Characterization of the enzymatic activity of Clostridium perfringens TpeL
Serge Pauillac, Jacques d’Allayer, Pascal Lenormand, Jean Claude Rousselle, Philippe Bouvet, Michel Popoff

To cite this version:

HAL Id: pasteur-01766020
https://pasteur.hal.science/pasteur-01766020
Submitted on 1 Aug 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - ShareAlike 4.0 International License
Abstract: TpeL is a toxin produced by Clostridium perfringens which belongs to the large clostridial glucosylating toxin family. It was shown that TpeL modifies Ras using UDP-glucose or UDP-N-acetylglucosamine as cosubstrates (Guttenberg et al., 2012; Nagahama et al., 2011). We confirmed that TpeL preferentially glucosaminates the three isoforms of Ras (cH-Ras, N-Ras, and K-Ras) from UDP-N-acetylglucosamine and to a lower extent Rap1a and R-Ras3. In contrast to previous reports, we observed that Rac and Ral are no substrates of TpeL. In addition, we confirmed by in vitro glucosylation and mass spectrometry that TpeL modifies cH-Ras at Thr35.
Highlights

- TpeL is a cytotoxin produced by *Clostridium perfringens* which belongs to the large clostridial glucosylating toxin family.

- TpeL uses both UDP-glucose and UDP-N-acetylglucosamine as cosubstrates but preferentially this latter.

- TpeL modifies mainly cH-Ras, N-Ras, K-Ras and to a lower extent Rap1a, R-Ras3, and Rac1.

- TpeL modifies cH-Ras at Thr35.
CHARACTERIZATION OF THE ENZYMATIC ACTIVITY OF 

*Clostridium perfringens* TpeL

Serge PAUILLAC¹, Jacques D’ALLAYER², Pascal LENORMAND², Jean Claude ROUSSELLE ², Philippe BOUVET¹, Michel R. POPOFF¹

¹Institut Pasteur, Unité des Bactéries anaérobies et Toxines, 75724 Paris cedex15, France
²Institut Pasteur, Plateforme Protéomique, 75724 Paris cedex15, France

Running title: *Clostridium perfringens* TpeL

Key words: *Clostridium perfringens*, toxin, TpeL, GTPase, Ras, Rho, Rac glucosylation

Correspondence to Michel R. Popoff, Institut Pasteur, Unité des Bactéries anaérobies et Toxines, 25 rue du Dr Roux, 75724 Paris cedex 15, France
Tel: 33 1 45688307
Fax: 33 1 45613123
mpopoff@pasteur.fr
Abstract

TpeL is a toxin produced by *Clostridium perfringens* which belongs to the large clostridial glucosylating toxin family. It was shown that TpeL modifies Ras using UDP-glucose or UDP-N-acetylglucosamine as cosubstrates (Guttenberg *et al.*, 2012; Nagahama *et al.*, 2011). We confirmed that TpeL preferentially glucosaminates the three isoforms of Ras (cH-Ras, N-Ras, and K-Ras) from UDP-N-acetylglucosamine and to a lower extent Rap1a and R-Ras3, and very weakly Rac1. In contrast to previous report, we observed that Ral was not substrate of TpeL. In addition, we confirmed by in vitro glucosylation and mass spectrometry that TpeL modifies cH-Ras at Thr35.

1. Introduction

*Clostridium perfringens* is an ubiquitous anaerobic bacterium which is responsible for mild to severe diseases in humans (gas gangrene, gastrointestinal diseases) and animals (mainly gastrointestinal and enterotoxemic diseases). The pathogenicity of this microorganism is based on protein toxins, which are secreted in the culture medium, gastrointestinal tract or tissues (Popoff and Bouvet, 2009). *C. perfringens* is among the bacteria which produce the greatest number of toxins. According to the toxins, which are synthesized, *C. perfringens* strains are divided into several toxinotypes (Petit *et al.*, 1999). Recently, a new toxin, called TpeL (for toxin *C. perfringens* large cytotoxin), was identified in several strains of *C. perfringens* type B and C. TpeL was found to be lethal for mice and cytotoxic for Vero cells and shows a significant homology of its N-terminal region with the catalytic domain of the large glucosylating clostridial toxins which encompass *Clostridium difficile* toxin A (TcdA) and toxin B (TcdB), *Clostridium sordellii* lethal toxin (TcsL) and hemorrhagic toxin (TcsH), and *Clostridium novyi* alpha toxin (TcnA) (Amimoto *et al.*, 2007).

