**Clostridium perfringens** TpeL Glycosylates the Rac and Ras Subfamily Proteins

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**Clostridium perfringens** type C has been identified as a causative agent of necrotizing enterocolitis associated with diarrhea and dysentery in infant animals (20, 23). In humans, the bacteria cause necrotic enteritis, which is termed “pig-bel” (20). Type C strains produce alpha-toxin, beta-toxin, beta2-toxin, and perfringolysin O. Beta-toxin is lethal, cytoxic, and related to the pathogenicity of *C. perfringens* type C (20). Beta2-toxin is also considered important for pathogenicity (22). Furthermore, many type C isolates produce a newly discovered toxin named TpeL, which is a truncated homologue of *Clostridium difficile* TcdA and TcdB (1). TpeL was identified in the culture supernatant of *C. perfringens* strain CP4 and is thought to be associated with necrotic enteritis (1).

TpeL is cytoxic, causing cell rounding (1). The molecular mass calculated from the deduced amino acid sequence was 191 kDa, and a signal peptide region was not found within the open reading frame (1). The deduced amino acid sequence exhibited 30 to 39% homology to *Clostridium difficile* toxins A (TcdA) and B (TcdB), *Clostridium sordelli* lethal toxin (TcsL), and *Clostridium novyi* alpha-toxin (TcnA). All of these toxins are called large clostridial toxins (LCTs) (1). At least four domains, “ABCD,” can be distinguished in LCTs. Respectively, they are putatively involved in N-terminal biological activity (A-domain), C-terminal receptor binding (B-domain), autoproteolytic cleavage during toxin-processing (C-domain), and delivery of the A-domain into the cytosol (D-domain) (2). LCTs bind with their B-domain to the membrane receptor of host cells (2, 24). After endocytosis, the toxin inserts into the endosome membrane, most likely via the D-domain. Cellular inositolhexaphosphate (InsP6) activates the protease C-domain (7, 18). This results in cleavage of the toxin and release of the A-domain into the cytosol. In the cytosol, small GTPases are glycosylated and thereby inactivated. The amino acid sequence of TpeL is shorter than that of other LCTs, and the large clostridial toxin A (TcdA) and B (TcdB) and *Clostridium sordellii* lethal toxin (TcsL). We report here the identification of the TpeL-catalyzed modification of small GTPases. A recombinant protein (TpeL1-525) derived from the TpeL N-terminal catalytic domain in the presence of streptolysin O (SLO) induced the rounding of Vero cells and the glycosylation of cellular Rac1. Among several hexoses tested, UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-glucose (UDP-Glc) served as cosubstrates for TpeL1-525-catalyzed modifications. TpeL1-525 catalyzed the incorporation of UDP-Glc into Ha-Ras, Rap1B, and RalA and of UDP-GlcNAc into Rac1, Ha-Ras, Rap1B, and RalA. In Rac1, TpeL and TcdB share the same acceptor amino acid for glycosylation, Thr-35. In Vero cells treated with TpeL1-525 in the presence of SLO, glycosylation leads to a translocation of the majority of Rac1 and Ha-Ras to the membrane. We demonstrate for first time that TpeL uses both UDP-GlcNAc and UDP-Glc as donor cosubstrates and modifies the Rac1 and Ras subfamily by glycosylation to mediate its cytoxic effects.

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UDP-GlcNAc as cosubstrates and mainly glycosylates the Ras subfamily.

