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Chimeric Clostridial Cytotoxins: Identification of the N-Terminal Region Involved in Protein Substrate Recognition

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***Clostridium sordellii* lethal toxin is a member of the family of large clostridial cytotoxins that glucosylate small GTPases. In contrast to *Clostridium difficile* toxins A and B, which exclusively modify Rho subfamily proteins, *C. sordellii* lethal toxin also glucosylates Ras subfamily proteins. By deletion analysis and construction of chimeric fusion proteins of *C. sordellii* lethal toxin and *C. difficile* toxin B, we localized the enzyme activity of the lethal toxin to the N terminus of the holotoxin and identified the region involved in protein substrate specificity. The toxin fragment of the N-terminal 546 amino acid residues of *C. sordellii* lethal toxin glucosylated Rho and Ras subfamily proteins, as the holotoxin did. Deletion of a further 30 amino acid residues from the C terminus of this active fragment drastically reduced glucotransferase activity and blocked glucohydrolase activity. Exchange of amino acid residues 364 through 516 of lethal toxin for those in the active toxin B fragment (1 to 546) allowed glucosylation of Ras subfamily proteins. In contrast, the chimera with amino acids 1 to 364 from toxin B, 365 to 468 from lethal toxin, and 469 to 546 from toxin B exhibited markedly reduced modification of Ras subfamily proteins, whereas modification of Rac and Cdc42 was hardly changed. The data indicate that the region of amino acid residues 364 through 516 primarily defines the substrate specificity of *C. sordellii* lethal toxin.**

Clostridium difficile produces two potent toxins (toxins A and B) which are causally involved in antibiotic-associated diarrhea and pseudomembranous colitis (5, 17, 26). Both toxins exert their cytotoxic effects on eukaryotic target cells by glucosylation of GTP-binding proteins of the Rho subfamily but not other small GTPases (1, 15, 16). With UDP-glucose as a cosubstrate, the toxins glucosylate Rho, Rac, and Cdc42 at threonine 37 and threonine 35, respectively. This modification inhibits the biological activity of the small GTPases. Because Rho subfamily proteins regulate the actin cytoskeleton (6, 21, 25), glucosylation results in depolymerization of the actin cytoskeleton and inhibition of signaling pathways which are controlled by these GTPases (15, 24).

Toxins A and B belong to the family of large clostridial cytotoxins, with masses of ~308 and ~270 kDa, respectively (4). By analogy with well-studied toxins like diphtheria toxin, it was suggested that the *C. difficile* toxins are constructed of three major domains (3, 18, 29). According to this model, the C-terminal part of repetitive oligopeptides participates in binding to the target cell while the small hydrophobic region in the middle of the protein was suggested to be involved in translocation and the N terminus was suggested to be involved in biological activity. Moreover, it was recently shown that a fragment of the N-terminal 546 amino acid residues of ToxB possesses full glucosyltransferase activity and is able to induce the typical cytotoxic effects after microinjection (10).

The LT from *Clostridium sordellii* is another member of the family of large clostridial cytotoxins (2, 4, 8). This toxin, with a molecular mass of ~270 kDa, is involved in gas gangrene in humans and diarrhea and enterotoxemia in domestic animals (9, 20). Also, LT possesses glucosyltransferase activity, sharing the cosubstrate UDP-glucose with the *C. difficile* toxins (11, 14, 23). In contrast to ToxB, LT exhibits a different protein substrate specificity. While *C. difficile* (VPI10463) ToxB modifies all Rho subfamily proteins studied so far, LT (from *C. sordellii* 6018) was shown to also modify Ras subfamily proteins like Ras, Ral, and Rap (11, 14, 23). To gain more insight into the structure-function relationship of the lethal *C. sordellii* toxin, we attempted to localize the protein regions of LT and ToxB involved in substrate specificity. To this end, we constructed various chimeric proteins of ToxB and LT covering the enzymatically active part of the N terminus. Here we report that the protein substrate specificities of LT and ToxB are primarily defined by the region between amino acid residues 364 and 516.