Large glucosylating clostridial toxins (LGCTs) contain at least three functional domains. 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. The central part contains a hydrophobic segment and probably mediates the translocation of the toxin across the membrane. The enzymatic site characterized by the DxD motif surrounded by a hydrophobic region, and the substrate recognition domain are localized within the 543 N-terminal residues corresponding to the natural cleavage site in TcdB (Hofmann *et al.*, 1998; Hofmann *et al.*, 1997; Rupnik *et al.*, 2005). The overall structure of the enzymatic domains of TcdB, TcsL and TcnA is conserved and consists of a β-strain
central core (about 235 amino acids) forming an active center pocket surrounded by numerous
α-helices (Jank and Aktories, 2008; Reinert et al., 2005; Ziegler et al., 2008). LGCTs catalyze
the glucosylation of Rho- and/or Ras-GTPases from UDP-glucose, except TcnA, which uses
UDP-N-acetylglucosamine as cosubstrate. The glucosylating activity supports the main
biological activities of these toxins. But, LGCTs differ in their substrates. Thereby, TcdA and
TcdB glucosylate Rho, Rac and Cdc42 at Thr-37, whereas LcsT glucosylates Ras at Thr-35,
Rap, Ral and Rac at Thr-37 (Just and Gerhard, 2004; Popoff and Bouvet, 2009). The
structural basis of the different recognition of substrates is not well known. Differences in α-
helices, insertions-deletions, probably account for the substrate specificity of each toxin
(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
(Hofmann et al., 1998; Jank et al., 2007). Since TpeL seems to be a natural variant of large
glucosylating clostridial toxins, the characterization of its enzymatic activity and substrate
specificity would permit to better understand the structure/function of this group of toxins.

In this study, we show that TpeL is a glucosylating toxin which targets Ras proteins
and to a lower extent Rap and R-Ras3, and preferentially uses UDP-N-acetylglucosamine as
cosubstrate. Similarly to C. sordellii TcsL, TpeL monoglucosylates Ras at Thr35. During the
progress of this work, related results have been published (Guttenberg et al., 2012).

2. Materials and methods

2.1 Reagents

Uridine-5-diphospho(UDP)-glucose, UDP-N-acetylglucosamine, UDP-galactose,
UDP-glucuronic acid, UDP-galacturonic acid, UDP-mannose, ADP-glucose, ADP-ribose,
CDP-glucose, GDP-glucose, TDP-glucose were from Sigma. Plasmids encoding Rho-, Rac-, Cdc42-GST proteins were from A. Hall.

2.2 Production and purification of recombinant TpeL N-terminus (rN-TpeL)

The DNA coding for the N-terminal part of TpeL was PCR amplified from C.
perfringens type B strain NCTC3181 with primers adding a BamHI site at the 5’ end and a
SalI site at the 3’ end. The PCR product was cloned into pCR2.1 vector (Invitrogen), and the
digested insert with BamHI-SalI was subcloned into pET28a (Novagen) at the corresponding
sites. The recombinant protein was fused to an N-terminal extension containing a 6-histidine
motif from pET28.

*E. coli* BL21 CodonPlus (DE3)-RIL (Stratagene) transformed with recombinant plasmids respectively were grown in LB medium containing kanamycine (50 μg/ml) at 26° C until a OD of 0.8. The expression was induced with IPTG (1 mM) and growth was continued overnight at 26° C. The bacteria were harvested by centrifugation, suspended in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, and protease inhibitors (Calbiochem), and sonicated. The cell debris were separated from the soluble fraction by centrifugation (18000g, 15 min). The soluble fraction was applied on a cobalt column (Talon, Qiagen). The column was washed with lysis buffer, and eluted with 2, 10 and 100 mM imidazole in the same buffer. The fractions containing highly purified recombinant proteins were pooled and dialyzed against 20 mM Hepes, pH 7.5, 150 mM NaCl.

