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Glycosylating Toxin of *Clostridium perfringens*

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1. Introduction

Clostridium perfringens type C strains that produce various toxins cause hemorrhagic noxious ulceration or mucousal necrosis of the small intestine in humans, pigs, cattle and chickens (Sakurai et al. 1997, Sakurai and Nagahama 2006). In humans, the bacteria cause necrotic enteritis, which is termed “pig-bel” (Sakurai and Nagahama 2006). *C. perfringens* has been classified into five types, A to E, according to the toxinogenicity of major extracellular toxins designated alpha-, beta-, epsilon- and iota-toxins. The *C. perfringens* strains defined as type C show alpha- and beta-, but not epsilon- and iota-toxinogenicities (Sakurai and Nagahama 2006). Type C strains produce alpha-toxin, beta-toxin, beta2-toxin, and perfringolysin O. Beta-toxin is known to be the primary pathogenic factor of necrotic enteritis in the type C strains (Tweten 2005, Sakurai and Nagahama 2006). Beta2-toxin is a toxin discovered in *C. perfringens* type C isolated from piglets with necrotic enteritis, and speculated to be important because its gene has been detected in most *C. perfringens* type C strains recovered from animals with clinical disease (Manteca et al. 2002, Waters et al. 2003).

Most of the toxins produced by *C. perfringens* type C are toxic to particular cells or cell lines. Beta-toxin possesses lethal, dermonecrotic, and pressor activities. Direct evidence concerning the biological activities of beta-toxin had been lacking due to the absence of a susceptible cell line for experiments *in vitro*. However, a cell line that is susceptible to the toxin was found (Nagahama et al. 2003). The toxin induced swelling and lysis on HL60 cells by binding to lipid rafts and forming a functional oligomer. Beta2-toxins is lethal for mice and cytotoxic for the cell line I407 cells inducing cell rounding and lysis without affecting the actin cytoskeleton (Gibert et al. 1997). On the other hand, delta-toxin is toxic to various rabbit immune cells, i.e. alveolar macrophages, peritoneal appendix cells, bone marrow cells, splenocytes and thymocytes (Jolivet-Reynaud et al. 1982). Theta-toxin is the prototypic member of the cholesterol-dependent cytolysin (CDC) family that includes listeriolysin O (LLO), streptolysin O (SLO), pneumolysin, and others (Tweten 2005). Theta-toxin exhibits cytotoxic effects on macrophages to escape from phagosomes in consort with alpha-toxin. Alpha-toxin is essential for growth and spread of infection in the host (Sakurai et al. 2004),

and it helps *C. perfringens* avoid the host defense mechanism by altering the normal traffic of the host phagocytes (Ochi et al. 2002, Oda et al. 2006). The role played by alpha-toxin in pathogenesis is dictated by its ability to interact with membranes, whether from outside the cell or while inside the phagosomes (Naylor et al. 1998). Alpha-toxin possesses phospholipase C (PLC), sphingomyelinase (SMase) and biological activities causing hemolysis, lethality and dermonecrosis (Sakurai et al. 2004). On the other hand, enterotoxin, which has molecular masses of 35 kDa as a monomer (Duffy et al. 1982) and 90–200 kDa as aggregate forms with eukaryotic proteins (Singh et al. 2001), is known to be highly cytotoxic to Vero and Caco-2 cells (Gao and McClane 2012). Among the *C. perfringens* type C toxins reported previously, only enterotoxin is toxic to Vero cells, and no toxin is known to have a molecular mass of around 200 kDa without eukaryotic proteins.

TpeL, a recently-described novel member of the family of large clostridial cytotoxins, was found in *C. perfringens* type C. TpeL (named TpeL: *C. perfringens* large cytotoxin) is a truncated homologue of *Clostridium difficile* TcdA and TcdB (Amimoto et al. 2007). TpeL was identified in the culture supernatant of *C. perfringens* strain CP4 and is thought to be associated with necrotic enteritis (Amimoto et al. 2007). Coursodon et al. (2012) reported that TpeL may potentiate the effect of other virulence attributes of necrotic enteritis caused by *C. perfringens*.

2. The family of clostridial glucosylating toxins

Cytosolic mono-O-glucosylation is an important molecular mechanism by which various bacterial protein toxins and effectors target eukaryotic cells. *C. difficile* TcdA and TcdB, *Clostridium sordellii* lethal toxin (TcsL) and *Clostridium novyi* alpha-toxin (TcnA) are important pathogenic factors of the family of large clostridial toxins (LCTs). The pathogenicity of *C. difficile* is based upon the action of at least one of the two major exotoxins (TcdA and TcdB) (Voth and Ballard 2005). TcdA and TcdB are the main cause of antibiotic-associated diarrhea and pseudomembranous colitis (Voth and Ballard 2005), as a consequence of treatment with antibiotics, which destroy the normal microflora of the gut and allow colonization and proliferation of *C. difficile* bacteria (Bartlett et al. 1977, Kelly and LaMont 2008). Although the precise pathogenic mechanisms of induction of diarrhea and colitis are not known, it is generally accepted that the toxin-induced glucosylation of Rho GTPases is central to the action of the *C. difficile* toxins (Kelly and LaMont 2008). TcsL is implicated in toxic shock syndrome after medical-induced abortion (Ho et al. 2009) and TcnA causes gas gangrene syndrome (Tsokos et al. 2008). All these toxins are 50 to 90% identical in their amino acid sequences. They are large proteins of 250 to 308 kDa. This family has now more than 30 members, including putative glycosyltransferases from *C. perfringens*, *Escherichia coli*, *Citrobacter rodentium*, *Photobacterium profundum*, *Pseudomonas fluorescens* and various species of *Chlamydia* and *Chlamidophila*.