MATERIALS AND METHODS

Materials. UDP-GlcNAc, UDP-Man, UDP-GlcNAc, UDP-Glc, UDP-Gal, and SLO were obtained from Sigma (St. Louis, MO). UDP-[14C]Glc and UDP-[14C]GlcNAc were obtained from Perkin-Elmer Life Sciences (Boston, MA). The anti-Rac1 (Mab23A8), Rac1 (Mab102), RhoA, Cdc-42, and Ras antibodies were from Millipore (Billerica, MA). BD biosciences (Franklin Lakes, NJ), Santa Cruz (Santa Cruz, CA), BD biosciences, and Cell signaling (Danvers, MA), respectively. Horseradish peroxidase-labeled anti-rabbit immunoglobulin G (IgG), horseradish peroxidase-labeled anti-mouse IgG, and an enhanced chemiluminescence kit were obtained from GE Healthcare (Tokyo, Japan). Dulbecco modified Eagle medium (DMEM) was purchased from Gibco-BRL (New York, NY). C. difficile TcdB was purchased from List Biol. Lab. (Campbell, CA). Plasmids encoding GST-RhoA, GST-Rac1, GST-Cdc42, and GST-Ha-Ras were provided by H. Horiguchi (Osaka University) and plasmids encoding GST-RaA and GST-Rap1B by M. Matsuda (Kyoto University). A monoclonal anti-TpeL antibody was prepared as previously described (1). All other chemicals were of the highest grade available from commercial sources.

Construction of TpeL1-525. To prepare the catalytic domain of TpeL, we constructed TpeL1-525, from which 1,125 amino acids have been from the C terminus of TpeL (residues 526 to 1651), by PCR using the DNA of the C. perfringens type C strain MC18 (1), a forward primer (5'-AGGGGATCTAGGGTTAATGTCAAAAAAG3') encoding a BamHI site (indicated in boldface), and a reverse primer (5'-TCTCCGTAGTTAATCTATGTTTTCAAAA3') encoding a downstream Xhol restriction site (indicated in boldface). PCR products were digested with BamHI and Xhol and ligated into BamHI- and Xhol-digested pGEX-4T-1 (GE Healthcare) so that the correct reading frame was maintained with the thrombin cleavage site under the glutathione S-transferase (GST) gene (pGEX-TpeL1-525). The construct was checked by sequencing with an ABI Prism dye terminator cycle sequencing ready reaction kit and ABI 310 cycle sequencer (Life Technologies, Carlsbad, CA).

Expression and purification of TpeL1-525. The recombinant TpeL1-525 was expressed as a protein fused with GST in E. coli BL21 as described previously (15). After growth (at 30°C) and induction (with IPTG [isopropyl-β-D-thiogalactopyranoside] to 0.5 mM) of a large culture, the cells were centrifuged and disrupted by a sonicator on ice in a short burst. Centrifugation of the lysate and rescaling, the cells were rinsed with 5 ml of ice-cold phosphate-buffered saline and scraped off in 300 µl of lysis buffer ([50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM MgCl2, 40 µg of aprotinin/ml, 0.1 mM phenylmethylsulfonyl fluoride, 20 µg of leupeptin/ml, 80 µg of benzamidine/ml] per dish. The cells were disrupted mechanically by sonication (five times on ice) and then centrifuged for 10 min at 1,000 x g to remove the nuclear fraction and intact cells. The supernatant (1 mg/ml) was used as the cell lysate (8).

Fractionation of cell lysates. Lysates were centrifuged at 100,000 x g for 1 h to prepare cytosolic and total particulate fractions. The high-speed pellet, which was maintained with the thrombin cleavage site under the glutathione S-transferase (GST) gene (pGEX-TpeL1-525), from which 1,125 amino acids have been from the C terminus of TpeL (residues 526 to 1651), by PCR using the DNA of the C. perfringens type C strain MC18 (1), a forward primer (5'-AGGGGATCTAGGGTTAATGTCAAAAAAG3') encoding a BamHI site (indicated in boldface), and a reverse primer (5'-TCTCCGTAGTTAATCTATGTTTTCAAAA3') encoding a downstream Xhol restriction site (indicated in boldface). PCR products were digested with BamHI and Xhol and ligated into BamHI- and Xhol-digested pGEX-4T-1 (GE Healthcare) so that the correct reading frame was maintained with the thrombin cleavage site under the glutathione S-transferase (GST) gene (pGEX-TpeL1-525). The construct was checked by sequencing with an ABI Prism dye terminator cycle sequencing ready reaction kit and ABI 310 cycle sequencer (Life Technologies, Carlsbad, CA).

