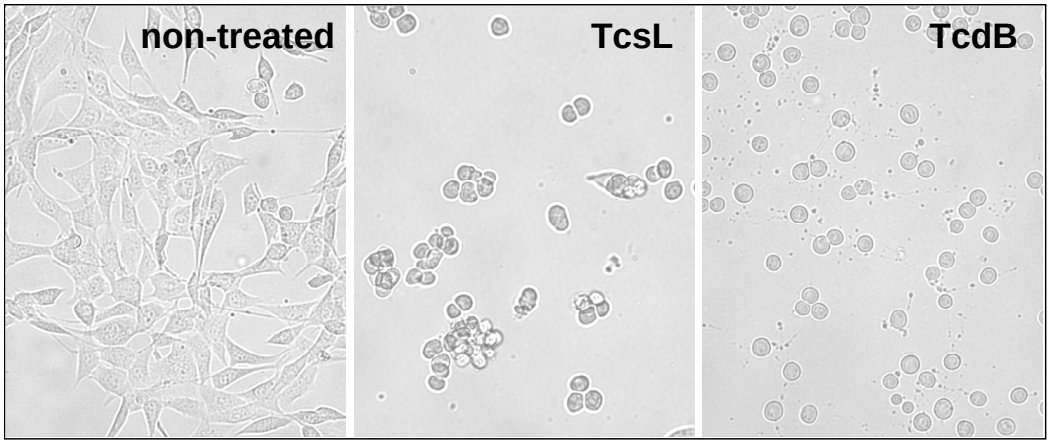

Fig. (3). Differences in the cytopathic effects of the glucosylating toxins. NIH3T3 fibroblasts were treated with TcsL and TcdB or left untreated as indicated for 4 h. Cell rounding was induced by TcdB and TcsL; TcsL-induced cell rounding was accompanied by clustering of the cells. This feature is exploited as a "diagnostic" marker to distinguish between TcsL and TcdB.

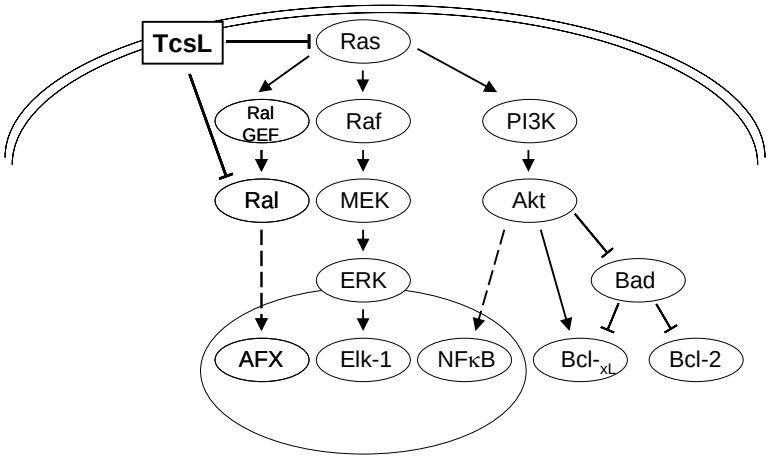

Fig. (4). Effects of TcsL on Ras-dependent signaling pathways. The three best characterized Ras effector proteins are the Ral guanine nucleotide exchange factor (RalGEF), the serine/threonine kinase Raf, and the PI3-K lipid kinases. PI3K/Akt signaling results in activation of the transcription factor NFkB, the inhibition of pro-apoptotic Bad, and the activation of anti-apoptotic Bcl_{xL}. RalGEF activates a Ral-dependent signaling pathway that results in activation of AFX forkhead transcription factor. TcsL-induced glucosylation (i.e. inactivation) of Ras and Ral results (among other effects) in Akt dephosphorylation (indicative of inactivation) and apoptosis induction.

(UDCA). It is an approved drug for the treatment of cholestasis and biliary cirrhosis [50]. TUDCA treatment prevents Akt dephosphorylation and expression of pro-apoptotic RhoB in TcsL-treated cells, indicating that TUDCA preserves PI3K/Akt signalling downstream of Ras glucosylation. As TUDCA prevents TcsL-induced apoptosis, this finding strongly suggests that PI3K/Akt signalling is critical for TcsL-induced apoptosis [28, 31]. It is conceivable that inhibition of either Raf/ERK or RalGEF/Ral signaling might further contribute to TcsL-induced apoptosis but evidence remains to be provided.