2.1. Structure of LCTs

LCTs are single protein chains containing four functional domains and share 26 to 76 % sequence identity and are structurally and functionally organized (Busch and Aktories

2000). LCTs are composed of four domains, the glucosylating enzymatic A-domain, the autocatalytic processing C-domain, the translocating D-domain and the binding B-domain (Fig. 1). The one third C-terminal part 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. Related carbohydrates could be involved as TcdA receptor. The gp96, a member heat shock protein family, has been proposed to bind TcdA to the plasma membrane of enterocytes (Na et al. 2008). Because this B-domain exhibits sequence similarity to the carbohydrate binding region of the glucosyltransferase from *Streptococcus mutans*, it was suggested early on that this part of the toxin is involved in binding to a carbohydrate-containing receptor. The crystal structure of the C-terminal binding domain of

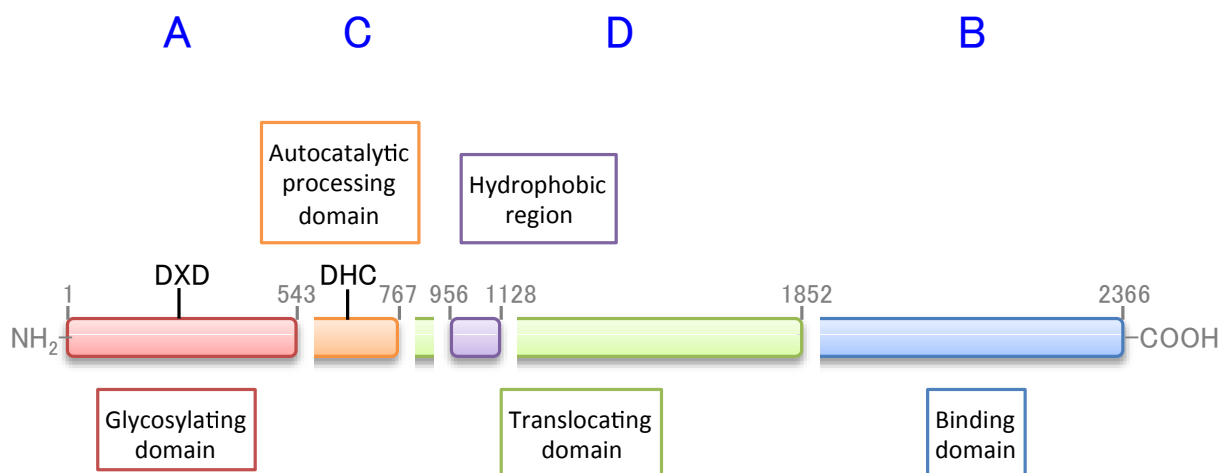


Figure 1. Domain organization of clostridial glucosylating toxins.

Clostridial glucosylating toxins are composed of four domains, the glucosylating enzymatic domain (A-domain), the autocatalytic processing domain (C-domain), the translocating domain (D-domain) and the binding domain (B-domain). The A-domain has the glucosyltransferase activity. The B-domain containing polypeptide repeats is responsible for specific receptor-binding. The C-domain participates in the auto-catalytic cleavage of LCTs and is a cysteine-protease with the catalytic residues DHC. InsP_6 is required for activation of the cysteine protease. The D-domain is likely involved in the delivery of the A-domain into the cytosol. This domain includes a hydrophobic region suggested to be important for insertion of the toxin into endosome membranes.

TcdA has been determined (Ho et al. 2005, Greco et al. 2006), showing a solenoid-like structure with 32 repeats consisting of 15–21 amino acid residues and seven repeats consisting of 30 residues. The repeats form β -hairpins, arranged in pairs with each adjacent pair of hairpins rotated by 120° to the next pair, resulting in a screw-like structure of a left-handed β -solenoid helix (Greco et al. 2006). Co-crystallization with a derivate of the trisaccharide α -Gal(1,3) β -Gal(1,4) β GlcNAc confirmed the carbohydrate binding capacity of the domain. In this complex there are two carbohydrate-binding regions. However, in the full-length C-terminal fragment there are seven of these potential binding domains that are

highly conserved, giving it a high binding capacity. Although there is little information about the binding domain of TcdB, it is believed that TcdB uses different receptors to bind to target cell surfaces than TcdA (Jank et al. 2007a).

The cysteine protease C-domain is located between residues 543 and 769 in TcdA and between residues 543 and 767 in TcdB (Egerer et al. 2007, Giesemann et al. 2008). It was shown that cleavage of the toxin occurs auto-catalytically by a cysteine protease activity, which is harbored in the C-domain, covering residues 544–955, directly down-stream of the glucosyltransferase domain (Egerer et al. 2007, Giesemann et al. 2008). Cys-698 and His-653 have been shown to be part of the catalytic dyad, which in addition to Asp-587 might participate in the auto-cleavage reaction (Egerer et al. 2007). The crystal structure of C-domain (543–809) from TcdA was resolved in the presence of inositol hexakisphosphate (InsP₆) (Pruitt et al. 2009). InsP₆ binds to the C-Domain, causing a conformational change that activates the auto-catalytic activity (Egerer et al. 2009). This locates between the enzymatic A-domain and the delivery D-domain, playing a role in proteolytic cleavage of the toxin. The toxins undergo autoproteolysis allowing only the enzymatic A-domain to be released into the cytosol in the presence of InsP₆. Once the target cell has taken up the LCT via receptor-mediated endocytosis at the B-domain, the toxin undergoes autoproteolysis in order to allow the A-domain to pass across the endosomal membrane into the cytosol (Kreimeyer et al. 2011).