RESULTS

Cytotoxic effect of TpeL1-525. TpeL was toxic to Vero cells (1). We tested the cytotoxicity of the recombinant TpeL1-525, a glycosyltransferase domain of TpeL, to Vero cells. TpeL1-525 alone was without any cytotoxic effects (Fig. 2C). This finding indicates that TpeL induces cytotoxic effects through the binding of its C-terminal region to cell surface receptors. To confirm this, TpeL1-525 was transported into Vero cells using the pore-forming toxin SLO as a delivery system (25). As shown in Fig. 2B, in the presence of SLO, TpeL1-525 caused the rounding of cells like the native toxin as reported previously (1). TpeL1-525 at 1 to 10 µg/ml in the presence of SLO induced cell rounding in a dose-dependent manner (Fig. 2C). 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 de-
creased in a dose-dependent manner (data not shown). The cytotoxicity induced by TpeL1-525 was completely neutralized by a monoclonal anti-TpeL antibody and heat-inactivated TpeL1-525 did not induce cell rounding.

Glycosylation of Rac1. Rac1 is the only substrate GTPase inactivated by all LCTs. We investigated whether TpeL1-525 in the presence of SLO glycosylates Rac1 in Vero cells. Genth et al. (9) reported that the recognition of Rac1 by anti-Rac1 (Mab102) is impaired by the glycosylation of Thr-35 in Rac1. The intracellular level of unmodified Rac1 in Vero cells treated with TpeL1-525 in the presence of SLO was determined using anti-Rac1 (Mab102) (Fig. 3). TpeL1-525 caused a decrease in the cellular level of unmodified Rac1 (Fig. 3A). In addition, a quantitative densitometric analysis indicated that TpeL1-525 in the range of 1 to 10 μg/ml reduced the relative band intensities applying the antibody Rac1 (Mab102) in a dose-dependent manner (Fig. 3B). We applied an alternative anti-Rac1 (Mab23A8) recognizing glycosylated and unglycosylated Rac1 (total Rac1) to analyze cellular Rac1 levels. As shown in Fig. 3A, cellular Rac1 levels did not decrease in the cells treated with TpeL1-525 in the presence of SLO. These observations demonstrated that the decreasing levels of Rac1 in Vero cells treated with TpeL1-525 in the presence of SLO observed with anti-Rac1 (Mab102) were due to impaired recognition by anti-Rac1 (Mab102), suggesting that TpeL1-525 glycosylates Rac1 in Vero cells.

Substrate specificity. LCTs use UDP-Glc or UDP-GlcNAc as a cosubstrate (2). The specificity of TpeL1-525 was examined by incubating UDP-[14C]Glc or UDP-[14C]GlcNAc and TpeL1-525 with various GTPases. As shown in Fig. 4, Ha-Ras, RaLA, and Rap1B, the members of the Ras subfamily, were substrates for TpeL1-525-catalyzed glucosylation, whereas other GTPases of the Ras superfamily, namely, RhoA, Cdc42, and Rac1, were not modified in vitro by TpeL1-525 in the presence of UDP-[14C]Glc. As illustrated in Fig. 4, Rac1, Ha-Ras, RaLA, and Rap1B were N-acetylglycosaminylated by TpeL1-525, but RhoA and Cdc42 were not. Heat denaturation of either GTPases or TpeL1-525 completely inhibited glycosylation and N-acetylglycosaminylation (data not shown), indicating that the native protein structure is essential for this type of modification. Thus, TpeL1-525 modifies the same recombinant substrates as do TcsL from C. sordellii and TcdB-1470 from C. difficile 1470 (13). To investigate the type of sugar incorporated...
into Ha-Ras and Rac1, several UDP-hexoses were tested. Because Ha-Ras was incubated with TpeL1-525 and UDP-[14C]Glc (A) or UDP-[14C]GlcNAc (B), respectively, in the presence of various nucleotide-hexoses at 37°C for 120 min. Labeled proteins were analyzed by SDS-PAGE and autoradiography. The amount of glycosylation was calculated as the percentage of the untreated control using densitometric analysis of the autoradiography. One representative experiment from three is shown. (C) Acceptor amino acid. Rac1 was glycosylated with either TpeL1-525 (20 μg/ml) or TcdB (5 μg/ml) in the presence of 30 μM UDP-[14C]GlcNAc (lane 1) or 30 μM UDP-[14C]Glc (lane 2). For sequential glycosylation, Rac1 was glycosylated with TpeL1-525 (lane 3) in the presence of 30 μM unlabeled UDP-GlcNAc for 60 min at 37°C and then with TcdB in the presence of 30 μM UDP-[14C]Glc. In lane 4, glycosylation with TcdB and 30 μM unlabeled UDP-Glc was followed by a second glycosylation with 30 μM UDP-[14C]GlcNAc and TpeL1-525. Labeled proteins were analyzed by SDS-PAGE and autoradiography.

