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Review

Bacterial toxin and effector glycosyltransferases

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ABSTRACT

Clostridial glucosylating cytotoxins, including *Clostridium difficile* toxins A and B, *Clostridium novyi* α-*toxin*, and *Clostridium sordellii lethal toxin*, are major virulence factors and causative agents of human diseases. These toxins mono-O-glucosylate (or mono-O-GlcNAcylate) a specific threonine residue of Rho/Ras-proteins, which is essential for the function of the molecular switches. Recently, a related group of glucosyltransferases from *Legionella pneumophila* has been identified. These *Legionella* glucosyltransferases modify the large GTPase elongation factor eEF1A at a serine residue by mono-O-glucosylation, thereby inhibiting protein synthesis of target cells. Recent results on structures, functions and biological roles of both groups of bacterial toxin glucosyltransferases will be discussed.

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1. Introduction

Cytosolic mono-O-glucosylation is an important molecular mechanism by which various bacterial protein toxins and effectors target eukaryotic cells. So far, two major groups of bacterial toxins have been described that possess glucosyltransferase activity. One group is comprised of the clostridial glucosylating cytotoxins, which are produced by *Clostridium difficile*, *Clostridium novyi*, and *Clostridium sordellii* [18,41,58,60,62,97,113]. These exotoxins are released by the clostridia into the environment and are able to enter eukaryotic target cells by an inherent cellular uptake mechanism. Once inside the target cell, the toxins modify Rho- and Ras-subfamily proteins by O-glucosylation at specific threonine residues. In addition, one member of this toxin family, *Clostridium novyi* α-toxin, modifies proteins by attachment of N-acetyl-glucosamine (GlcNAc). The second group of bacterial glucosyltransferases is produced by *Legionella pneumophila*, and these enzymes modify eukaryotic elongation factor EF1A by mono-O-glucosylation at a specific serine residue. Instead of being termed *toxins*, these bacterial glucosyltransferases are often called *effectors*, because translocation of the bacterial enzymes into the cytosol of eukaryotic target cells depends on direct contact of the pathogen with host cells. The present review covers both groups of glucosyltransferases and their eukaryotic targets, their structure, function, and intracellular consequences of their actions, as well as the medical impact and role in host–pathogen interaction during infection.

2. The family of clostridial glucosylating toxins

Members of the family of clostridial glucosylating toxins are *Clostridium difficile* toxins A and B (TcdA, TcdB), *C. sordellii* lethal toxin (TcsL) and hemorrhagic toxin, and the α-toxin from *C. novyi* that catalyzes a GlcNAcylation. In addition to the *C. difficile* prototype toxins A and B, several isoforms have been described for *C. difficile* toxins [92,93]. All these toxins are 50 to 90% identical in their amino acid sequences. They are large proteins of 250 to 308 kDa. Hence, they are also called large clostridial cytotoxins.

These toxins are groups in the *carbohydrate-active enzyme* (CAZY) family GT44. This family has now more than 30 members, including putative glycosyltransferases from *Clostridium perfringens*, *Escherichia coli*, *Citrobacter rodentium*, *Photobacterium profundum*, *Pseudomonas fluorescens* and various species of *Chlamydia* and *Chlamidophila* (http://www.cazy.org/fam/GT44.html).

2.1. Glucosylating toxins are important virulence factors

From a medical point of view the *C. difficile* toxins A and B are the most important in terms of prevalence and pathogenicity. In the late 1970s, it was recognized that *C. difficile* toxins A and B are the causative agents of antibiotic-associated diarrhea and pseudomembranous colitis as a consequence of treatment with antibiotics, which destroy the normal microflora of the gut and allow colonization and proliferation of *C. difficile* bacteria [7,8,67]. Although the precise pathogenetic mechanisms of induction of diarrhea and colitis are not known, it is generally accepted that the toxin-induced glucosylation of Rho GTPases is central

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to the action of the *C. difficile* toxins [67]. Elderly patients are particularly at risk for severe sequelae, prolonged stay in hospitals and even lethal outcomes of *C. difficile* infections. *C. difficile*-induced diseases received additional attention recently, when hypervirulent strains of *C. difficile* were isolated, that produced more than 10 times higher concentration of toxins than previous isolates [79].

The larger amount of toxin production is apparently due to changes in the pathogenicity locus of the toxins. At least 5 *tcdA–E* genes are responsible for toxin production: *tcdA* and *tcdB* are the structural genes of both toxins, *tcdC* encodes a negative regulator of toxin expression, *tcdD* appears to be responsible for activation of toxin gene expression and *tcdE* encodes a holin-like protein likely involved in toxin release. In the hypervirulent strain the negative regulator is deleted, which results in higher toxin production.

Historically, toxin A was designated as an enterotoxin, whereas toxin B was termed a cytotoxin. This distinction was based on findings in animal models showing that *C. difficile* toxin A but not toxin B caused disease after intragastral application [75]. In contrast, toxin B was in general 100 to 1000-fold more potent in inducing cytotoxic effects in cell culture than toxin A, most likely due to differential receptor binding [113]. Later however, it was shown that toxin B is also able to potently induce destruction of mucosal gut tissue in humans [91]. Recently it was reported in an excellent study using genetic approaches that toxin B, but not toxin A, is crucial for virulence of *C. difficile* in diverse animal models [76].

The lethal toxin produced by *C. sordellii* and the α-toxin from *C. novyi*, appear to be involved in gas gangrene syndrome [13,95,105]. The same may be true for the less characterized *C. sordellii* hemorrhagic toxin. Lethal toxin may also play a role in toxic shock syndrome associated with abortion and gynecological infections [25] and deaths of drug users [68].

2.2. Multidomain structure of the toxins

All members of the glycosyltransferase toxin family share 26 to 76% sequence identity and are structurally and functionally organized in a similar module-like manner [18]. At least four domains "ABCD" can be distinguished in toxins A and B of *C. difficile* [58], which are putatively involved in biological activity (A-domain), receptor binding (B-domain), auto-proteolytic cleavage during toxin-processing (C-domain), and delivery of the A-domain into the cytosol (D-domain) (Fig. 1).

The A-domain, which harbors the glucosyltransferase activity, is located at the N-terminus of the toxins. This domain is most well characterized and will be described in more detail below. The receptor-binding domain (B), which consists of polypeptide repeats, is located at the C-terminus [111,112]. 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 [111]. Accordingly, antibodies generated against this part of the molecule blocked cytotoxic activity [38], and toxin fragments containing this domain are able to compete with the holotoxin and block the toxin effects [39]. A part of the polypeptide repeats of the C-terminus of *C. difficile* toxin A has been crystallized recently [44,50], 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 [44]. Co-crystallization with a derivate of the trisaccharide α-Gal(1,3)β-Gal(1,4)βGlcNAc confirmed the carbohydrate binding capacity of the domain [44].