D-domain, which is located between residues 955 and 1852, is a large hydrophobic region that makes up almost 50% of the total size of the toxin (Barth et al. 2001, Qa'Dan et al. 2000). However, the exact function of D-domain is unknown. It is characterized by a hydrophobic stretch which is most probably responsible for membrane penetration (transmembrane prediction) (von Eichel-Streiber et al. 1992). Therefore, this region is speculated to as the translocation domain. Deletion studies proved the importance of the hydrophobic region for toxin activity (Barroso et al. 1994). A small region in the primary sequence between residues 965 and 1128 is characterized by hydrophobic amino acids and is suggested to play a role in formation of transmembrane structure during pore formation and translocation of the toxin into the cytosol (Voth and Ballard 2005). Pore formation induced by the toxin has been shown in artificial lipid membranes (Barth et al. 2001, Giesemann et al. 2006). However, so far it is not clear how pore formation relates to the delivery of the toxin into the cytosol.

The biologically active domain, A-domain, harboring the glucosyltransferase activity, is translocated into the cytosol, comprises the first 543 aa (Pfeifer et al. 2003, Rupnik et al. 2005). Therefore, cleavage of the toxin is required. The 3D-structure of A-domain showed that it was closely related to other bacterial glucosyltransferases belonging to the glucosyltransferase A family (Reinert et al. 2005). The catalytic core consists of 234 aa and is formed by a mixed α/β -fold with mostly parallel β -strands as the central part. The more than 300 additional residues are mainly helices, of which the first four N-terminal helices are most probably involved in membrane association, therefore assuring close proximity of the enzyme with its substrates. The structure of the central core is similar to that of glucosyltransferase A family (Reinert et al. 2005, Ziegler et al. 2008). Characteristic for glucosyltransferase A family members is the DXD motif involved in complexation of

manganese ions, UDP and glucose. Mutation of these essential aspartate residues leads to inactivation of the toxin (Giesemann et al. 2008). The first aspartic residue of the DXD motif binds to ribosyl and glucosyl moieties of UDP-glucose and the second aspartic residues binds to divalent cation (mainly manganese ions) which increases the hydrolase activity and/or the binding of UDP-glucose (Just et al. 2000). Other amino acids in TcdB having an essential role in the enzymatic activity have been identified such as Trp-102 which is involved in the binding of UDP-glucose, Asp-270, Arg-273, Tyr-284, Asn-384, Trp-520, as well as Ile-383 and Glu-385 being important for the specific recognition of UDP-glucose (Busch et al 2000a and 2000b, Jank et al. 2005, Jank et al. 2007a,). Differences in α -helices probably account for the substrate specificity of each toxins (Reinert et al. 2005, Ziegler et al. 2008). Chimeric molecules between TcdB and TcsL have been used to identify the sites of Rho-GTPase recognition. Amino acids 408 to 468 of TcdB ensure the specificity for Rho, Rac and Cdc42, whereas in TcsL, the recognition of Rac and Cdc42 is mediated by residues 364 to 408, and that of Ras proteins by residues 408 to 516 (Voth and Ballard 2005, Jank et al. 2007a). The four N-terminal helices which mediates the binding of TcsL to phosphatidylserine, are possibly involved in membrane interaction (Mesmin et al. 2004). Amino acids 22-27 of Rho and Ras GTPase which are part of the transition of the α 1-helix to the switch 1 region, are the main domain recognized by the glucosylating toxins (Müller et al. 1999). The cosubstrate for the bacterial glucosyltransferases is UDP-glucose; only TcnA utilizes UDP-N-acetylglucosamine (UDP-GlcNAc) (Selzer et al. 1996). This difference in cosubstrate specificity is based on sterical hindrance by bulky amino acids (e.g. Ile383/Gln385 in TcdB) blocking the catalytic pocket for the larger UDP-GlcNAc. In TcnA, small serine and alanine residues at the corresponding positions allow UDP-GlcNAc to enter the catalytic cleft (Jank et al. 2005). Little is known so far about the molecular/structural determinants underlying the differences in substrate recognition by different glucosylating toxins.

2.2. Internalization of LCTs

LCTs enter eukaryotic target cells through receptor-mediated endocytosis according to the 'short trip model' of bacterial exotoxin uptake (Sandvig et al. 2004). The cytotoxicity of the toxins are blocked by endosomal and lysosomal acidification inhibitors (monensin, bafilomycin A1, ammonium chloride) and the inhibiting effects can be by-passed by an extracellular acidic pulse (Popoff et al. 1996, Qa'Dan et al. 2000, Barth et al. 2001, Popoff and Bouvet 2009). As shown in Fig. 2, on binding to host cell receptors (Karlsson 1995, Giesemann et al. 2008), the toxins are endocytosed (Voth and Ballard 2005). After endocytosis, the acidification of early endosomes by the vesicular H⁺-ATPase induces a conformational change characterized by an increase in hydrophobicity, leading to membrane insertion (Qa'Dan et al. 2000, Barth et al. 2001, Voth and Ballard 2005). It reported that at low pH, LCTs induce channel formation in cell membranes and artificial lipid bilayers (Qa'Dan et al. 2000 and 2001, Giesemann et al. 2006). Membrane cholesterol seems critical for TcdA pore formation (Giesemann et al. 2006). Then, the hydrophobic region enables to form a pore through which the catalytic domain can translocate into the cytosol. Pore formation under acidic conditions has been demonstrated for TcdA and B (Barth et al. 2001,