PI3K/Akt signaling is linked to the mitochondrial cell death pathway, as Akt phosphorylates the Bad component of the Bad/$Bcl\text{-}_{xL}$ complex (Fig. **4**). Although Bad phosphorylation is not changed in TcsL-treated Hela cells, $Bcl\text{-}_{xL}$ is cleaved in a caspase-dependent manner [43]. This finding does not exclude a critical role of Akt, as Akt may regulate $Bcl\text{-}_{xL}$ independently of Bad [51]. $Bcl\text{-}_{xL}$ cleavage converts the protein into its pro-apoptotic form, which inserts into the mitochondrial membrane and may cause TcsL-induced cytochrome C release [44]. Finally, HL-60 cells stably over-expressing anti-apoptotic Bcl-2 are insensitive to TcsL-induced apoptosis [44], further confirming involvement of the mitochondrial cell death pathway in TcsL-induced apoptosis.

ROLE OF TcsL-INDUCED APOPTOSIS IN *C. SORDELLII*-ASSOCIATED DISEASE

In some cases, *C. sordellii* remains localized to the site of infection [52]. To survive at those sites and to escape eukaryotic phagocytosis, bacteria produce virulence factors that induce apoptosis of professional phagocytotic cells. One example is *Pseudomonas aeruginosa* that injects the effector protein exoenzyme S into phagocytotic cells to kill them [53]. TcsL may exert a comparable activity in *C. sordellii* infection [28]. This hypothesis is supported by the observation that cells from myeloid origin are more susceptible than epithelial or endothelial cells (at least in cell culture) to TcsL-induced apoptosis [44]. Apoptosis of phagocytic cells may further explain why *C. sordellii* infections are often associated with massive necrosis at the sites of infection. Dead cells may remain at sites of infection and liberation of factors from these cells may alert the innate immune system, leading to local inflammation that in turn triggers necrosis, as phagocytosis is blocked.

CONCLUSIONS

The biological activity of TcsL has two important aspects (Fig. **5**):

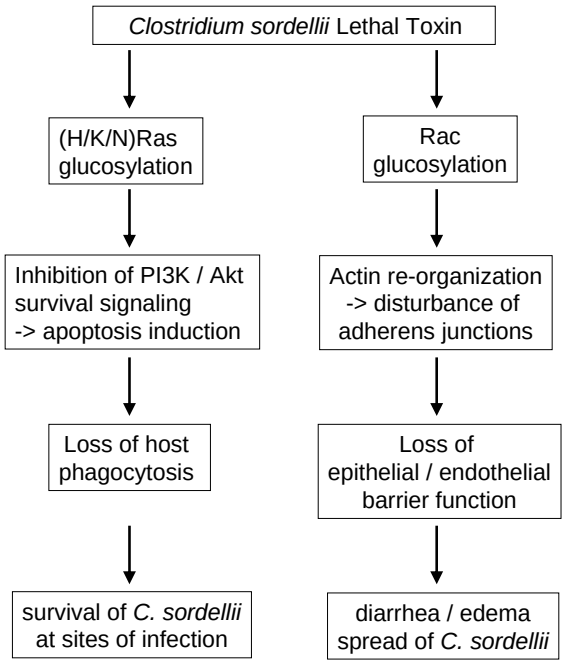

Fig. (5). Model on the functional outcome of Rac1 glucosylation and Ras glucosylation by TcsL in *C. sordellii*-associated disease. TcsL-induced apoptosis that depends on Ras glucoslyation inhibits host phagocytosis to ensure the survival of *C. sordellii* at sites of infection. TcsL-induced actin re-organization that depends on Rac1 glucosylation results in the loss of epithelial / endothelial barrier function.

1) Glucosylation of Rho proteins results in actin re-organization, which results in perturbation of adherens junctions of epithelial and endothelial cells and finally in the loss of barrier function ("cytopathic effects"). This mechanism is the basis of diarrhoea and extravasation of blood fluid in the course of *C. sordellii*-associated disease in livestock.

2) Glucosylation of Ras proteins results in inhibited survival signalling and subsequent induction of apoptotic cell death ("cytotoxic effects"). Especially, induction of apoptosis of phagocytic cells may be interpreted as a strategy of *C. sordellii* to escape eukaryotic phagocytosis and to survive.

ACKNOWLEDGEMENT

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ABBREVIATIONS

PI3K	=	Phosphoinositide 3-kinase
RBL cells	=	Rat basophilic leukemia cells
TcdA	=	Toxin A from *Clostridium difficile* strain-VPI10463
TcdB	=	Toxin B from *Clostridium difficile* strain-VPI10463
TcsH	=	Hemorrhagic toxin from *Clostridium sordellii*
TcsL	=	Lethal toxin from *Clostridium sordellii*
TUDCA	=	Tauroursodeoxycholic acid

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