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MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: GST, glutathione *S*-transferase; ToxB, *C. difficile* toxin B; LT, *C. sordellii* lethal toxin; CDB1, N-terminal *C. difficile* toxin B fragment (amino acid residues 1 to 900); 1-546B, N-terminal *C. difficile* toxin B fragment (amino acid residues 1 to 546); 1-516B, N-terminal *C. difficile* toxin B fragment (amino acid residues 1 to 516); CS1, N-terminal *C. sordellii* lethal toxin fragment (amino acid residues 1 to 900); 1-546LT, N-terminal *C. sordellii* lethal toxin fragment (amino acid residues 1 to 546); 1-517LT, N-terminal *C. sordellii* lethal toxin fragment (amino acid residues 1 to 517); 1-468LT, N-terminal *C. sordellii* lethal toxin fragment (amino acid residues 1 to 468); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Materials. ^{14}C-labeled UDP-hexoses were obtained from DuPont, NEN (Dreieich, Germany). PCR primers were from MWG Biotech (Ebersberg, Germany). All other reagents were of analytical grade and were purchased from commercial sources. LT of *C. sordellii* 6018 was purified as described for ToxB of *C. difficile* VPI10463 (13).

PCR amplification. Amplification of the ToxB fragment CDB1 and construction of C-terminal-truncated fragments 1-546ToxB and 1-516ToxB were done as described previously (10).

C. sordellii 6018 (8) was used as the source for preparation of the chromosomal DNA as described previously (10). Amplification of the LT 6018 toxin fragment CS1 was performed with PCR System 2400 from Perkin-Elmer (Norwalk, Conn.) and the primer pair CS1C-CS1N (5′-AGATCTATGAACTTAGTTAACAAAG CC-3′ and 5′-GGATCCGAACCTTATCCTAAATCC-3′). The reaction was carried out with 300 nmol of each primer and 250 ng of chromosomal DNA for 30 cycles (denaturation, 94°C, 10 s; annealing, 48°C, 30 s; elongation, 68°C, 3 min) in a total volume of 100 μl. The amplified DNA fragments were cleaved with *Bgl*II/*Bam*HI and cloned in the pGEX2T (Pharmacia, Freiburg, Germany) expression vector.

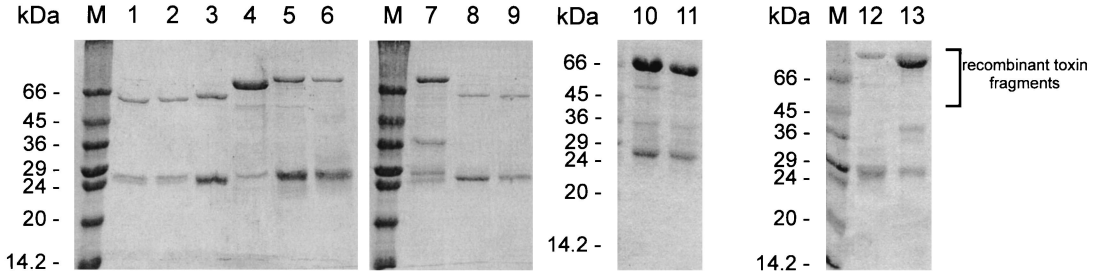


FIG. 1. Purification of recombinant toxin chimeras. The N-terminal toxin chimeras were constructed as GST fusion proteins, expressed in *E. coli*, and purified by affinity chromatography and subsequent thrombin cleavage. Lanes 1 to 3 and 8 to 9, toxin chimeras 1-516LT517-546ToxB (lane 1), 1-134ToxB135-516LT517-546ToxB (lane 2), 1-134LT135-516ToxB517-546LT (lane 3), 1-364ToxB365-516LT517-546ToxB (lane 8), and 1-364LT365-516ToxB517-546LT (lane 9) after thrombin cleavage of the fusion proteins. Lanes 4 to 7 and 10 to 13, GST fusion proteins of 1-468LT469-546ToxB (lane 4), 1-468ToxB469-546LT (lane 5), 1-271ToxB272-546LT (lane 6), 1-271LT272-546ToxB (lane 7), 1-546LT (lane 10), 1-517LT (lane 11), 1-364ToxB365-468LT469-546ToxB (lane 12), and 1-364LT365-468ToxB469-546LT (lane 13). Two micrograms of protein was loaded on lanes 1 to 3 and lanes 5, 6, 8, and 12; 4 μg of protein was loaded on lanes 4, 7, 10, 11, and 13. The band at 26 kDa represents GST, which does not interfere with the assays.

C-terminal truncation of CS1. Further C-terminal deletions of CS1 were done by restriction enzyme cleavage with *Bsa*BI/*Eco*RI for 1-546LT, *Spe*I/*Eco*RI for 1-517LT, and *Afl*II/*Sma*I for 1-468LT. Religation of the resulting truncated fragments was performed after treatment with DNA polymerase I large (Klenow) fragment (NEBiolabs, Schwalbach, Germany).