Gieseemann et al. 2006). The exact mode of translocation remains to determine. The translocation-ligand domain remains associated with endosomal membranes and only the catalytic-DXD domain penetrates into the cytosol (Pfeifer et al. 2003, Rupnik et al. 2005). The N-terminal catalytic domain (A-domain) is then delivered from the early endosomes into the cytosol by auto-proteolytic activity stimulated by InsP_6 (Reineke et al. 2007, Egerer et al. 2007). This autoproteolytic activity is induced by InsP_6 and/or dithiothreitol and is responsible for the separation of the catalytic domain from the holotoxin (Egerer et al. 2007, Reineke et al. 2007). A cysteine protease domain (C-domain containing putative catalytic residues, DHC) has been identified close to the cutting site in TcdB (amino acid 544-955), which is conserved in all LCTs (Egerer et al. 2007, Reineke et al. 2007, Egerer et al. 2009). Cys-700, His-655 and Asp-589 have been identified as the catalytic triad. It reported that a cysteine protease catalytic triad is involved in processing of the toxin and auto-cleavage is essential for toxin activity (Egerer et al. 2007). Following its translocation and release, the catalytic-DXD fragment (A-domain) acts on its cytosolic targets, the GTPases of the Rho/Rac family, leading to the observed blockade of signal transduction processes and, consequently, the disaggregation of the cytoskeleton and cell death (Just and Gerhard 2004, Belyi and Aktories 2010). TcdA and B target Rho GTPases (Rho, Ras and Cdc42), which are molecular switches involved in numerous signal processes, in particular, the regulation of the actin cytoskeleton. Once the toxins enter the cytosol, they catalyse the addition of UDP-Glc (UDP-glucose) to Thr-37 (monoglucosylation) in Rho GTPase leading to depolymerization of actin filaments, disruption of the cytoskeleton and eventually cell rounding and cell death (Jank et al. 2007b, Belyi and Aktories 2010) (Fig. 2).

2.3. Glucosylation of Rho GTPase by LCTs

In the cytosol, LCTs glucosylate small GTPases of the Rho and Ras superfamily (Popoff et al. 1996, Selzer et al. 1996, Belyi and Aktories 2010). Small GTP-binding proteins involve organization of the cytoskeleton and control cellular activity of numerous other cellular enzymes. Rho proteins are molecular switches involved in various signal processes, including actin cytoskeleton regulation, cell cycle progression, gene transcription, and control of the activity of many enzymes like protein and lipid kinases, phospholipases, and nicotianamide adenine dinucleotide-oxidase (Etienne-Manneville and Hall 2002, BurrIDGE and Wennerberg, 2004). In respect to their role in host-pathogen interactions, Rho proteins essentially participate in epithelial barrier functions and cell-cell contact, in immune cell

LCTs bind with their B-domain to the receptor of target cells. After endocytosis, the toxin inserts into the endosome membrane most likely involving the hydrophobic part of the D-domain. The acidic pH of the endosome triggers the first conformational change and results in pore formation of the ligand-translocation domain. Cytosolic InsP_6 interacts with the cysteine protease C-domain and induces a second conformational change, activating the protease function. This results in cleavage of the toxin and release of the glucosyltransferase A-domain into the cytosol. In the cytosol, Rho GTPases are glucosylated and thereby inactivated. Inactive Rho can not interact with a numerous variety of effectors and induce multiple signaling events.