2.3 *In vitro glucosylation*

Glucosylation of small GTPases was achieved as followed according to (Popoff et al., 1996). 2 μl of UDP-[14C]Glucose or UDP-[14C]-N-acetylglucosamine in ethanol (0.2 μCi, 300 mCi/mmol; DuPont NEN, les Ulis France) was dried under vacuum; 1 μg of GST-GTPase protein in a final volume of 20 μl of glucosylation buffer (50 mM triethanolamine pH 7.5, 2 mM MgCl2, 0.3 mM GDP ) were added to the dried UDP-[14C]Glucose. One μl of rN-TpeL (2 mg/ml) was added to start the reaction, which was carried out for 1 h at 37°C. The reaction was stopped by adding 5 μl of 2 x SDS sample buffer, boiled and electrophoresed on a 15% SDS-polyacrylamide gel. After staining with Coomassie blue, followed by distaining, the gel was dried and autoradiographed.

2.4 *Peptide analysis by HPLC, sequencing and mass spectrometry*

cH-Ras (5 μg) was incubated without or with rN-TpeL (10⁻⁷ M) in glucosylation buffer containing UDP-N-acetylglucosamine (5 mM) for 3 h at 37°C. Ras was the subjected to SDS-(14%)PAGE and stained with Amido Black. Ras bands were cut from the gel, washed with distilled water, dried, and resuspended in 150 μl of 50 mM Tris-HCl (pH 8.6) containing 0.001% Tween 20 and 0.2 μg of trypsin (Promega). Following 18 h at 30°C, the preparations were centrifuged, the pellets were washed with 100 μl of 50 mM Tris-HCl (pH 8.6) containing 0.05% SDS and then with 100 μl 60% acetonitrile. The supernatants were recovered and concentrated to 100 μl by Speed Vac. Samples were injected onto a DEAE-HPLC column (Interchim Hema Bio 1000 DEAE, 33 x 1 mm) linked to a C18 reverse phase
HPLC column (Interchim Uptisphere ODBD, 150 x 1 mm) eluted with 2-70% acetonitrile gradient in 0.1% trifluoroacetic acid and monitored at 214 nm.

Separated peptides (1-2 µl on a ProteinChip gold array) were identified by their mass using SELDI-TOF-MS (PCS4000) from Bio-Rad (focus mass = 4000 daltons; laser intensity = 1100 nJ). The matrix was α-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile, 0.5% trifluoroacetic acid.

2.5 MS and MS/MS analysis

Purified HPLC fractions were diluted in 10 µl of CHCA matrix (5 mg/ml in 70% ACN / 30% H₂O / 0.1% TFA) and 0.5 µl of them were spotted on the MALDI plate.

MS spectra were acquired in reflector positive mode using the 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, USA) within a mass range of 800 to 4000 m/z. MS calibration was first carried out to a final mass accuracy of 1 ppm with a mixture of 5 standard peptides: des-Arg¹ Bradykinin, [M+H]⁺=904.468; Angiotensin, [M+H]⁺=1296.685; Glu1-Fibrinopeptide, [M+H]⁺=1570.677; ACTH 1-17, [M+H]⁺=2093.087; ACTH 18-39, [M+H]⁺=2465.199. MS spectra of the purified fractions were then acquired (3000 laser shots were averaged) and calibrated using the external default calibration (10 ppm mass accuracy).

MS/MS were performed in CID positive ion mode with a 2 kV collision energy of and air as collision gas. MS/MS calibration was performed using 4 CID fragments of the Glu1-Fibrinopeptide ([M+H]⁺= 175.119, 684.346, 1056.475 and 1441.634) (mass accuracy of 10 ppm). 4000 to 10 000 laser shots were averaged to acquired MS/MS spectra of the selected precursors. Each spectrum was then calibrated using the external default calibration (mass accuracy of 100 ppm).