As mentioned above only the A-domain, harboring the glucosyltransferase activity, is translocated into the cytosol [84,94]. Therefore, cleavage of the toxin is required. Recently, 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 downstream of the glucosyltransferase domain [33]. 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 [33]. The cysteine protease activity is enhanced by inositol hexakisphosphate (InsP6) [89]. InsP6 binds to the C-Domain, causing a conformational change that activates the auto-catalytic activity [32].

Structure and function of the D-domain, which is located between residues 955 and 1852, are least understood. A small region in the primary sequence between residues 965 and 1128 is characterized by hydrophobic amino acids and is suggested to participate in formation of transmembrane structure during pore formation and translocation of the toxin into the cytosol [113]. Pore formation induced by the toxin has been shown in artificial lipid membranes, as well as by the release of radioactive rubidium ions (^{86}Rb) from preloaded cells under low pH conditions, which mimic the pH of endosomes [6,43]. However, so far it is not clear how pore formation relates to the delivery of the toxin into the cytosol.

2.3. Toxin up-take

Clostridial glucosylating toxins bind to cell membrane receptors and are subsequently endocytosed as a receptor–toxin complex [37,39] (Fig. 2). Our knowledge about the receptors involved in uptake of the toxins is still very limited. Studies using enterocytes have shown that toxin A acts from the apical side of cells, whereas toxin B targets cells

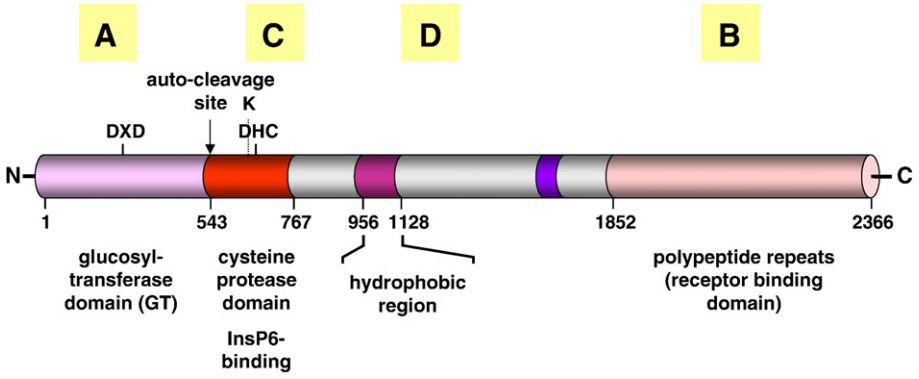

Fig. 1. ABCD-model of clostridial glucosylating toxins. The clostridial glucosylating toxins are constructed of at least 4 domains. The A-domain covers the glucosyltransferase activity (note, *C. novyi* α-toxin possesses GlcNAcylation activity). The B-domain consisting of polypeptide repeats is involved in receptor-binding. The C-domain is responsible for the auto-catalytic cleavage of the toxins (arrow: cleavage site) and is a cysteine-protease with the catalytic residues DHC. Lysine-600 (K) was identified to be involved in InsP6-binding. InsP6 is necessary for activation of the cysteine protease. The D-domain is likely involved in the delivery of the A-domain into the cytosol. This domain contains a hydrophobic region (indicated) suggested to be important for insertion of the toxin into endosome membranes.

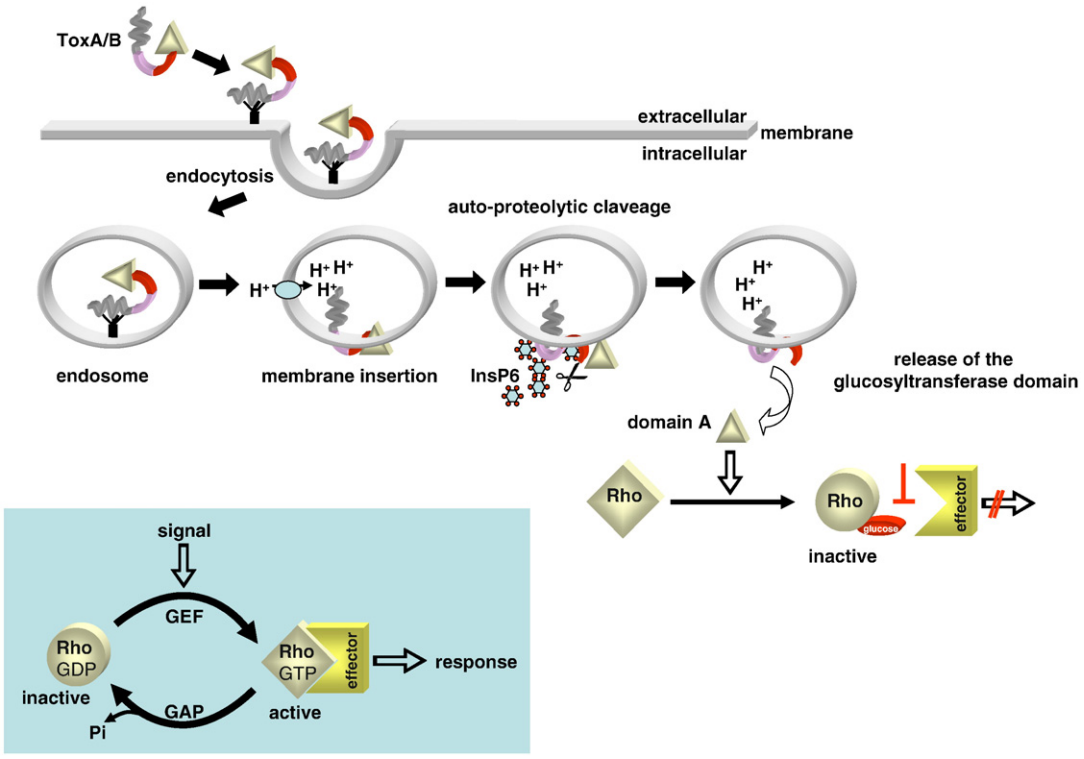

Fig. 2. Up-take of clostridial glucosylating toxins. The clostridial glucosylating toxins bind with their C-terminal B-domain to the membrane receptor of target cells. After endocytosis, the toxin inserts into the endosome membrane most likely involving the hydrophobic part of the D-domain. Cellular InsP6 activates the cysteine protease C-domain. 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. Insert: Rho proteins are GTP binding proteins, which are active in the GTP-bound state. The nucleotide exchange is facilitated by guanine nucleotide exchange factors (GEFs). In the GTP-bound form the Rho GTPases interact with a large variety of effectors and induce multiple signaling events. The active state of Rho proteins is terminated by GTP hydrolysis stimulated by GTPase-activating proteins (GAPs).

from the basolateral side [102]. It has been suggested that toxin A binds to carbohydrates [23,71,104,106], including α-Gal(1,3)β-Gal (1,4)βGlcNAc on rabbit erythrocytes and hamster brush border membranes. Proteins have also been discussed as receptors for toxin A, including sucrase-isomaltase in rabbit gut [86] and glycoprotein 96 (gp96) in human colonocytes [81]. However, it is not clear whether they are relevant as receptors of toxin A in humans. The receptor of toxin B is completely unknown and the same is true for the other clostridial glucosylating toxins.