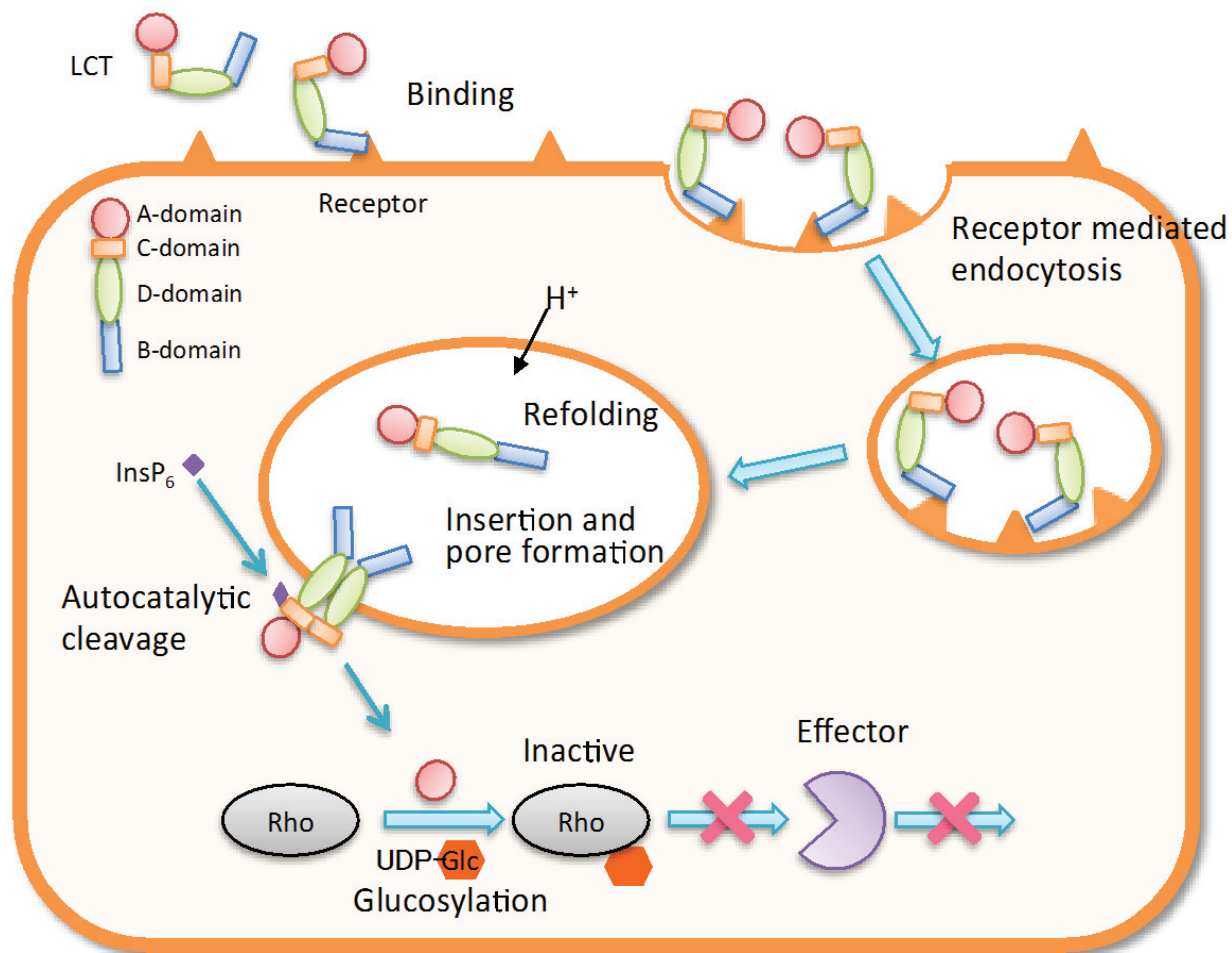


Figure 2. Model of entry and intracellular modification of LCTs

Migration, phagocytosis, cytokine production, wound repair, immune cell signaling, and superoxide anion production. Modification of small GTP-binding proteins by LCTs arises at a Thr-35/37, depending on the Rho GTPase isoforms (Fig. 3) (Belyi and Aktories 2010). Marked differences in substrate specificity have been recognized among the various LCTs. TcdA and B glucosylate Rho, Rac and Cdc42 at Thr-37, whereas TcsL glucosylates Ras at Thr-35, Rap, Ral and Rac at Thr-37 and TcsH glucosylates Rho, Rac, Cdc42 (Fig. 4). LCTs catalyze the glucosylation of 21 kDa small GTP-binding proteins from UDP-glucose, except TcnA which uses UDP-N-acetylglucosamine as cosubstrate. TcnA glucosylates Rho, Rac, Cdc42 (Fig. 4). LCTs cleave the cosubstrate and transfer the glucose moiety to the acceptor amino acid of the Rho proteins (Popoff et al. 1996, Popoff and Bouvet 2009, Belyi and Aktories 2010) (Fig. 3). The conserved Thr, which is glucosylated, is located in switch 1. Thr-35/37 is involved in the coordination of Mg²⁺ and subsequently to the binding of the two phosphates of GTP. The hydroxyl group of Thr-35/37 is exposed to the surface of molecule in its GDP-bound

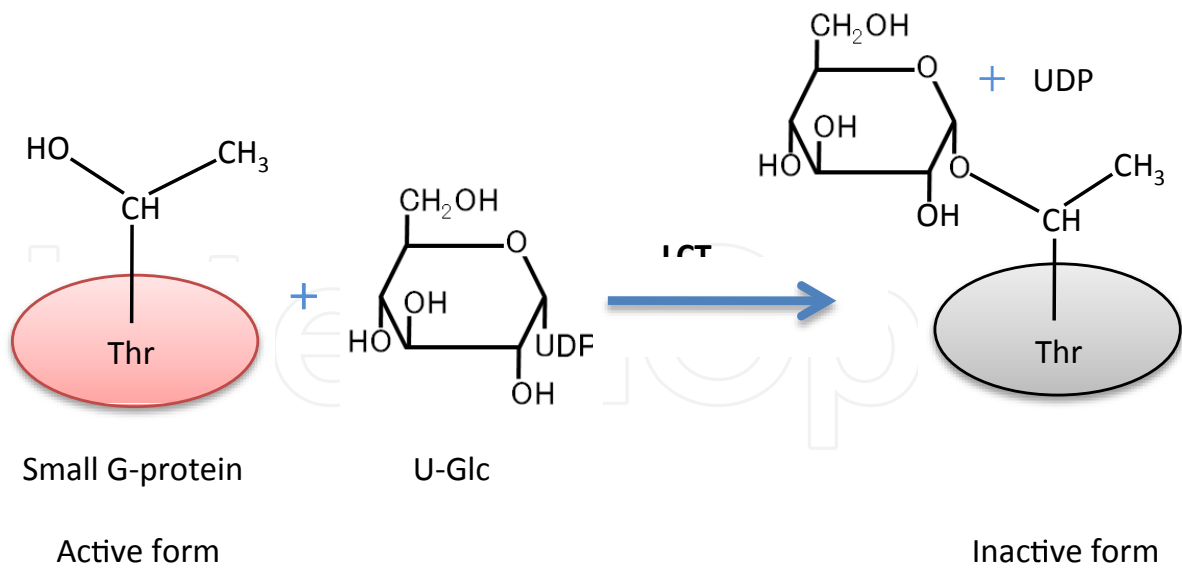


Figure 3. Model of glucosyltransferase activity of LCTs.

LCT	MW(kDa)	Sugar donor	Protein substrate
TcdA	308	U-Glc	Rho, Cdc42, Rac1
TcdB	270	U-Glc	Rho, Cdc42, Rac1
TcsL	271	U-Glc	Rac1, H-Ras, Rap, Ral
TcsH	300	U-Glc	Rho, Rac, Cdc42
TcnA	250	U-Glc, U-NAG	Rho, Rac, Cdc42
TpeL	191	U-Glc, U-NAG	Rac1, H-Ras, Rap, Ral

Figure 4. Protein substrates and cosubstrates of the LCTs.