MS/MS queries were performed using the MASCOT search engine 2.1 (Matrix Science Ltd., UK) and the SwissProt 57.4 database (470369 sequences; 166709888 residues) with the following parameters: 50 ppm mass tolerance for the precursor and 0.3 Da for MS/MS fragments, trypsin cleavage. Hexose-N-acetylglucosamine (HexNAc) and oxidation of methionins were used as variable modifications. In these conditions, the minimal MASCOT score was 28 for a peptide confidence index ≥ 95%.

3. Results

3.1 TpeL is a glucosyltransferase which targets Ras proteins
As previously found (Amimoto et al., 2007), TpeL shows a significant overall sequence homology with the LGCTs and contains a conserved active enzymatic site (DxD) in the N-terminal part, except that the total protein length is shorter (190 kDa) than that of the glucosylating toxins from *C. difficile*, *C. sordellii* or *C. novyi* (250 to 300 kDa) (Jank and Aktories, 2008; Just and Gerhard, 2004; Popoff and Bouvet, 2009; Rupnik and Just, 2006). The N-terminal domain (amino acid 1 to 543) of LGCTs is the intracellular active domain, which is released into the cytosol through an autoproteolytic process during the toxin endocytosis (Jank and Aktories, 2008). Interestingly, the TpeL N-terminal domain shows a high level of sequence similarity with that of the other LGCTs with an intermediate position between the N-terminal domain of *C. novyi* alpha toxin and those of the *C. difficile* and *C. sordellii* glucosylating toxins (Fig. 1). Therefore, we produced and purified the N-terminal domain of TpeL as recombinant protein (Fig. 3) to check its enzymatic activity. In a first series of experiments we used Rho, Rac, Cdc42 and cH-Ras, which are the representative substrates of LGCTs, in a glucosylation assay with TpeL. As shown in Fig. 2B, TpeL N-terminus glucosylated only cH-Ras and no RhoA, Rac1 or Cdc42. In addition, the glucosylation of cH-Ras was more efficient by using UDP-N-acetylglucosamine than UDP-glucose as cosubstrate. Thereby, recombinant TpeL N-terminal part contains a glucosyltransferase activity specific of cH-Ras protein but not of Rho and Cdc42.

3.2 **TpeL preferentially uses UDP-N-acetylglucosamine as cosubstrate**

In order to determine the cosubstrate requirement for TpeL (10^{-7} M) activity, kinetics of cH-Ras glucosylation was performed in the presence of UDP-[^{14}C]-glucose or UDP-[^{14}C]-N-acetylglucosamine (Fig. 3 A). An almost complete glucosylation of cH-Ras was obtained with TpeL in the presence of UDP-[^{14}C]-N-acetylglucosamine, whereas the reaction with UDP-[^{14}C]-glucose had longer kinetics (Fig. 3A and B). The level of cH-Ras glucosylation was about 100 fold less with UDP-glucose compared to UDP-N-acetylglucosamine. Even after longer incubation period (until 2 h), a 100-fold difference in enzymatic level was observed between both cosubstrates (not shown).

Then, we investigated if UDP-N-acetylglucosamine was the only efficient cosubstrate of TpeL by a competition assay using cH-Ras, UDP-[^{14}C]-N-acetylglucosamine, and various non-radioactive hexose derivatives. As expected, non-radioactive UDP-N-acetylglucosamine totally blocked the transfer of [^{14}C]-N-acetylglucosamine from UDP-[^{14}C]-N-acetylglucosamine on cH-Ras, whereas N-acetylglucosamine was inefficient (Fig. 3C and D).
UDP-glucose and UDP-mannose, a close isoform of UDP-glucose, were also as efficient as UDP-N-acetylglucosamine to block the TpeL-dependent glucosylation of cH-Ras. The other UDP derivatives (UDP-galactose, UDP-glucuronic acid, and UDP-galacturonic acid) also inhibited the glucosylation of cH-Ras but to a lower extent. Among the other nucleoside-diphosphate hexose derivatives, GDP-glucose partially prevented cH-Ras glucosylation. TDP-glucose which contains a pyrimidine base as UDP, was not effective to compete the reaction. This suggests that UDP is a major structure which is recognized by TpeL.