Construction of ToxB-LT chimeras. (i) 1-516LT517-546ToxB. CS1 and CDB1 were digested with *Spe*I/*Sma*I or *Nhe*I/*Sma*I, respectively. The mobilized *Nhe*I-*Sma*I fragment of CDB1 was cloned into the pGEX2T vector containing the truncated CS1(Δ*Spe*I/*Sma*I). The resulting chimera was cleaved by *Bsa*BI/*Sma*I double digestion and religated.

(ii) 1-134ToxB135-516LT517-546ToxB and 1-134LT135-516ToxB517-546LT. After the digestion of 1-546LT and 1-546B with the restriction enzymes *Nsi*I/*Spe*I or *Nsi*I/*Nhe*I, respectively, the resulting fragments were swapped and ligated.

(iii) 1-468ToxB469-546LT and 1-468LT469-546ToxB. CDB1 and CS1 were cleaved with *Sma*I/*Afl*II, and the resulting fragments were exchanged and ligated. Further truncation of the resulting chimeras was performed by a double digestion with *Bsa*BI/*Sma*I.

(iv) 1-364ToxB365-516LT517-546ToxB and 1-364LT365-516ToxB517-546LT. A *Dra*III site in 1-546LT was constituted by site-directed mutagenesis of position 1091 within LT from A to G. Mutagenesis was done with the QuickChange kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions with the primer pair QC1 and QC2 (5′-GAAATTTTTTTGCCACTTGGTGATATAAAAG-3′ and 5′-CTTTTATATCACCAAGTGGCAAAAAAATTTC-3′). The mutated 1-546LT D364G, which showed full enzymatic activity, and 1-546ToxB were digested with *Dra*III/*Spe*I or *Dra*III/*Nhe*I, respectively, and the fragments were exchanged and ligated.

(v) 1-271ToxB272-546LT and 1-271LT272-546ToxB. The Seamless Cloning kit (Stratagene) was used to generate these chimeras. Primers were constructed in order to locate the junction at the 5′ terminus at position 811 of the cloned toxin fragments and to locate the junction at the 3′-terminus at position 991 in pGEX2T. Amplification was done with the primer pairs SC1 and SC2 (5′-GGGGCTCTTCAATATTAAGAATATCTGCATTAAAAGAAATTG-3′ and 5′-GGGGTCTTCACCGTCATCACCGAAACGGGCGGA-3′) for the insert 271-546ToxB, SC3 and SC4 (5′-GGGGCTCTTCATATATCTGATGCAGCTGCTAAATTATTTG-3′ and 5′-GGGGCTCTTCACGGTGAAAACCTCTGACACATGCAG-3′) for the pGEX2T vector including 1-270ToxB, SC7 (5′-GGGGCTCTTCAATATTACGAATATCTATGTTAAAAGAAATG-3′) and SC2 for the insert 271-545LT, and SC5 (5′-GGGGCTCTTCAQTATATCAGAAGCAGCAGCCAAATTCCA-3′) and SC4 for the pGEX2T vector including 1-270LT. Procedures for cloning of the resulting fragments were carried out in accordance with the manufacturer's instructions.

Sequencing. Sequencing of CS1 and all its truncated derivatives was done with the ABI PRISM dye terminator cycle-sequencing ready-reaction kit (Perkin-Elmer) to check for both correct cloning and mutations due to PCR amplification. Sequencing was performed at least twice with overlapping DNA fragments.

Expression of recombinant proteins. The recombinant GTP-binding proteins RhoA, Rac, Cdc42, and Ha-Ras were prepared from their fusion proteins as described previously (12). The recombinant toxin fragments were expressed and purified as GST fusion proteins in accordance with the manufacturer's instructions (Fig. 1). GST fusion proteins from the *Escherichia coli* expression vector pGEX2T were isolated by affinity chromatography with glutathione-Sepharose (Pharmacia) followed by cleavage of the toxin fragment proteins from the GST fusion protein by thrombin treatment (100 μg/ml for 30 min at 22°C). Removal of thrombin was achieved by binding to benzamidine-Sepharose.

Glucosylation reaction. Rat brain tissue lysate (1 mg/ml) or recombinant GTP-binding proteins (50 μg/ml) were incubated with LT 6018 (100 nM; 1 μg/ml), recombinant toxin fragments (100 nM), or chimeric fragments of ToxB and LT (100 nM) in a buffer containing 50 mM HEPES (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 1 mM $MnCl_2$, 100 μg of bovine serum albumin/ml, and 10 to 30 μM [^{14}C]UDP-glucose for 30 min at 37°C. The total volume was 20 μl. Labeled proteins were analyzed by SDS-PAGE and subsequently by phosphorimaging (Molecular Dynamics).