After receptor-binding, the toxins are taken up by endocytosis through pathways that are still not clear and end up in acidic endosomes, from where they translocate into the cytosol [6,87]. The translocation mechanism is still largely enigmatic, but appears to depend on the low pH of endosomes, because bafilomycin, a specific inhibitor of the vacuolar H^+-ATPase, blocks toxin up-take. Most likely the low pH of endosomes induces conformational changes, that favor insertion of the toxin into the membrane [6,87]. Interestingly, a pH-pulse (pH<5.5) at the cell membrane allows membrane insertion and pore formation by toxins without prior endocytosis [6,43]. Pore formation and toxin up-take appear to be cholesterol-dependent [43]. It is still not clear, where or at what point in the uptake process the above-mentioned auto-catalytic processing of the toxins occurs (e.g., in the vesicles or in the cytosol), which finally results in release of the glucosyltransferase domain. Inositol-hexakisphosphate, which is required for auto-catalysis is present at relatively high concentrations (~100 μM) in the cytosol, but the concentration in endosomes is unknown.

2.4. Glucosylation of Rho GTPases by clostridial toxins

Once in the cytosol, the clostridial toxins glucosylate small GTPases of the Rho and Ras superfamily [64,65,85,100]. The structure, function and roles of these substrates in cellular processes have been described in detail in several excellent recent reviews and will be mentioned only briefly here [17,35,48,57,114]. The ~20 different Rho GTPases are molecular switches involved in several cellular signaling pathways. The GTPases are active in the GTP-bound form and inactive with GDP bound. Hydrolysis of GTP is caused by inherent GTPase activity which can be facilitated by numerous GTPase-activating proteins (GAPs). Activation of the GTPases occurs after nucleotide exchange induced by guanine nucleotide exchange factors (GEFs), which release GDP and allow rebinding of GTP. The best studied small GTPases of this family are RhoA, Rac and Cdc42 isoforms. They control organization of the cytoskeleton and regulate cellular motility, and participate in the regulation of transcription, cell cycle progression, apoptosis, transformation and the activity of numerous other cellular enzymes. Modification of low-molecular GTPases by the toxins occurs at a Thr35/37, depending on the Rho GTPase isoforms [64]. Important differences in substrate specificity have been detected among the various clostridial glucosyltransferases. Whereas *C. difficile* toxins A and B and *C. novyi* α-toxin modify most Rho, Rac and Cdc42 isoforms *C. sordellii* lethal toxin glucosylates Rac but not RhoA or Cdc42 in intact cells [63,85]. However, *C. sordellii* lethal toxin also glucosylates Ras GTPases, including Rap, Ral und Ras isoforms. All clostridial glucosylating toxins with the exception of *C. novyi* α-toxin use UDP-glucose as a cosubstrate, whereas the α-toxin uses UDP-GlcNAc as a sugar donor [99].

Glucosylation of Rho or Ras GTPases inhibits activation of the GTPases by GEFs and blocks interaction with their effectors [98,109] as well as the cycling of Rho GTPases between the membrane localization and cytosolic localization [40] (Fig. 3). Glucosylated Rho proteins are located at the membrane. Most importantly, the toxin-induced glucosylation inhibits the active conformation of Rho/Ras GTPases [42,109]. This fact also explains why the stimulation of the GTPase activity by GAPs is also blocked after glucosylation. Although not studied in detail, it is thought that attachment of N-acetylglucosamine causes the same functional consequences.

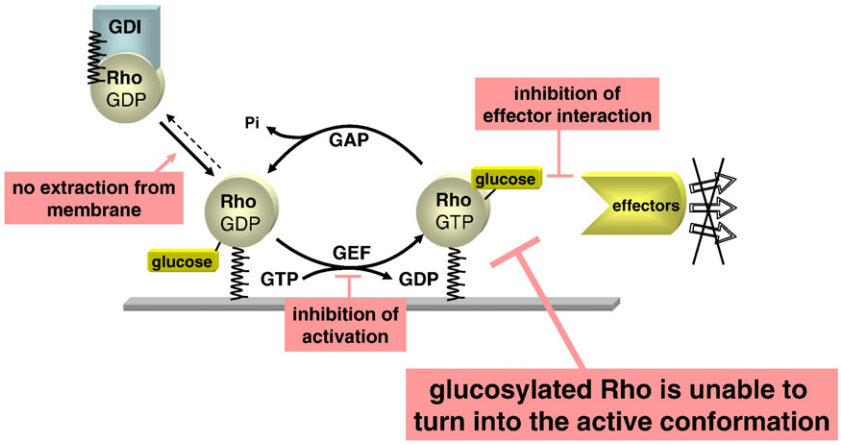

Fig. 3. Functional consequences of the glucosylation of Rho GTPases. Glucosylation of Rho proteins by clostridial glucosylating toxins has several consequences for Rho GTPases. The glucosylation inhibits the active conformation of Rho proteins. This causes: (1) blockade of Rho activation by GEFs; (2) inhibition of the interaction of Rho proteins with effectors; (3) inhibition of the stimulation of the GTP hydrolyzing activity of Rho proteins by GAPs; and (4) blockade of the cycling of Rho proteins between membranes and the cytosol (in the cytosol Rho proteins are bound to guanine nucleotide dissociation inhibitors (GDIs)). Ras proteins are modified by some clostridial glucosylating toxins (e.g., *C. sordellii* lethal toxin) with the same consequences. Modified from [60].

Because Rho GTPases have major effects on the cytoskeleton, efforts have been made to differentiate toxin effects on cell morphology from those on cell survival. Results from these studies suggested that the morphological changes induced by toxin B are largely dependent on Rac protein because cells expressing non-glucosylatable Rac1-Q61L were protected against toxin B, whereas this was not the case when RhoA-Q63L was expressed [47]. On the other hand, cell death by toxin B induced apoptosis appears to depend on RhoA, because a specific toxin B isoform (from *C. difficile* 1470 serotype F), which does not accept RhoA as a substrate but instead only modifies Rac, does not cause apoptosis [54]. Similarly, lethal toxin from *C. sordellii*, which modifies Rac but not RhoA, is able to induce apoptosis but in this case toxin-induced apoptosis appears to depend on glucosylation of Ras and modulation of phosphatidylinositol-3-kinase [31].