3.3 Ras is the main substrate of TpeL

Next, we investigated the substrates of TpeL. Using a large set of recombinant small GTPases and UDP-N-acetylglucosamine as cosubstrate, we checked the TpeL activity in the in vitro glucosylation assay. As shown in Fig. 4A, Ras proteins including cH-Ras, K-Ras, and N-Ras were the main substrates which were efficiently glucosylated by TpeL. In addition, R-Ras3 and Rap1a, which also belong to the Ras family of proteins, were glucosylated by TpeL, but to a lower extent. The glucosylation level of R-Ras3 was 16% from that of Ras, and that of Rap1a 6% (Fig. 4B). No proteins from the Rho family nor Rab6 and Arf1, representative proteins from the Rab and Arf families, were substrates of TpeL, except Rac1 which was very weakly modified compared to cH-Ras (Fig. 4A). Thereby, TpeL contains a glucosyltransferase activity targeting Ras proteins, and predominantly cH-Ras, K-Ras, and N-Ras.

3.4 TpeL glucosylates cH-Ras at Thr35

In an effort to identify the cH-Ras residue or residues that are modified by TpeL, cH-Ras (5 µg) was incubated with TpeL (10^{-7} M), non-radioactive UDP-N-acetylglucosamine (10 mM) in glucosylation buffer during 3 h at 37°C and then analyzed by SELDI-TOF using a Q10 chip (strong anionic exchanger). cH-Ras prepared in the same conditions but without TpeL was used as reference. As shown in Fig. 5, cH-Ras treated with TpeL in the presence of UDP-N-acetylglucosamine has an increase in mass of 205, which correlates with an addition of a one HexNAc molecule (MW 203). This indicates that TpeL catalyzes the transfer of one HexNAc molecule per cH-Ras molecule.

Next, cH-Ras treated with rN-Tpel or untreated was run on a SDS-PAGE, trypsin digested, and the resulting peptides were separated by HPLC. As shown in Fig. 6A, fraction 14 from rN-Tpel-treated cH-Ras has an increased retention time which corresponds to an increased mass of 203 daltons compared to fraction 15 of untreated cH-Ras as determined by
SELDI-TOF (Fig. 6B). N-terminal sequencing of fractions 14 and 15 showed that the peptides with the sequence SALT and SALTIQ, respectively. This indicates that fraction 14 from rN-TpeL treated cH-Ras and fraction 15 from untreated cH-Ras contained the same trypsin cleavage peptide SALTIQLQNHFVDEYDPTIEDSYR. These results show that the residue modified by rN-TPEL and resulting from an addition of an HexNAc is part of the peptide SALTIQLQNHFVDEYDPTIEDSYR which contains two Thr residues.

Fractions 14 and 15 were further analyzed by MS and MSMS followed by MASCOT searching (Fig. 7 and supplementary Fig. 1 and 2). HexNAc modification was identified on Thr19 of the peptide which corresponds to Thr35 of cH-Ras.

4. Discussion

It is reported that TpeL is produced by several C. perfringens types including types A, B and C and its gene localization on plasmid accounts for horizontal transfer between C. perfringens strains (Amimoto et al., 2007; Sayeed et al., 2010). TpeL synthesis seems to be dependent of the sporulation (Paredes-Sabja et al., 2011) and this novel toxin likely represents an additional virulence factor involved in necrotic enteritis in poultry (Coursodon et al., 2012). Indeed, TpeL is cytotoxic to various cell lines by inducing apoptosis via impairment of the Ras signaling pathway (Guttenberg et al., 2012). However some C. perfringens strains, like MC18, seems to contain a truncated tpel gene resulting in a protein lacking 128 C-terminal amino acids which is not cytotoxic (Guttenberg et al., 2012). In our experience, only a few C. perfringens strains type C isolated from necrotic enteritis in piglets, and a C. perfringens type A strain isolated from food contain tpel gene.