Glucohydrolase reaction. ToxB, LT 6018, 1-546ToxB (100 nM each), 1-546LT (50 nM), and 1-516ToxB and 1-517LT (1 μM each) were incubated with 20 μM [^{14}C]UDP-glucose and 100 μM unlabeled UDP-glucose in a buffer containing 50 mM HEPES (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 100 μM bovine serum albumin, and 1 mM $MnCl_2$. The total volume was 20 μl. For the time course, 1.5-μl samples were taken out at each time point and subjected to thin-layer chromatography with polyethyleneimine-cellulose plates (catalog no. 1.05579; Merck, Darmstadt, Germany) and 0.2 M LiCl as a mobile phase to separate hydrolyzed glucose from UDP-glucose. The plates were dried and analyzed by phosphorimaging.

RESULTS

We have shown that the N-terminal 546 amino acids of ToxB are sufficient for full glucosyltransferase activity (10). To test whether this also holds true for the LT of *C. sordellii*, we cloned a fragment consisting of the 546 N-terminal amino acids of LT in the expression vector pGEX2T. Then we compared the purified recombinant protein with the holotoxin in a glucosylation assay. As shown in Fig. 2, the toxin fragment 1-546LT glucosylated Rac, Cdc42, Ras, Ral, Rap, and, to a minor extent, Rho. Thus, this fragment exhibited the same substrate specificity as the LT holotoxin. In contrast, a fragment consisting of the N-terminal 517 amino acid residues glucosylated only Ras and Rap to a minor extent but not other GTPases. Further deletions resulted in a fragment of 468 N-terminal amino acid residues that was totally inactive (not shown). To get more quantitative data on the enzyme activity of the toxin fragments, a time course of the glucosylation of Ras was performed. As shown in Fig. 3, whereas LT holotoxin and 1-546LT exhibited very similar glucosyltransferase activities, the activity

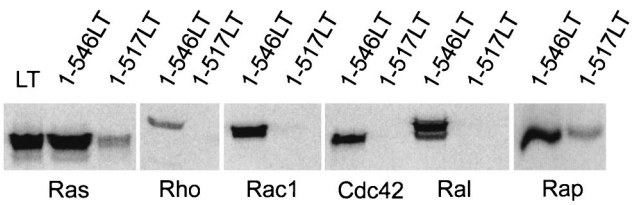


FIG. 2. Protein substrate specificity of glucosylation by LT, 1-546LT and 1-517LT. Recombinant Rho, Rac1, Cdc42, Ras, Ral, and Rap (1 μg of each) were glucosylated by LT (100 nM) and 1-546LT and 1-517LT (100 nM each) in the presence of [^{14}C]UDP-glucose for 30 min. Then, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (shown).

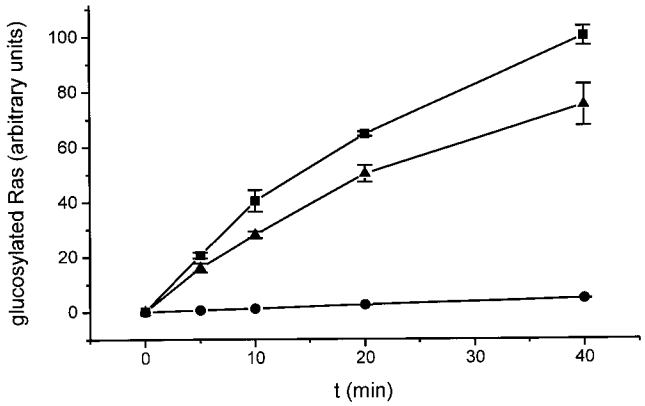


FIG. 3. Time course of the glucosylation of Ras by LT and by the N-terminal toxin fragments 1-546LT and 1-517LT. Ras (1 μg) was incubated with LT (1 nM; ▲) or purified N-terminal toxin fragments 1-546LT (1 nM; ■) and 1-517LT (1 μM; ●), respectively, in the presence of [^{14}C]UDP-glucose (10 μM) for the indicated times (t). Then, labeled proteins were analyzed by SDS-PAGE and phosphorimaging. Time points represent the averages of three experiments.

of the fragment 1-517LT was reduced about 20-fold. To test the cosubstrate specificity of 1-546LT, we studied the ability of this fragment to glucosylate Ras in the presence of [^{14}C]UDP-glucose, [^{14}C]UDP-galactose, or [^{14}C]UDP-*N*-acetylglucosamine, respectively. Figure 4 shows that only UDP-glucose served as a cosubstrate for the 1-546LT fragment.