2.5. Structure of the catalytic A-domain of clostridial glucosyltransferases

The crystal structures of the catalytic A-domains of toxin B, lethal toxin and α-toxin have been solved [90,116]. These studies show that all these toxins belong to the GT-A family of glycosyltransferases [15,73,107], which is characterized by a common catalytic core (243 amino acids in length for toxin B) with a mixed α/β-fold and a central 6-stranded β sheet (Fig. 4). As is typical for the GT-A family, all but one β strand are parallel; strand 5 is antiparallel. The catalytic core of the clostridial toxins is surrounded by helical structures (309 residues in length for toxin B). The four N terminal helices appear to form a subdomain, which might be involved in membrane binding. Indeed, it has been suggested that this part of lethal toxin binds to phosphatidylserine at membranes [80].

Another typical feature of the GT-A-family is the so-called DXD motif [19,73,115], which is involved in coordination of manganese (Mn^{2+}). The motif, which includes Asp286 and Asp288 in toxin B, also interacts with the pyrophosphate of the sugar donor UDP-glucose [90] (Fig. 4). Asp288 directly binds to Mn^{2+}, whereas Asp286 coordinates Mn^{2+} via a water molecule. Asp286 together with Arg273 and Asp270 forms a tight network of hydrogen bonds to the hydroxyl groups (positions 3″, 4″ and 6″) of the glucose molecule. The ribose moiety of UDP-glucose is fixed by Tyr284, Ser269 and also by Asp286. Among other residues, Trp102 is important for positioning of the uracil ring of UDP by aromatic stacking [90]. All these residues have been shown to be essential for enzyme activity [60]. Exchange of these residues with alanine caused a several hundred fold drops in glucosyltransferase and glucohydrolase activities. Comparison of the structures of the catalytic domains of different clostridial glucosyltransferases with or without UDP-glucose bound suggests that Trp520 is of major importance for the glucosylation reaction (Fig. 5). In the absence of Mn^{2+} and without UDP-glucose, Trp520 and the mobile chain segment of residues 519–525 exhibit a large shift of ~15 Å from an open conformation to a closed UDP-glucose-binding conformation [116], allowing bonding of the glycoside oxygen of UDP-glucose with Trp520-Nε1. Accordingly, substitution of Trp520 with alanine or even phenylalanine inhibits glucosyltransferase activity.

The glucosylation of Rho/Ras GTPases by clostridial glucosyltransferases causes retention of the α-anomeric configuration of UDP-glucose [42,109]. Whereas the molecular mechanism of *inverting* glucosylation reactions (e.g., conversion of the α-anomeric configuration of UDP-glucose to the β-anomeric configuration) is well-understood, e.g., by a direct S_N2-like displacement reaction [73], the *retaining* reaction is less well-understood. As deduced from comparison of the crystal structures obtained from toxin A, lethal toxin and α-toxin, a model for the glucosylation reaction has been proposed, which largely depends on Trp520 and suggests a circular type of reaction [116].

2.6. Substrate specificity

The crystal structures also provide an explanation for the cosubstrate specificity of the clostridial toxins, e.g., it is now very well-understood why α-toxin from *C. novyi* uses UDP-GlcNAc but not UDP-glucose as a sugar donor [99]. It appears that two amino acids in the vicinity of the catalytic cleft are responsible for the cosubstrate specificity. Toxins A, B and lethal toxin, which all use UDP-glucose, have isoleucine and glutamine in equivalent positions (Ile-383 and Glu-385 in toxin B), whereas *C. novyi* α-toxin has serine and alanine residues in the respective positions [61,116]. Substitution of Ile-383 with serine or Glu385 with alanine favored the acceptance of UDP-GlcNAc for glycosylation by toxin B, but change of both residues to that of α-toxin completely converted the donor specificity from UDP-glucose to UDP-GlcNAc. Apparently, the bulkier side chains of Ile383 and Glu385 limit the space of the catalytic cleft for binding of UDP-GlcNAc and the exchange of these side chains with smaller groups causes a dramatic drop in the Km-value for UDP-GlcNAc from ~900 μM to ~25 μM.

Although some specific regions and residues of the Rho GTPases have been identified to play a role in substrate–enzyme interaction, the protein substrate specificity is less clear. Arg455, Asp461, Lys463 and Glu472 as well as helixα17 are involved in protein substrate recognition