TpeL is related to the LCGTs based on amino acid sequence identity and shows the highest similarity with TcnA. We have analyzed the enzymatic properties of TpeL, from which most of them have been published during the progress of this work (Guttenberg et al., 2012; Nagahama et al., 2011). Albeit confirmatory in a large part, our investigations bring complementary data. TpeL is a glucosytransferase, which retains the enzymatic site DxD of LCGTs and with a global amino acid sequence identity more related to TcnA than to the other LCGTs (Fig. 1). In addition, TpeL displays the same motif, A\textsuperscript{383}NQ\textsubscript{385}, of co-substrate specificity than TcnA, which uses UDP-N-acetylglucosamine as sugar donor, instead of I\textsuperscript{383}NQ\textsubscript{385} in the other LCGTs which catalyzes the transfer of glucose from UDP-glucose (Guttenberg et al., 2012). Using the recombinant catalytic domain (1-543), we showed that TpeL exhibited both UDP-glucoosylation and UDP-N-acetylglocosamination (GlcNAcylation) activities towards cH-Ras, but with a 100 fold more efficiency of GlcNAcylation. UDP is the
major binding determinant since the rN-TpeL GlcNAcylation was impaired by all UDP-hexoses tested in competition assay (Fig. 3C and D). However, UDP-glucose and UDP-N-acetylglucosamine were more efficient competitors than the other UDP-hexoses, indicating a stringent specificity for these two cosubstrates. In contrast, Nagahama et al. report a TpeL enzymatic competition only with UDP-glucose and UDP-N-acetylglucosamine and no with other UDP-hexoses (Nagahama et al., 2011). The bases of these discrepant results are not clear, in our study, the recombinant N-terminal TpeL 1-543 amino was used instead TpeL 1-525 by Nagahama et al. (Nagahama et al., 2011).

As previously found, the three isoforms of Ras including cH-Ras, N-Ras and K-Ras are the main substrates of Tpel. Using a large panel of small GTPases, we observed that rN-TpeL modified cH-Ras, N-Ras and K-Ras and to a lower extent Rap1a and R-Ras3. In contrast to previous findings [Guttenberg, 2012 #3074; Nagahama, 2011 #3076], we showed that Rac1 was a very poor substrate of TpeL in the presence of UDP-N-acetylglucosamine. Only a weak glucosamination was evidenced after a long exposure of the autoradiographies and no Rac glucosylation was observed with rN-TpeL and UDP-glucose. In agreement with Guttenberg et al. (Guttenberg et al., 2012) we showed no modification of Ral by TpeL in the presence of UDP-N-acetylglucosamine or UDP-glucose, whereas Ral has been reported to be a TpeL substrate by Nagahama and coworkers (Nagahama et al., 2011). These different observations might result from the distinct recombinant TpeL fragments used in each study. We confirmed by in vitro glucosylation and mass spectrometry thet TpeL modifies Ras at Thr35. In our conditions, TpeL displays glucosyltransferase activity only with Ras proteins, mainly the three isoforms of Ras and weakly Rap1a and R-Ras3, and is not active with Rho proteins. This opens the way to delineate specific tools directed towards Ras for therapeutic purpose and cell biology investigations.

Acknowledgement

This was supported by Institut Pasteur funding.
References

Aimoto, K., Noro, T., Oishi, E., Shimizu, M., 2007. A novel toxin homologous to large
clostridial cytotoxins found in culture supernatant of Clostridium perfringens type C.
Microbiology 153, 1198-1206.

Coursodon, C.F., Glock, R.D., Moore, K.L., Cooper, K.K., Songer, J.G., 2012. TpeL-
producing strains of Clostridium perfringens type A are highly virulent for broiler
chicks. Anaerobe 18, 117-121.

Guttenberg, G., Hornei, S., Jank, T., Schwan, C., Lu, W., Einsle, O., Papatheodorou, P.,
Aktories, K., 2012. Molecular characteristics of Clostridium perfringens TpeL toxin and
consequences of mono-O-GlcNAcylation of Ras in living cells. J Biol Chem 287,
24929-24940.

the N-terminal region involved in protein substrate recognition. Infect. Immun. 66,
1076-1081.

glucosyltransferase activity of Clostridium difficile Toxin B to the N-terminal part of
the holotoxin. J. Biol. Chem. 272, 11074-11078.