In the absence of a protein substrate, large clostridial cytotoxins possess glucohydrolase activity to split UDP-glucose into UDP and glucose (16). Therefore, we studied the glucohydrolase activities of LT and ToxB holotoxins and compared them with those of the respective toxin fragments. As shown in Fig. 5A, fragment 1-546LT exhibited the same glucohydrolase activity as the holotoxin. In contrast, the small fragment of 517 amino acid residues was inactive even when a 20-fold-higher concentration of the protein fragment was applied in the assay. The same results were obtained with ToxB and its fragments (Fig. 5B).

As mentioned above, LT and ToxB differ in their protein substrate specificities. Whereas the substrate specificity of ToxB is limited to the Rho subfamily, LT is capable of modifying Ras subfamily proteins (11, 14, 23). To define the structural basis of these differences in protein substrate specificity, we constructed chimeras covering the N-terminal 546 amino acid residues of the toxins, which apparently harbor glucosyltransferase activity. All these chimeras were expressed as GST

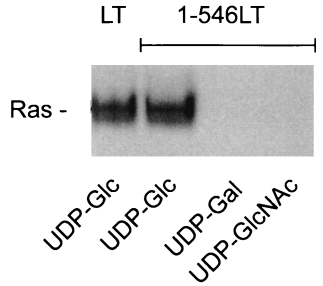


FIG. 4. Cosubstrate specificity of glucosylation by LT and 1-546LT. Recombinant Ras (1 μg) was incubated with LT and 1-546LT in the presence of [^{14}C]UDP-glucose (UDP-Glc), [^{14}C]UDP-galactose (UDP-Gal), and [^{14}C]UDP-*N*-acetylglucosamine (UDP-GlcNAc) for 30 min. Thereafter, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (shown).

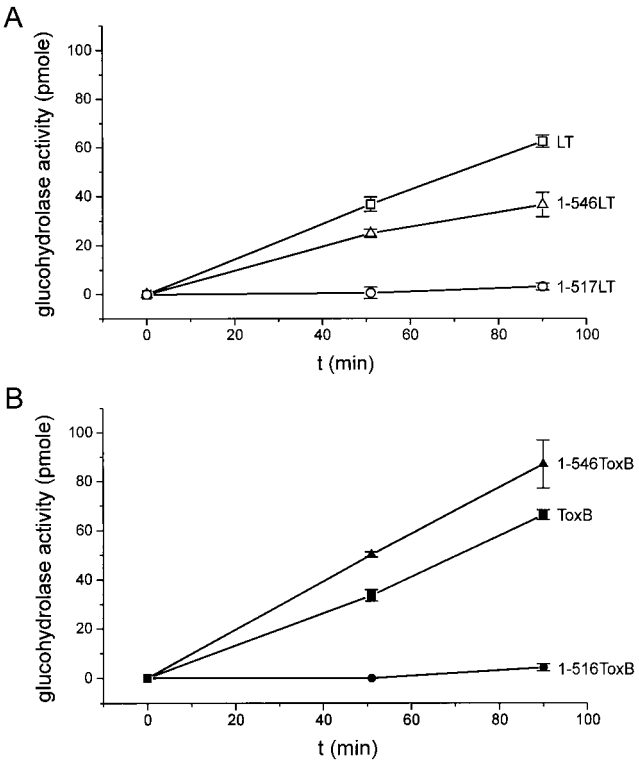
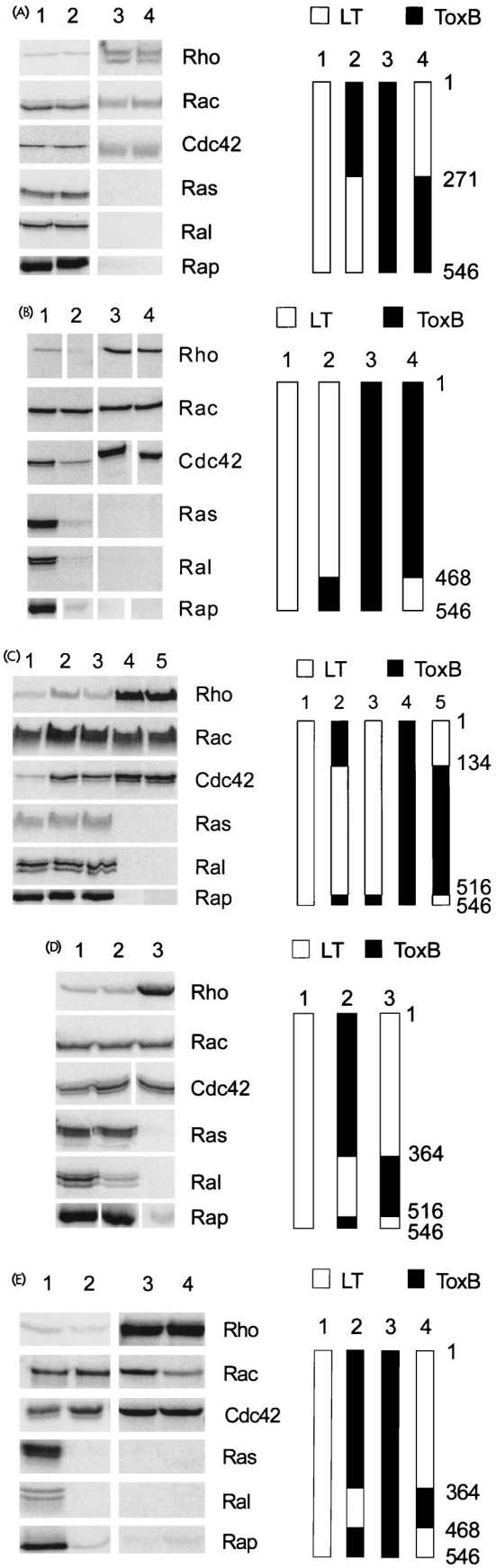