Membrane binding of glycosylated GTPase. We investigated whether the glycosylation of cellular small GTPases occurred in the cells treated with TpeL1-525 in the presence of SLO. It was reported that GTPases glycosylated by LCTs are accumulated in the cell membranes (2, 8). Therefore, we studied the membrane binding of glycosylated GTPases. After incubation of the cells with SLO alone or SLO plus TpeL1-525, the membranes were prepared from the cells. The membrane levels of Rac1, Ha-Ras, RhoA, and Cdc42 were determined by using anti-Rac1 (Mb23A8), anti-Ha-Ras, anti-RhoA, and anti-Cdc42 antibodies, respectively. As shown in Fig. 6, Rac1 and Ha-Ras in the membranes increased in TpeL1-525-treated cells in the presence of SLO compared to the level in the cells treated with SLO alone. TpeL1-525 caused a 2- to 3-fold increase in membrane levels of Rac1 and Ha-Ras. RhoA and Cdc42 levels were not altered by SLO plus TpeL1-525.
DISCUSSION

TpeL is the first LCT shown to utilize UDP-GlcNAc and UDP-Glc to modify small GTP-binding proteins. The substrate specificity of TpeL is similar to that of C. sordellii TcsL. TpeL glycosylates Ha-Ras, RaLA, Rap1B, and Rac1.

TpeL-525 in the presence of SLO induced cell rounding and the detachment of cells from the dish. The cytotoxicity was inhibited by the anti-TpeL antibody, and heat-inactivated TpeL-525 was not cytotoxic. On the other hand, TpeL-1-525 by itself did not have the cytotoxic effects. The results indicated that the N- and C-terminal regions 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 (14, 17). 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 (14, 17). 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 (14, 17). 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 (14, 17).

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 (2, 13). TpeL inactivates Rac1 through the glycosylation of Thr-35.

TpeL glycosylated Rac1, as well as the Ras subfamily consisting of Ha-Ras, RaLA, and Rap1B, but not RhoA and Cdc42. Important differences in substrate specificity have been detected among the various LCTs. Whereas TcdA, TcdB, and TcsA modify most RhoA, Rac1, and Cdc42 isoforms, TcsL glycosylates Rac1 but not RhoA or Cdc42 (24). On the other hand, TcsL also modifies the Ras subfamily, including Ras, Rap, and Ral isoforms (24). Thus, TpeL modifies similar substrates to TcsL. It was reported that Arg-455, Asp-461, Lys-463, and Gly-472 and residues of helix α17 (e.g., Glu-449) of TcdB are essential for enzyme-RhoA recognition (12). Changing the respective amino acid residues in TcsL to those of TcdB reduced glycosylation of Ras by TcsL (12). Furthermore, the introduction of helix α17 of TcdB into TcsL caused a reduction in the glycosylation of Ras subfamily proteins but permitted the glycosylation of RhoA, indicating that helix α17 is involved in RhoA’s recognition by TcdB (12). Glu-449, Lys-463, and Gly-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 (1). 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.

In conclusion, 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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