Jank, T., Aktories, K., 2008. Structure and mode of action of clostridial glucosylating toxins:
the ABCD model. Trends Microbiol 16, 222-229.

Jank, T., Giesemann, T., Aktories, K., 2007. Clostridium difficile glucosyltransferase toxin B-

152, 23-47.

Nagahama, M., Ohkubo, A., Oda, M., Kobayashi, K., Amimoto, K., Miyamoto, K., Sakurai,
J., 2011. Clostridium perfringens TpeL glycosylates the Rac and Ras subfamily

Paredes-Sabja, D., Sarker, N., Sarker, M.R., 2011. Clostridium perfringens tpeL is expressed
during sporulation. Microb Pathog 51, 384-388.

Trends Microbiol. 7, 104-110.


Popoff, M.R., Chaves-Olarte, E., Lemichez, E., Von Eichel-Streiber, C., Thelestam, M.,
Chardin, P., Cussac, D., Antonny, B., Chavrier, P., Flatau, G., Giry, M., de Gunzburg,
J., Boquet, P., 1996. Ras, Rap, and rac small GTP-binding proteins are targets for
*Clostridium sordellii* lethal toxin glucosylation. J. Biol. Chem. 271, 10217-10224.

Reinert, D.J., Jank, T., Aktories, K., Schulz, G.E., 2005. Structural basis for the function of
*Clostridium difficile* toxin B. J Mol Biol 351, 973-981.

Rupnik, M., Just, I., 2006. Large clostridial cytotoxins modifying small GTPases, in: Alouf,
J.E., Popoff, M.R. (Eds.), The Sourcebook of Bacterial Protein Toxins, 3° ed. Elsevier,

Characterization of the cleavage site and function of resulting cleavage fragments after
limited proteolysis of *Clostridium difficile* toxin B (TcdB) by host cells. Microbiology
151, 199-208.

among *Clostridium perfringens* type B isolates. Infect Immun 78, 495-504.

Ziegler, M.O., Jank, T., Aktories, K., Schulz, G.E., 2008. Conformational changes and
**Figure legends**

**Figure 1.** Phylogenetic tree of the whole toxin sequence (A) and enzymatic domain (543 N-terminal amino acids) (B) of TpeL (AB262081), TcdA (YP_001087137), TcdB (CAJ67492), TcdBF (CAC19891), TcsL6018 (X82638) and TcnA (CAA88565.1) according to the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

**Figure 2.** TpeL N-terminal part glucosylates Ras but not Rho, Rac, and Cdc42. (A) SDS-PAGE of recombinant N-terminal part of TpeL stained with Blue Coomassie R-250. (B) Glucosylation of Ras, Rho, Rac, or Cdc42 (1 μg recombinant protein) by TpeL N-terminal part (10^{-7} M) in the presence of UDP-[\textsuperscript{14}C]Glucose or UDP-[\textsuperscript{14}C]-N-acetylglucosamine for 1 h at 37°C. The proteins were run on a SDS-PAGE and autoradiographed.

**Figure 3.** TpeL uses preferentially UDP-N-acetylglucosamine as cosubstrate. A kinetics of Ras glucosylation by rN-TpeL. Ras (1 μg) was incubated with rN-TpeL (10^{-7} M) for 1, 5, 10, 15 or 30 min at 37°C in glucosylation buffer containing 7 μM UDP-[\textsuperscript{14}C]-N-acetylglucosamine or UDP-[\textsuperscript{14}C]-glucose. The samples were electrophoresed on a SDS-(14%)PAGE, autoradiographed, and band intensity was quantified by ImageJ (NIH, USA). Experiments were done in triplicate. Means +/- standard deviation are shown. (B) Kinetics of Ras glucosylation by rN-Tpel with UDP-[\textsuperscript{14}C]-glucose as in A but with a different scale of Y axis. Note that Ras glucosylation with UDP-[\textsuperscript{14}C]-glucose is about 100 fold less than with UDP-[\textsuperscript{14}C]-N-acetylglucosamine. (C) Competition assay of Ras glucosylation with various hexose derivatives. Ras (1 μg) was incubated in glucosylation buffer with rN-Tpel (10^{-7} M) and each of the various hexose derivatives (5 mM) for 30 min at 37°C. Then UDP-[\textsuperscript{14}C]-N-acetylglucosamine was added and the preparations were incubated at 37°C for additional 30 min and processed as described above. (D) Quantification of the competition assays.
Figure 4. TpeL recognizes specifically Ras. (A) Glucosylation of recombinant small GTPases (1 μg) with rN-Tpel (10^{-7} M) and UDP-[^{14}C]-N-acetylglucosamine was performed as described in Experimental Procedures for 1 h at 37°C. (B) Quantification of the glucosylated small GTPases. Glucosylation of Ras was defined as 100%.