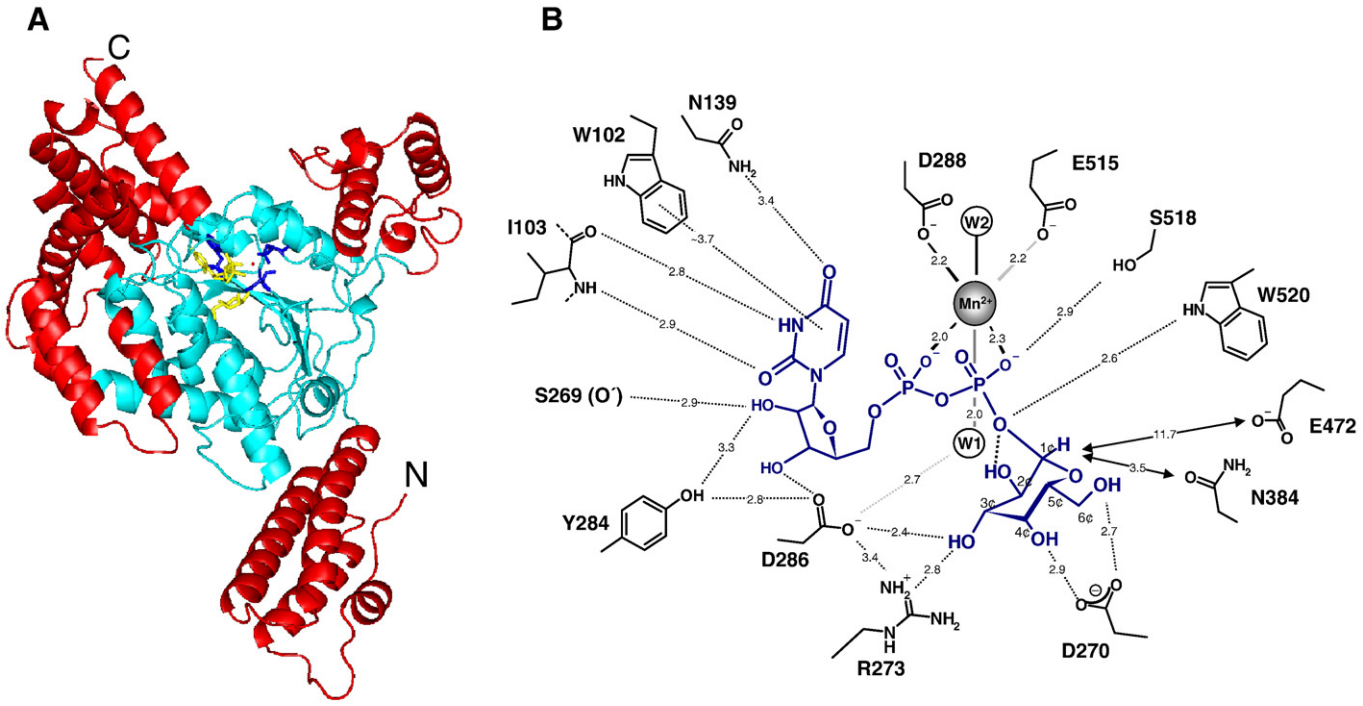

Fig. 4. A. Structure of the glucosyltransferase A-domain of toxin B. The catalytic core of the glucosyltransferase A-domain is given in blue. Additional amino acids and regions of the A-domain, likely not directly involved in catalysis are given in red. B. Amino acid involved in the interaction with the sugar donor UDP-glucose. Data are from [90,116].

by toxin B. Of particular interest is the finding that introduction of helixα17 from toxin B into *C. sordellii* lethal toxin inhibits modification of Ras but allows glucosylation of RhoA [59]. Of course, co-crystallization of the catalytic domain of the toxin with the substrate GTPase is necessary for a complete understanding of the molecular basis for the enzyme–substrate specificities.

3. The family of *L. pneumophila* glucosyltransferases

3.1. Intracellular biology of L. pneumophila

Legionella is a fastidious gram-negative bacterium, causing severe often fatal pneumonia in humans, known as Legionnaires' disease. This

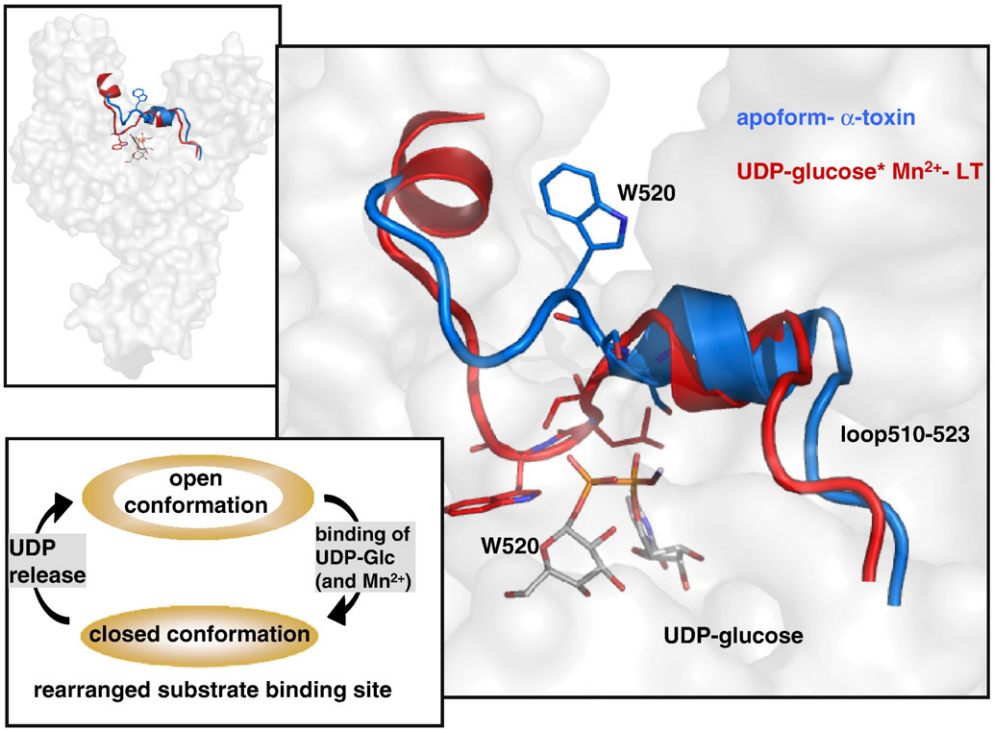

Fig. 5. Conformational changes occurring after binding of UDP-glucose and Mn^{2+} to the the apo-enzyme. Deduced from a series of crystal structures, it is suggested that Trp520 undergoes a major conformational change after binding of UDP-glucose and Mn^{2+} to the apo-enzyme resulting in an open or closed conformation of the glucosyltransferase domain.

infection ranks among the most common causes of severe pneumonia in the community setting, and its infectious agent is isolated in up to 40% of the cases of hospital-acquired pneumonia. Among the more than 50 known species of *Legionella*, the most important human pathogen is *L. pneumophila*, strains of which account for up to 90% of morbidity records due to legionellosis [30].

L. pneumophila is able to multiply in phagocytes — either in free-living unicellular eukaryotes (amoebae and ciliated protozoa) or in mammalian cells (macrophages and monocytes). This property is a direct prerequisite for survival of this bacterium in natural aquatic systems and for proliferation in lung tissues of infected macroorganisms [36].

In terms of its subcellular compartmentalization, *L. pneumophila* is a vacuolar pathogen. After penetration into eukaryotic cells legionellae reside and multiply within the phagosome. This is in obvious contrast to some other intracellular microorganisms, such as *Listeria* or *Shigella*, which lyse the membrane of a phagosome and multiply freely in cytoplasm of a target cell [46]. Subsequent to uptake, the legionellae-containing vacuole (LCV) is subjected to specialized biogenesis steps, leading to transformation of this organelle into a "cozy" niche that supports multiplication of the bacteria and hence, this LCV is also termed "replicative phagosome" [24,56].

Prominent characteristics of LCV development include the delay in maturation of a phagosomal membrane, the avoidance of the degrading lysosomal pathway, the malfunction in proton pump activity and, subsequently, impaired acidification of the vacuole, the attraction of mitochondria and components of rough endoplasmic reticulum, and the interception of early secretory vesicles, containing host cell membranous material and nutrients [28,51,52,66]. Concurrently, global changes in target cell metabolism become evident, including inefficient oxidative burst generation, shift in apoptotic–antiapoptotic equilibrium, drop in general protein synthesis, modulation of ubiquitination processes and NF-κB activity [5,72,74,78,96,103].