FIG. 5. Time course of glucohydrolase activity of ToxB, LT, and recombinant toxin fragments. (A) LT (100 nM), 1-546LT (100 nM), and 1-517LT (1 μM) were incubated with 20 μM [^{14}C]UDP-glucose and 100 μM UDP-glucose in a total volume of 20 μl. At the indicated time points (t), 1.5-μl samples were taken and analyzed by thin-layer chromatography and phosphorimaging. Time points represent the averages of three experiments. (B) ToxB (100 nM), 1-546ToxB (100 nM), and 1-516ToxB (1 μM) were incubated with 20 μM [^{14}C]UDP-glucose and 100 μM UDP-glucose in a total volume of 20 μl. At the indicated time points, 1.5-μl samples were taken and analyzed by thin-layer chromatography and phosphorimaging. Glucohydrolase activity is given as picomoles of hydrolyzed UDP-glucose per total UDP-glucose (180 pmol).

fusion proteins in *E. coli*, purified, analyzed by SDS-PAGE, and then used in the glucosylation assay. At first, we constructed chimeric proteins from the N-terminal 271 amino acid residues of LT or ToxB fused to amino acid residues 272 through 546 of ToxB and LT, respectively. Figure 6A shows the substrate specificities of these two chimeras. The protein substrate specificities of both chimeras were clearly defined by their C-terminal parts. Thus, the fusion protein was LT-like when amino acid residues 271 through 546 were from LT and the chimera was ToxB-like when these residues were from ToxB. Next we reduced the lengths of these C-terminal parts of the fusion toxins and made chimeras from 468 amino acid residues of LT and ToxB fused to amino acid residues 469 through 546 of ToxB and LT, respectively. In these cases, the chimeras did not show corresponding properties. Whereas the chimera 1-468ToxB469-546LT exhibited the typical substrate specificity of ToxB, with modification of the Rho subfamily proteins but not Ras proteins, the reverse chimera, 1-468LT469-546ToxB, clearly showed reduced activity on Ras subfamily proteins, suggesting that the region of amino acid residues 468 through 546 is important for defining the substrate specificity of LT (Fig. 6B). The chimera 1-134ToxB135-516LT517-546ToxB exhibited the typical LT substrate specificity, and the complementary chimera, 1-134LT135-516ToxB 517-546LT, behaved like ToxB (Fig. 6C). To further delimit



the region defining the LT substrate specificity, the chimera 1-364ToxB365-516LT517-546ToxB was constructed (Fig. 6D). This fusion protein showed a substrate specificity very similar to that of control LT, with a reduction in the modification of Ral. The complementary chimera, 1-364LT365-516ToxB517-546LT, exhibited properties of control ToxB in that it catalyzed an increased modification of Rho. However, this fusion protein also showed a minor modification of Rap and Ras, which we never observed with the control ToxB. We also reduced the internal LT or ToxB part of the chimeras. The resulting 1-364ToxB365-468LT469-546ToxB chimera showed no modification of Ras and Ral, and modification of Rap was clearly reduced (Fig. 6E). The modification of Rho by this chimera was poor, similar to that by control LT. The complementary 1-364LT365-468ToxB469-546LT chimera was mainly characterized by modification of Rho subfamily proteins, with a minor glucosylation of Rap. A summary of the glucosylation patterns of the different chimeras is given in Fig. 7.