Figure 5. Mono-N-acetylglicosaminilation of cH-Ras by TpeL. cH-Ras untreated and treated by rN-TpeL (10^{-7} M) in glucosylation buffer containing 5 mM UDP-N-acetylglucosamine for 3 h at 37°C was analyzed by SELDI-TOF mass spectrometry with a Q10 array. Ras treated with rN-TpeL shows an increased mass of 205 Da.

Figure 6. Identification of Ras peptide containing the TpeL modification. (A) Ras untreated and treated by rN-TpeL (10^{-7} M) in glucosylation buffer containing 5 mM UDP-N-acetylglucosamine for 3 h at 37°C was trypsin digested and analyzed by HPLC on a DEAE column linked to a C18 reverse phase column. Fraction 15 from untreated Ras and fraction 14 from rN-TpeL treated Ras were found to contain the same peptide SALTQLQHNFVDEYDPTIEDSYR by N-terminal sequencing. (B) SELDI-TOF mass spectrometry showing that the peptide from fraction 14 (rN-TpeL-treated Ras) has an increased mass of 203 Da versus that of the parental peptide (fraction 15) from untreated Ras.

Figure 7. TpeL mono-N-acetylglicosaminates Ras at Thr35. MS and MS-MS spectrum of fraction 14 from rN-TpeL-treated Ras (A) and fraction 15 from untreated Ras (B).

Supplementary Figure 1. Mascot search results of MS-MS residues of peptide from fraction 14 (rN-TpeL-treated Ras).

Supplementary Figure 2. Mascot search results of MS-MS residues of peptide from fraction 15 (untreated Ras).
Figure 1
Figure 2
Figure 3
Figure 4
Figure

Click here to download high resolution image

Figure 6

Mass (m/z)
Figure 7

A Ras treated with TpeL fraction 14

MS spectrum

[M+H]+ 3170.4993

MSMS spectrum
B

Ras wt fraction 15

[MS spectrum with peaks labeled [M+H]^+ and mass 2967.3958]

MSMS spectrum

Figure 7 (followed)
Mascot Search Results
Peptide View
MS/MS Fragmentation of SALTIQLQHVFDEYDPTIEDSR
Found in **RASH_HUMAN**, GTPase HRas OS=Homo sapiens GN=HRAS PE=1 SV=1
Match to Query 1: 3169.491724 from(3170.499000,1+)

Monoisotopic mass of neutral peptide Mr(calc): 3169.5091
Variable modifications:
T19 : HexNac (T)
Ions Score: 160 Expect: 3.3e-015
Matches (grey background): 54/439 fragment ions using 85 most intense peaks

Supplementary Figure 1
RasH wt fraction 15

Mascot Search Results
Peptide View
MS/MS Fragmentation of SALTQILQNFHVDFYEYDPTIEDSYR
Found in RASH_HUMAN, GTPase HRas OS=Homo sapiens GN=HRAS PE=1 SV=1
Match to Query: 2966.388724 from(2967.396000,1+)

Monoisotopic mass of neutral peptide Mr(calc): 2966.4297
Ions Score: 191 Expect: 2.9e-018
Matches (grey background): 72/457 fragment ions using 83 most intense peaks

Supplementary figure 2