Infection of phagocytes by *L. pneumophila* is accompanied by numerous alterations in cellular processes, leading to transformation of normally hostile intracellular environment into friendly niche suitable for bacterial proliferation. A specialized *dot/icm* type 4 secretion system (T4SS) and an array of its substrates/effectors have been shown to participate in adaptation of a target cell to inhabitation by *L. pneumophila* [83]. The estimated number of such substrates/effectors reaches 30 [83], 85 [56], or almost 130 [49], but the general consensus is that even these figures are an underestimation. In most instances the molecular mechanism of the activities of the effectors and their substrates is not known. But in certain cases detailed information is available. Recent data demonstrate that several effector proteins target the small GTPases Arf1 and Rab1 [55,77,82]. These GTP-binding proteins play critical roles in tethering ER-derived vesicles and therefore manipulation of their activities by *Legionella* effectors apparently allows the bacteria to redirect early secretory traffic.

3.2. Identification of glucosyltransferases in L. pneumophila

Recent studies have shown that among the various bacterial effectors, which are translocated from *Legionella* into the host cytosol are bacterial glucosyltransferases. Initially it was observed that in the presence of *L. pneumophila* cellular extract and UDP-[^{14}C]glucose labeling of a ~50 kDa cytoplasmic component of eukaryotic cells was detectable [9]. Further purification of the extract of *L. pneumophila* Philadelphia-1 resulted in isolation of a ~60 kDa glucosyltransferase, which was subsequently named "Lgt1". The enzyme was present in all tested members of *L. pneumophila*, but was absent in strains of certain non-*pneumophila* species, such as *L. longbeachae*, *L. gormanii* and *L. steigerwaltii*. Whether all *pneumophila* species or only a subset of these bacteria contain glucosylation activity is not known. The enzymatic activity was sugar-specific, i.e. only UDP-glucose, but not glucose, UDP-galactose, UDP-N-acetyl-galactosamine, UDP-N-acetylglucosamine, UDP-glucuronic acid or GDP-mannose served as co-substrates in the reaction [10].

Primary amino acid sequence of the glucosylating proteins shared little homology with known proteins in the NCBI database. The most notable hit was similarity between central region of Lgt1 and the DXD-containing domains of clostridial glucosylating toxins (Fig. 6). In this sequence region several stretches of identical amino acid residues could be identified, including two residues found to be critically important for catalysis and apparently representing part of a DXD motif (D_{246} and D_{248} in Lgt1) [10].

Subsequent database searches in the sequenced genomes of four *L. pneumophila* strains (Philadelphia-1, Corby, Lens and Paris) disclosed altogether nine open reading frames with significant sequence homology (Fig. 7). Based upon the level of identity, these gene products have been grouped into three families: Lgt1 through Lgt3 (type representatives of Philadelphia-1 strain had gene IDs *lpg1368*, *lpg2862* and *lpg1488* for Lpg1, Lpg2 and Lpg3, respectively). Only one copy of each gene family could be found in the corresponding genome. Philadelphia-1 contained the full set of genes (i.e. *lgt1*, *lgt2* and *lgt3*), whereas the other three strains included only *lgt1* and *lgt3*. Representatives within each family demonstrated more than 85% amino acid identity, whereas differences between the groups were in the range of 15–25%. Interestingly, whereas proteins from Lgt1, Lgt2 and Lgt3 groups demonstrated interfamily homology with the majority of identical amino acid residues grouped around the DXD motifs, two additional translated sequences (*lpg1491* in Philadelphia-1 and *lpp1447* in Paris strains) displayed considerable similarity only to the C-terminal region of the Lgt3 members and did not contain the canonical DXD motif. Due to these data they were not included into this scheme.

In order to establish the enzymatic activity of these putative glucosyltransferases, the coding sequences from the Philadelphia-1 strain of *L. pneumophila* were expressed in *E. coli* as recombinant proteins and tested *in vitro* in the UDP-[^{14}C]glucose assay. Additionally, Lgt2 and Lgt3 proteins from several other *L. pneumophila* strains belonging to different serogroups were purified in recombinant form and tested in glucosylation assays. Representatives of Lgt1, Lgt2 and Lgt3 did possess glucosylation activity and modified a ~50 kDa component in mammalian cell extracts.

In a recent study another putative glycosyltransferase produced by *L. pneumophila* has been identified [49]. The protein caused delay in vacuolar trafficking and was termed therefore SetA (subversion of eukaryotic vesicle trafficking A). Closer inspection of the amino acid sequences disclosed moderate homology to that of clostridial glycosylating toxins, as well as to *Legionella* glucosyltransferases. In particular, SetA possessed a DSD motif resembling the DXD motif of the active domain of other bacterial glycosylating enzymes [19,73,115]. Although the SetA DXD motif contains polar amino acid residue serine in the middle of the triplet in contrast to typical non-

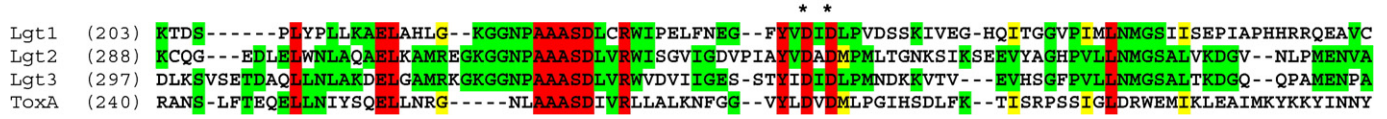

Fig. 6. Partial alignment of amino acid sequences of Lgt1/2/3 with that of toxin A from *C. difficile*. Amino acid residues, which are identical in 2 or 3 sequences are marked by green or yellow, whereas residues, which are identical in all 4 sequences are marked by red. The conserved DXD motif is indicated by asterisks. Identification codes of *lgt1*, *lgt2*, *lgt3* and toxin A gene are *lpg1368*, *lpg2862*, *lpg1488* and M30307, respectively.

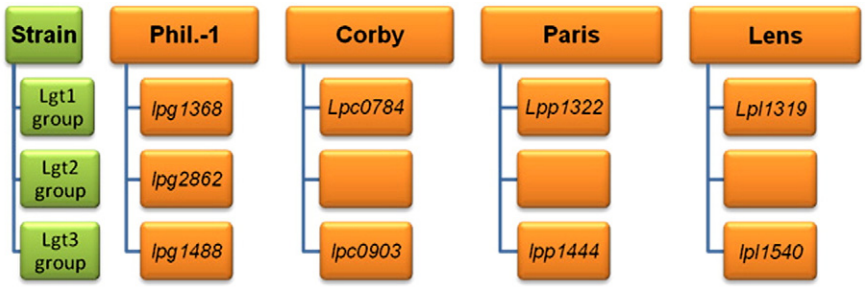

Fig. 7. Schematic view of the *L. pneumophila* glucosyltransferase family. Identification code of each Lgt gene is shown as deposited in the corresponding *L. pneumophila* sequenced genome database. Empty rectangles indicate lack of Lgt2 group in Corby, Paris and Lens strains.

polar residues in the case of large clostridial cytotoxins or glucosyltransferases from *L. pneumophila*, point mutations of both aspartic acid residues with substitutions to alanine attenuated growth suppression and specific vacuolar transport phenotypes in yeast [49]. However, molecular functions of SetA or experimental evidence that it possesses glycosylating activity are still missing.