DISCUSSION

In this paper we present evidence that the glucosyltransferase activity of the LT from *C. sordellii* is located at the N-terminal part of the holotoxin. Very similar to what has been shown for the related ToxB from *C. difficile* (10), the N-terminal 546 amino acid residues of LT harbor the glucosyltransferase activity of the toxin and are sufficient for full in vitro glucosyltransferase activity. This active fragment of 546 amino acid residues also showed the same substrate and cosubstrate specificity as the holotoxin. When a further 30 amino acid residues at the C terminus of this fragment were deleted, the enzyme activity was reduced at least by a factor of 1,000. Again, similar data were recently obtained with ToxB (10). This decrease in enzyme activity is apparently not caused by loss of the transferase-GTPase interaction, because the glucohydrolase activities of both LT and ToxB were also dramatically reduced with these fragments of 517 or 516 amino acid residues.

Although ToxB and LT are about 76% identical and about 90% similar in their amino acid sequences (8), the toxins differ in substrate specificity and biological activity (4, 19, 22). Whereas ToxB glucosylates only members of the Rho subfamily, the protein substrate spectrum of LT is extended to include modification of Ras subfamily proteins (11, 14, 23). To gain further insight into the structural basis of substrate recognition by these glucosyltransferases, we constructed various chimeras of ToxB and LT covering the N-terminal active fragments (546 amino acid residues) of the toxins. A chimera consisting of the N-terminal 271 amino acid residues of ToxB and, at the C terminus, of the amino acid residues 272 through 546 from LT

FIG. 6. Glucosylation patterns of recombinant GTPases by LT and ToxB fragments and chimeric toxin constructs. Recombinant Rho, Rac, Cdc42, Ras, Ral, and Rap (1 μg of each) were glucosylated by the indicated toxin fragments or chimeric constructs (100 mM each) in the presence of [^{14}C]UDP-glucose for 30 min. Then, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (shown). (A) Glucosylation patterns of 1-546LT (lane 1), 1-271ToxB272-546LT (lane 2), 1-546ToxB (lane 3), and 1-271LT272-546ToxB (lane 4). (B) Glucosylation patterns of 1-546LT (lane 1), 1-468LT469-546ToxB (lane 2), 1-546ToxB (lane 3), and 1-468ToxB469-546LT (lane 4). (C) Glucosylation patterns of 1-546LT (lane 1), 1-134ToxB135-516LT517-546ToxB (lane 2), 1-516LT517-546ToxB (lane 3), 1-546ToxB (lane 4), and 1-134LT135-516ToxB517-546LT (lane 5). (D) Glucosylation patterns of 1-546LT (lane 1), 1-364ToxB365-516LT517-546ToxB (lane 2), and 1-364LT365-516ToxB517-546LT (lane 3). (E) Glucosylation patterns of 1-546LT (lane 1), 1-364ToxB365-468LT469-546ToxB (lane 2), 1-546ToxB (lane 3), and 1-364LT365-468ToxB469-546LT (lane 4). The diagrams at the right of each panel show summaries of the chimeric constructs in the corresponding lanes. The numbers at the right of the diagrams are amino acid residues.