TcdA: *Clostridium difficile* toxin A, TcdB: *Clostridium difficile* toxin B, TcsL: *Clostridium sordellii* lethal toxin, TcsH: *Clostridium sordellii* hemorrhagic toxin, TcnA: *Clostridium novyi* alpha-toxin, TpeL: *Clostridium perfringens* large cytotoxin. U-Glc: UDP-glucose, U-NAG: UDP-N-acetylglucosamine form, which is the only accessible substrate of LCTs. Glucosylation of Rho or Ras GTPases inhibits activation of the GTPases by GEFs and blocks interaction with their effectors (Sehr et al. 1998, Vetter et al. 2000) as well as the cycling of Rho GTPases between the membrane localization and cytosolic localization (Belyi and Aktories 2010). Glucosylated Rho proteins are located at the membrane. Most importantly, the toxin-induced glucosylation inhibits the active conformation of Rho/Ras GTPases (Vetter et al. 2000, Geyer et al. 2003). Glucosylation of Thr-35 completely

prevents the recognition of the downstream effector, blocking the G-protein in the inactive form (Popoff and Bouvet 2009).

2.4. Cellular effects of LCTs.

The inactivation of Rho proteins by LCTs-induced glucosylation causes extensive morphological changes, with loss of actin stress fibers, reorganization of the cortical actin, disruption of the intercellular junctions and thus increase in cell barrier permeability. The actin cytoskeleton of intoxicated cells is redistributed, causing shrinking and rounding up of most cell types, which is initially accompanied by the formation of neurite-like retraction fibers. Finally, the retraction fibers disappear and the cells detach from the dishes (Ottlinger and Lin 1988, Popoff and Bouvet 2009). Inactivation of Rac assumes to be critical player in disorganization of actin cytoskeleton (Halabi-Cabezon et al. 2008). Numerous other cellular responses to the inactivation of Rho and Ras proteins by LCTs have been described, which are all caused by inhibition of the various functions of the small GTPase. They include inhibition of secretion (Prepens et al. 1996), phospholipase D activity (Schmidt et al. 1996), apoptosis (Brito et al. 2002, Voth and Ballard 2005), chemoattractant receptor signaling (Servant et al. 2000), phagocytosis (Caron and Hall 1998) and alteration of endothelial barrier function (Hippenstiel et al. 1997). However, the role of LCTs on pathogenesis including diarrhea and pseudomembranous colitis are still unknown.

Tissue damage and inhibition of the barrier function of enterocytes might explain the fluid response in toxin-induced diarrhea. TcdA and B affect the morphology and function of tight junctions and associated proteins (ZO-1, ZO-2, occludin, claudin) to decrease transepithelial resistance, whereas E-cadherin junctions show little alteration (Nusrat et al. 2001, Chen et al. 2002, Aktories and Barbieri 2005). F-actin reconstruction is accompanied by the dissociation of occluding, ZO-1 and ZO-2 from lateral tight junctions without affecting adherens junctions. These data indicate that Rho proteins play an important role in tight junction regulation. On the other hand, TscH, which mainly modifies Rac, alter the permeability of intestinal cell monolayers causing a redistribution of E-cadherin whereas tight junctions are not significantly affected (Boehm et al. 2006).

TcdA and B induce apoptosis as a consequence of Rho glucosylation and caspase activation (Nottrott et al. 2007, Gerhard et al. 2008) or possibly cell necrosis (Genth et al. 2008). TcdB and TscH cause apoptosis by targeting mitochondria (Petit et al. 2003, Matarrese et al. 2007). In addition, the inactivation of Rho blocks various cellular functions including exocytosis, endocytosis, activation of lymphocyte, phagocytosis in macrophages, control of NADPH oxidase, activation of phospholipase D, contraction of smooth muscle, activation of the pro-apoptotic RhoB, and transcriptional activation via JNK/or p38 (Just and Gerhard 2004, Gerhard et al. 2005, Huelsenbeck et al. 2007, Popoff and Bouvet 2009). TcdA and B induce a large inflammatory response in the gut. There is massive infiltration of neutrophils, and release of many cytokines (Jefferson et al. 1999).

TcdA-caused p38 activation induces the production of IL-8 and IL-1 β , necrosis of monocytes, and inflammation of intestinal mucosa (Popoff and Bouvet 2009). TcsL glycosylates Ras and inhibits the MAP-kinase cascade and PLD regulation. On the other hand, the role of those inhibition on cytotoxicity is still unclear (Schmidt et al. 1998, El Hadj et al. 1999).

3. Characterization of TpeL

C. perfringens type C has been identified as a causative agent of necrotizing enterocolitis associated with diarrhea and dysentery in infant animals (Tweten 2005, Sakurai and Nagahama 2006). Type C strain produces various toxins responsible for the pathogenesis (Sakurai and Nagahama 2006). Amimoto et al. (2007) fractionated the culture filtrate of *C. perfringens* type C strain MC18, and they discovered an unknown toxin (TpeL) that was lethal to mice. The toxin was cytotoxic to Vero cells, and its molecular mass was estimated to be about 180 kDa by SDS-PAGE analysis (Amimoto et al. 2007). These characteristics completely differed from the previously reported toxins. TpeL was purified with HPLC and affinity column coupled with the mAb. Coursodon et al. (2012) reported that TpeL-producing strains of *C. perfringens* type A are highly virulent for broiler chicks. Paredes-Sabja et al. (2011) reported that TpeL is also expressed during sporulation and is a sporulation-regulated *C. perfringens* toxin.