3.3. Expression and translocation of L. pneumophila glucosyltransferases

To effectively accomplish their functions, effector proteins should be expressed during the appropriate phase of the bacterial growth. The typical timing interval for production of T4SS substrates by *Legionella* is usually the stationary phase of broth culture [16,118]. At this stage bacterial cells become remarkably virulent and execute transmission phenotype program [20]. In line with this view, it was observed that Lgt1 and Lgt2 production peaked at stationary phase of *in vitro*-growth of *Legionella* [12]. Similar *in vitro* behavior was reported for SetA [49]. In contrast, Lgt3 could be detected predominately in pre-logarithmic period of cultivation. These results were confirmed in infection studies with *L. pneumophila* Philadelphia-1 in the model of the amoeba *Acanthamoeba castellanii*. During *L. pneumophila* and *A. castellanii* co-cultivation mRNA levels of *lgt1* were maximal at late phase of infection, while *lgt3* was expressed mainly at initial stage of bacterium–amoeba interaction [12]. These experiments suggested distinctive regulation of the glucosyltransferases and implied differential roles of Lgt1/Lgt2 and Lgt3 during *L. pneumophila* infection. Based on the results obtained one can speculate that Lgt3 is important for initiation of infection cycle, while Lgt1/Lgt2 is necessary for egress of *Legionella* from the host cell.

Because infection of epithelial cells with *Legionella* is accompanied by glucosylation of an Lgt substrate [10], it is evident that during intracellular proliferation of bacterial cells glucosyltransferases gain access to their eukaryotic targets. Definite information about delivery mechanisms of *Legionella* enzymes has been obtained during a search for molecules transported into host cell by *Legionella* using adenylate cyclase-tagged or β-lactamase-tagged protein constructions [26,27]. These experiments identified Lgt2 (under the name "LegC8") and Lgt3 (under the name "LegC5") as T4SS-translocated substrates. However, there is still no information concerning active translocation (if any) of Lgt1 into the host cell. One should keep in mind that some proteins, which act outside microbial cells (e.g., enterotoxin of *C. perfringens* or LT toxin of *E. coli*) are released simply by lysing of bacteria [101]. Whether such a mechanism is operative in the case of Lgt1 is not known.

3.4. Substrates of glucosyltransferases Lgt

A question of exceptional importance for understanding the role of *Legionella* glucosyltransferases includes the nature of their substrate (s). While clostridial glucosylating toxins target small GTPases of the Rho and Ras family, the *Legionella* enzymes glucosylate ~50 kDa proteins. Analysis of the 50-kDa target of Lgt1 was accomplished by tandem mass-spectrometry of tryptic products isolated by SDS-PAGE from Lgt1-treated mammalian cell lysate. This approach identified the human elongation factor 1A (eEF1A) as a target for glucosylation, in which serine-53 was modified by mono-O-glucosylation [10].

Elongation factor eEF1A (prokaryotic homolog is EF-Tu) represents one of the key-players in ribosome-dependent synthesis [88]. It possesses GTPase activity and is necessary for recruitment of aminoacylated tRNA to the A-site of ribosomes charged with translated mRNA. In addition, eEF1A appears to be involved in several other cellular processes, including translational control, assembling/folding of newly synthesized proteins and proteosomal degradation of incorrectly-folded peptides [22,53], lipotoxic cell death [14] and regulation of the actin cytoskeleton and cell morphology [34,45]. No structural data for mammalian eEF1A are available, however the very similar yeast elongation factor 1A from *Saccharomyces cerevisiae* has been crystallized [3]. The structure showed that eEF1A consists of three domains: domain 1 is the G domain of ~240 residues, which is characterized by a Ras-like fold and harbors consensus sequences of typical G proteins [69]. Domains 2 and 3, which consist of 89 and 107 residues respectively, have both a six-stranded β-barrel structure and are involved in aminoacyl-tRNA binding [3].

Serine-53 of eEF1A, which is modified by Lgt1, Lgt2 and Lgt3, is located in domain I near the switch-1 region of the GTPase [10,12]. For prokaryotic EF-Tu, it is known that the switch-1 region undergoes major conformational changes from β- to α-structure, depending on its GDP- or GTP-bound form respectively [1,110]. However in eEF1A the switch-1 region is not well defined, because two additional helices (A* and A′) are inserted into this region, and no structural changes in the switch-1 region have been reported for eEF1A so far. In addition, Ser-53 of eEF1A is lacking in the prokaryotic factor, but can be compared functionally with Ala-42 in *E. coli* EF-Tu, which yet again shows no major structural alteration upon nucleotide exchange [1]. However, it is clear that the structures of eEF1A and EF-Tu are not identical and, therefore, complete extrapolation of data obtained on prokaryotic elongation factor onto eukaryotic molecule is obviously difficult.

3.5. Substrate recognition by glucosyltransferases from L. pneumophila

To clarify the substrate specificity of Lgt enzymes, recombinant eEF1A deletions were constructed and after protein expression, the fragments of eEF1A were used as substrates in the glucosylation assay. Surprisingly, deletions of considerable portions of the elongation factor were still able to serve as substrates for glucosylation. Neither domain 2 nor 3 of eEF1A was necessary for glucosylation (Fig. 8A). Moreover, also proper folding of the G-domain was dispensable for modification by Lgt. Finally, it turned out that a decapeptide comprising residues 50-GKGSFKYAWV-59 served as substrate for the bacterial glucosyltransferase (Fig. 8B). This peptide belongs to the loop of the helix–loop–helix region formed by helices A* and A′ of eEF1A and is part of the first turn of helix A′. Substitution of Ser-53, Phe-54, Tyr-56 or Trp-58 with alanine prevented or largely decreased glucosylation. Even more surprising is the finding that the decapeptide is a more efficient substrate for glucosylation than the full length eEF1A. This suggests that a specific