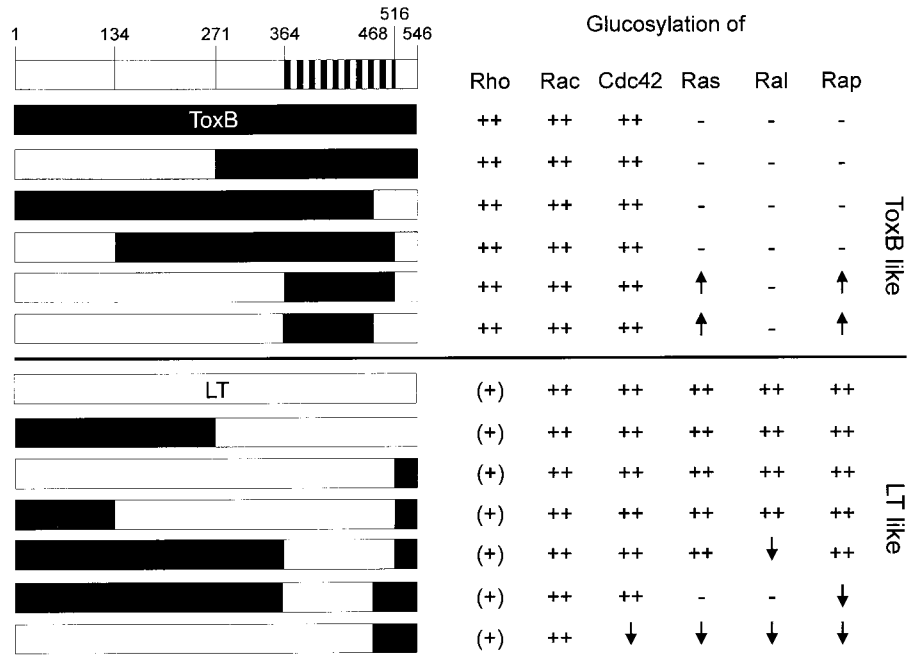


FIG. 7. Summary of LT-ToxB chimeric constructs and their substrate specificities. ToxB parts of chimeras are shown in black and LT parts are shown in white. Symbols: ++, marked labeling; (+), poor labeling; ↑, increase in labeling compared to control LT or ToxB; ↓, decrease in labeling compared to control LT or ToxB; –, no labeling. The hatched part of the top bar represents the region that is essential for glucosylation of Ras subfamily proteins by LT. The numbers represent amino acid residues. Data are from Fig. 6. From top to bottom, the bars represent 1-546ToxB, 1-271LT272-546ToxB, 1-468ToxB469-546LT, 1-134LT135-516ToxB517-546LT, 1-364LT365-516ToxB517-546LT, 1-364LT365-468ToxB469-546LT, 1-546LT, 1-271ToxB272-546LT, 1-516LT517-546ToxB, 1-134ToxB135-516LT517-546ToxB, 1-364 ToxB365-516LT517-546ToxB, 1-364ToxB365-468LT469-546ToxB, and 1-468LT469-546ToxB.

showed substrate specificity identical to that of LT holotoxin or its active fragment, residues 1 to 546. However, the complementary chimera, with the LT fragment at the N terminus and the ToxB fragment at the C terminus, exhibited ToxB-like characteristics with respect to its protein substrate specificity. These studies showed that the substrate specificity of LT is clearly defined by the C-terminal half of the protein fragment consisting of amino acid residues 1 to 546. Other chimeras allowed us to narrow the extended substrate specificity of LT down to the region of amino acid residues 364 through 516. When this region of ToxB was exchanged with that of LT, the resulting chimeric protein revealed a substrate specificity typical of LT with the exception that the modification of Ral was slightly reduced. The complementary chimera of LT with an insert of ToxB exhibited efficient glucosylation of Rho (which is typical of ToxB [15]) but also a slight modification of Ras subfamily proteins like Rap. Therefore, it appears that the region between amino acid residues 364 and 516 largely defines the substrate specificity of LT. However, additional regions may be involved because the complementary LT chimera, with amino acid residues 364 through 516 from ToxB, also modified Rap to some extent. This modification was never observed with the control ToxB holotoxin or fragment. Further reduction in the length of this LT insert into ToxB (e.g., chimera 1-364 ToxB365-468LT469-546ToxB) significantly inhibited modification of Ras proteins, suggesting that the region between amino acid residues 468 and 516 contains structural features important for the acceptance of Ras proteins as substrates for LT. Interestingly, this chimera still showed reduced modification of Rho, which is typical of LT (14, 23). Therefore, it is assumed that the interaction and/or modification of Rho depends on structures defined by residues between amino acids 364 and 468.

The cytotoxic effects of *C. sordellii* LT differ from those of *C. difficile* ToxB (2, 7, 19, 22). For example, ToxB induces massive retraction and arborization of CHO cells whereas LT causes rounding up without major retraction and arborization (22). On the other hand, it was shown that LT but not ToxB inhibits growth factor signaling via the MAP kinase pathway (14, 23). These differences in the morphological features and the biochemical consequences of the toxins' actions were ascribed to their different protein substrate specificities. ToxB variants have been described that differ in their biological activity from the reference ToxB produced by *C. difficile* VPI10463. For example, ToxB variants from *C. difficile* 1470 (28) and 8864 (27) were reported to induce LT-like morphological features. The structural gene of the ToxB variant 1470 (28) encodes a toxin that is 93% identical with the reference ToxB (VPI10463). However, this variant ToxB shows a cluster of amino acid changes (28) in the region defined above as being pivotal for LT substrate specificity and is only 61% identical with the reference ToxB between amino acid residues 316 and 516. Therefore, we suggest that changes in this region are responsible for the LT-like cytotoxic activity of this variant ToxB.

In summary, we describe the location of the glucosyltransferase and glucohydrolase activities of LT from *C. sordellii* at the N terminus of the holotoxin. Moreover, by constructing various chimeras of LT and ToxB, we were able to identify a region (amino acid residues 364 to 516) of LT which is essential for the extended substrate specificity of this large clostridial cytotoxin.

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