3.1. Toxicity of TpeL

TpeL was lethal to mice and toxic to Vero cells (Amimoto et al. 2007). The lethal activity of the purified TpeL in mice was determined as 62 MLD/mg (one was 16 μ g) and 91 LD₅₀/mg (one LD₅₀ was 11 μ g) by intravenous injection (Amimoto et al. 2007). TpeL showed obvious cytotoxicity in Vero cells, and the specific activity was 6.2×10^5 CU/mg (one CU was 1.6 ng) (Amimoto et al. 2007). Morphological changes induced by TpeL in Vero cells. The cytopathic effect induced by a low dose of TpeL was characterized by the enlargement of cells and appearance of rounded cells. Vero cells treated with a high dose of TpeL initially manifested similar changes to those treated with the low dose, then formed aggregates, and eventually detached from the well surface (Amimoto et al. 2007).

Vero cells were incubated with SLO (100 ng/ml) alone (A) or a combination of TpeL1-525 (10 μ g/ml) with SLO (100 ng/ml) (B) at 37 °C for 15 min. Pictures were taken after 120 min of resealing. (C) Vero cells were incubated with various amounts of TpeL1-525 with SLO (100 ng/ml) at 37 °C for 15 min. After 120 min of resealing, pictures of cells were taken and the percentage of rounded cells was determined. Value of three experiments were given a mean \pm the standard deviation (SD).

To clarify the biological activity of TpeL, we prepared a recombinant glycosyltransferase domain, TpeL1-525 (covering amino acids 1 to 525) because native TpeL is labile and is difficult to purify from the culture supernatant of *C. perfringens* type C, and the recombinant

full-length TpeL was poorly expressed in *Escherichia coli* (Nagahama et al. 2011). As TpeL1-525 did not possess binding domain, we used the streptolysin O (SLO) delivery systems (Nagahama et al. 2011). As shown in Fig. 5(B), in the presence of SLO, TpeL1-525 caused the cell rounding like the native toxin. TpeL1-525 at 1 – 10 µg/ml in the presence of SLO induced cell rounding in a dose-dependent manner (Fig. 5(C)). The cells finally detached from the wells. On the other hands, the cells eventually detached from the well. Furthermore, when TpeL1-525 at a concentration of 1 to 10 µg/ml was delivered to the cells by SLO, cell viability decreased in a dose-dependent manner. The cytotoxicity was inhibited by the anti-TpeL antibody, and heat-inactivated TpeL1-525 was not cytotoxic. On the other hand, TpeL1-525 by itself did not have the cytotoxic effects. The results indicated that the N-terminal region of TpeL plays a role in the cytotoxicity and the C-terminal region is responsible for the binding of cells. The morphological alteration of cultured cells induced by TpeL is similar to that caused by TcdB and TcsL.

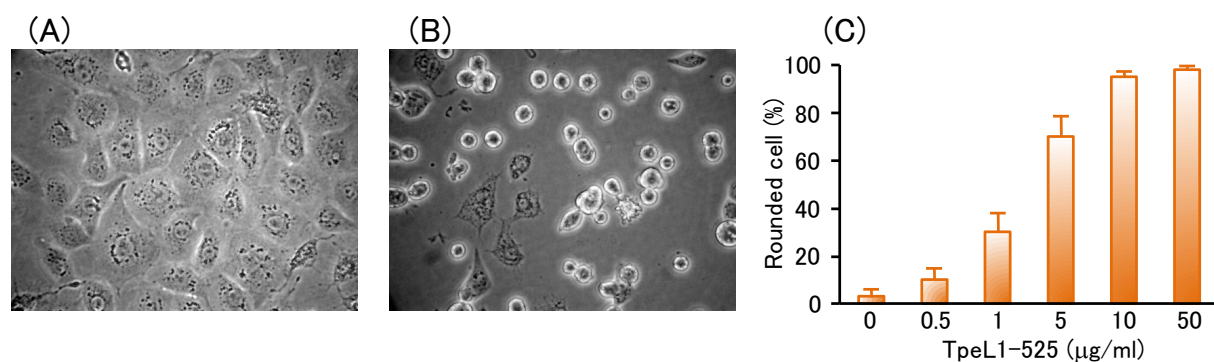


Figure 5. Morphological changes of Vero cells upon treatment with SLO plus TpeL1-525.

3.2. *tpeL* gene

The gene encoding TpeL revealed the presence an ORF of 4953 bases (Amimoto et al. 2007). The *tpeL* gene encoded 1651 amino acid residues and the molecular mass of TpeL calculated from the deduced amino acid sequence was 191 kDa. A signal peptide region was not found within the ORF. The deduced amino acid sequence shared homology with TcdA, TcdB, TcsL and TcnA, called LCTs. The homology scores were 39 % to TcdA, 38 % to TcdB, 39% to TcsL and 30% to TcnA. The amino acid sequence of TpeL is shorter than that of any of these toxins, and the homologous region was located at the N-terminal site of the LCTs. A DXD motif in N-terminal region of LCTs is essential for glycosyltransferase activity, and W-102 in TcsL is an essential amino acid residue for the enzyme activity (Busch et al. 2000a). TpeL conserved the DXD motif and W-102 of TcsL. However, the C-terminal carbohydrate-binding sites of LCTs (von Eichel-Streiber et al. 1992) were not conserved.