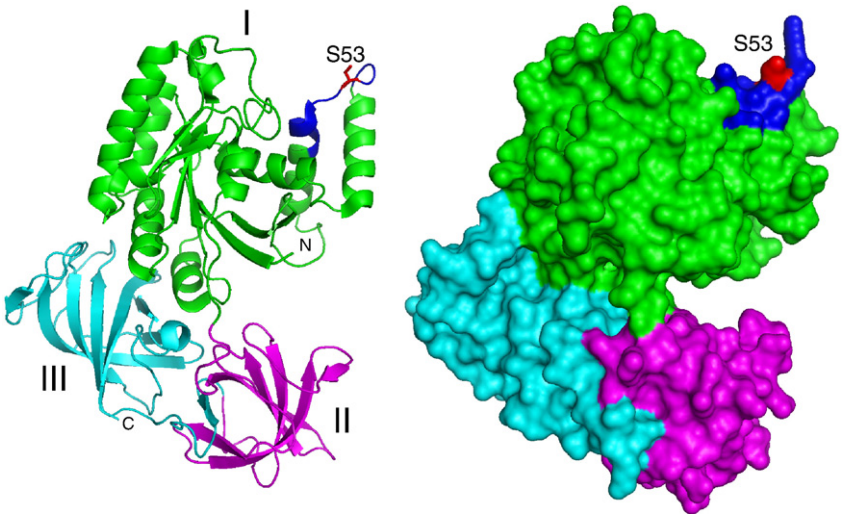

Fig. 8. Structural view of yeast elongation factor eEF1A (PDB ID: 1F60). Left, ribbon plot; right, surface representation. eEF1A consists of 3 domains (I (green), II (magenta) and III (cyan)). Domain I is the GTPase domain. The decapeptide (GKGSFKYAWV), which is an optimal substrate for glucosylation by Lgt, is shown in blue. Serine-53 (red), which is glucosylated by Lgts, is indicated. The complexed eEF1Bα molecule, which is present in the original scheme, is not shown.

conformation of eEF1A is the preferred substrate of Lgt1. Even shorter peptides than the decapeptide are glucosylated, although with lower efficiency [11]. Thus, the structural requirements for modification of eEF1A peptides by Lgt1 are rather low as compared to clostridial glucosylating toxins. The latter transferases probably recognize large regions of the surface of their substrates and require an intact protein fold of Rho/Ras GTPases for efficient modification [58].

In line with the low structural stringency of substrate recognition by *Legionella* glucosyltransferases, a GeneBank search on the basis of the substrate decapeptide for similar peptide sequences retrieved Hsp70 subfamily B suppressor 1 (Hbs1) as a possible target for glucosylation by Lgt1 *in vitro*. Recombinant Hbs1 and the corresponding fragment 303-GKASFAYAWV-312 were glucosylated by Lgt1. So far it is not known whether Hbs1 is substrate for *Legionella* glucosyltransferases in intact cells [11].

3.6. Lgt1 catalyzes a retaining glucosylation reaction

The finding that a small decapeptide was the efficient substrate of the *Legionella* enzymes allowed NMR structure analysis of the glucosylated peptide [11]. These studies identified an α-anomeric structure of the glucose-serine-53 bond and revealed that Lgt1 glucosylates the eEF1A peptide as a *retaining* glucosyltransferase. Thus, the *Legionella* glucosyltransferases have not only a significant sequence similarity with clostridial glucosylating toxins in the proposed region of the catalytic core around the DXD motif but also share a *retaining* type of reaction.

3.7. Functional consequences of the glucosylation of eEF1A by Legionella glucosyltransferases

Since the target of *Legionella* glucosyltransferases is a major component of the eukaryotic translation machinery, studies have focused on the effects of Lgt-catalyzed glucosylation on protein synthesis. Accordingly, it was shown that Lgt1, Lgt2 and Lgt3 effectively inhibit the *in vitro* transcription/translation process. Moreover, delivery of glucosylating enzymes into eukaryotic cells also stopped protein synthesis and caused death of intoxicated cells [10,12].

There are at least two feasible explanations for the translational arrest. First, glucosylation of serine-53 may prevent a conformational change in the switch-1 region, which is essential for the function of the elongation factor, and second, attachment of glucose onto serine-53, prevents protein–protein interaction by steric hindrance, while not directly influencing the structure of the switch-1 region. However, so far no information is available about changes in GTP-binding or alteration of GTPase activity of eEF1A after glucosylation by Lgt.

Termination of peptide synthesis by *Legionella* glucosyltransferases leads to death of intoxicated cell. However, the death rate due to protein synthesis inhibition is relatively slow (order of days), compared to death induced by cytoskeleton-targeting or cytolytic (i.e. membrane-targeting) toxins. The latter two act in the timeframe of hours and minutes, respectively. It is noteworthy that one glucosyltransferase, Lgt3, is produced early in the course of amoeba infection, while another enzyme, Lgt1 — at later time points. One can speculate that the action of Lgt3 transforms host cells into a state of "defenselessness" for subsequent proliferation of invading bacteria. On the other hand, at final stages of the intracellular life cycle *Legionella* has to kill and escape the eukaryotic cell, and Lgt1 may participate in such a task.

4. Conclusion

Our knowledge about virulence-related bacterial glycosyltransferases mostly comes from thorough experimental analysis of large clostridial cytotoxins. Whereas the role of the clostridial glucosylating toxins in disease induced by the toxin-producing pathogens is unequivocally established, the impact of the glucosyltransferases in host–pathogen interaction of *Legionella* is still unclear. The many questions regarding the action of these *Legionella* glucosyltransferases remain unanswered partly because of the complex life cycle of this bacterium in its eukaryotic host. Moreover, the situation appears to be even more complicated due to the fact that *Legionella* is known to produce bacterial effectors, which are likely involved in infectious processes, at high redundancy. Notable illustration to this phenomenon is the presence in *L. pneumophila* cultures of three enzymes (Lgt1/2/3) targeting single molecule — eEF1A.

Concerning modification of eEF1A by *Legionella*-induced glucosylation, one should keep in mind that the elongation factor is posttranslationally modified by various types of reactions, including phosphorylation [108], methylation of lysine residues, attachment of glycerylphosphorylethanolamine [29] and carboxymethylation [117]. It is interesting to know whether eEF1A is also the substrate for glycosylation by some eukaryotic enzyme.

Cytosolic glycosylation of eukaryotic target proteins by bacterial toxins or effectors appears to be of increasing importance in host–pathogen interactions of various bacteria. The number of glycosyltransferases with toxic activity has been increasing during the last decades. In addition to toxins of *Clostridia* and *Legionella* the list of

putative glycosylating enzymes includes, for example, virulence factors from *C. perfringens*, *E. coli*, *C. rodentium* and *Chlamydia* (lymphostatin LifA and related proteins) [2,4,21,70]. It is likely that thorough study of various infectious agents aiming at identification of eukaryotic proteins-targeting glycosyltransferases will disclose novel enzymes and hence new mechanisms used by invading bacteria to tyrannize eukaryotic host.

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