The *tpeL* gene was detected not only in type C strains isolated in recent years but also ATCC 3626, a type B strain preserved for many years. It therefore suggest that the *tpeL* gene has been conserved in *C. perfringens* DNA for a long time. Interestingly, beta-toxin-gene-positive strains completely coincided with *tpeL*-positive strains among the 18 strains examined

(Amimoto et al. 2007). Complete chromosomal and plasmid sequences of *C. perfringens* type A strain 13 are available (Shimizu et al. 2002). Also, there is no *tpeL* gene sequence within the data. It has been pointed out that beta- and epsilon-toxin genes carried by plasmids are sometimes lost during the passage of the strains (Katayama et al. 1996, Gibert et al. 1997). So, when the strain loses the plasmids, it changes to type A. In genetic study, total DNAs were used in the cloning and detection of the *tpeL* gene (Amimoto et al. 2007). Sayeed et al. (2010) reported that *tpeL* gene is located approximately 3 kb downstream of the plasmid-borne *cpb* gene. Gurjar et al. (2010) also reported that *tpeL* gene is localized to the plasmids containing *cpb* gene of *cpe*-negative type C isolates.

3.3. Glucosylation of small G proteins by TpeL

Rac1 is the only substrate GTPase inactivated by all LCTs. When the cells were treated with TpeL in the presence of SLO, glycosylation of cellular Rac1 was confirmed by Western blotting with the glycosylation-sensitive anti-Rac1 (Mab102) (Genth et al. 2006, Nagahama et al. 2011). TpeL and TcsL (Voth and Ballard 2005) act on Rac1 and the Ras subfamily but not RhoA. Furthermore, the isomeric TcdB from the variant *C. difficile* serotype F strain 1470 (TcdBF) that glucosylates Rac1 but not RhoA, has a cytopathic effect (Jank et al. 2007b). Halabi-Cabezón et al. (2008) reported that the glucosylation of Rac1 rather than RhoA correlates with the cytopathic effect of TcdB. It has been reported that Rac1 plays a critical role in the organization of the actin cytoskeleton (Bosco et al. 2009). These results strongly suggest that glycosylation of Rac1 is critical for the cytopathic effect of TpeL.

TpeL uses UDP-Glc and UDP-GlcNAc as sugar donors (Nagahama et al. 2011). All other LCTs use a single UDP-hexose (Belyi and Aktories 2010). The crystal structure provides evidence that two amino acids in the vicinity of the catalytic cleft are responsible for the specificity (Jank et al. 2005). TcdA and B, which both use UDP-Glc, have isoleucine and glutamine in the equivalent positions (Ile-383 and Gln-385 in TcdB), whereas TcnL, which uses UDP-GlcNAc, has serine and alanine residues at the respective positions (Jank et al. 2005). It has been reported that the bulkier side chains of Ile-383 and Gln-385 in TcdB limit the space of the catalytic cleft for the binding of UDP-GlcNAc and the exchange of these side chains with smaller groups changes the cosubstrate specificity from UDP-Glc to UDP-GlcNAc (Jank et al. 2005). TpeL has the smaller side chain Ala-383 and the bulkier side chain Gln-385 at the respective positions (Amimoto et al. 2007). We speculate that Ala-383 and Gln-385 in TpeL may stabilize the binding of UDP-Glc and UDP-GlcNAc and favor the acceptance of UDP-Glc and UDP-GlcNAc as the cosubstrates (Nagahama et al. 2011).

The sequential glycosylation of Rac1 by TpeL followed by TcdB, and vice versa indicates that both toxins share the same acceptor amino acid in Rac1. The acceptor amino acid of TcdB-glycosylated Rac1 has been determined as Thr-35 (Belyi and Aktories 2010). TpeL inactivates Rac1 through the glycosylation of Thr-35 (Nagahama et al. 2011).

TpeL glycosylated Rac1, as well as the Ras subfamily consisting of Ha-Ras, RalA, and Rap1B, but not RhoA and Cdc42 (Fig. 4). Important differences in substrate specificity have been detected among the various LCTs. Whereas TcdA, TcdB, and TcnA modify most RhoA, Rac1, and Cdc42 isoforms, TcsL glucosylates Rac1 but not RhoA or Cdc42 (Voth and Ballard 2005). On the other hand, TcsL also modifies the Ras subfamily, including Ras, Rap, and Ral isoforms (Voth and Ballard 2005). Thus, TpeL modifies similar substrates to TcsL. It was reported that Arg-455, Asp-461, Lys-463, and Glu-472 and residues of helix $\alpha 17$ (e.g., Glu-449) of TcdB are essential for enzyme-RhoA recognition (Jank et al. 2007b). Changing the respective amino acid residues in TcsL to those of TcdB reduced glycosylation of Ras by TcsL (Jank et al. 2007a). Furthermore, the introduction of helix $\alpha 17$ of TcdB into TcsL caused a reduction in the glycosylation of Ras subfamily proteins but permitted the glycosylation of RhoA, indicating that helix $\alpha 17$ is involved in RhoA's recognition by TcdB (Jank et al. 2007b). Glu-449, Lys-463, and Glu-472 in TcdB correspond to Lys, Arg, and Gly residues in TcsL and TpeL. Arg-455 in TcdB corresponds to Lys in TcsL and Gly in TpeL (Amimoto et al. 2007). The difference in those amino acid residues may be involved in recognizing small GTPases by TpeL. Additional residues in LCTs are needed for the recognition of small GTPases.

4. Conclusion

Infection with TpeL-positive *C. perfringens* strains may yield disease with a more rapid course and higher case fatality rate in broiler chicks. Thus, TpeL may potentiate the effect of other virulence attributes of necrotic enteritis strains of *C. perfringens*. TpeL from *C. perfringens* has been identified as a glycosyltransferase using UDP-GlcNAc and UDP-Glc as cosubstrates. The substrates of TpeL are confined to Rac1 and Ras subfamily proteins. The modification of Thr-35 on Rac1 induces cytopathic effects.

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