**Clostridium difficile** Glucosyltransferase Toxin B-essential Amino Acids for Substrate Binding*1

Thomas Jank, Torsten Giesemann, and Klaus Aktories

From the Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität Freiburg, 79104 Freiburg, Germany

Recently the crystal structure of the catalytic domain of ***Clostridium difficile*** toxin B was solved (Reinert, D. J., Jank, T., Aktories, K., and Schulz, G. E. (2005) *J. Mol. Biol.* 351, 973–981). On the basis of this structure, we studied the functional role of several amino acids located in the catalytic center of toxin B. Besides the 280DXD288 motif and Trp102, which were shown to be necessary for Mn2+ and UDP binding, respectively, we identified by alanine scanning Asp270, Arg273, Tyr284, Asn384, and Trp520 as being important for enzyme activity. The amino acids Arg455, Asp461, Lys463, and Glu472 and residues of helix α17 (e.g. Glu449) of toxin B are essential for enzyme-protein substrate recognition. Introduction of helix α17 of toxin B into ***Clostridium sordellii*** lethal toxin inhibited modification of Ras subfamily proteins but enabled glucosylation of RhoA, indicating that helix α17 is involved in RhoA recognition by toxin B. The data allow the design of a model of the interaction of the glucosyltransferase domain of toxin B with its protein substrate RhoA.

**Clostridium difficile** toxin B is, besides toxin A, the major pathogenicity factor of antibiotic-associated diarrhea and pseudomembranous colitis. Whereas toxin A is a potent enterotoxin in animal models, toxin B is designated as a cytotoxin, obtained from ***Clostridium difficile*** VPI 10463 and lethal toxin (LT)*2 from ***Clostridium sordellii*** 6018 and their recombinant catalytic domains were expressed in Escherichia coli (14). Glucosylation of Rho proteins inhibits the function of the switch proteins, most likely by inhibiting the conformational change into the active form of the GTPases. Therefore, Rho-dependent signaling is blocked (12).

The crystal structure of the catalytic domain of toxin B has been solved recently (13) and the structural requirements of the sugar substrate specificity have been determined (14). Here we studied the functional role of several amino acids located in or near the active center of toxin B and identified amino acid residues that are involved in protein substrate recognition.

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-[14C]glucose (287.4 mCi/mmol) were obtained from PerkinElmer Life Science; PCR primers were from Biochip Technologies (Freiburg, Germany). Toxin B from ***C. difficile*** VPI 10463 and lethal toxin (LT)*2 from ***C. sordellii*** 6018 and their recombinant catalytic domains were expressed and purified as described previously (14). All other reagents were of analytical grade and purchased from commercial sources.

**Polymerase Chain Reactions**—Amplification of the glucosyltransferase domain of toxin B of ***C. difficile*** VPI 10463 and the glucosyltransferase domain of ***C. sordellii*** 6018 was performed as described previously (5).

**Site-directed Mutagenesis of Toxin Fragments**—QuickChange™ kit (Stratagene, La Jolla, CA) in combination with *Pfu* Turbo DNA polymerase was used for the replacement of one to five nucleotides in the pGEX2T-LTcat or pGEX2T-Bcat plasmids. Primers were constructed as shown in the supplemental data. Corresponding plasmids were selected in *Escherichia coli* XL1-Blue followed by transformation into *E. coli* BL-21 (DE3) (Invitrogen) for expression.

**Sequencing**—All constructs of toxin B and LT fragments were checked by sequencing with ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit and ABI 310 Cycle Sequencer (Perkin-Elmer).

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental primers.

1 To whom correspondence should be addressed: Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albertstr. 25, D-79104 Freiburg, Germany. Tel.: 49-761-2035301; Fax: 49-761-2035311; E-mail: Klaus.Aktories@pharmakol.uni-freiburg.de.

2 The abbreviations used are: LT, lethal toxin of *C. sordellii*; PEI, polyethyleneimine; EBL, embryonic bovine lung.
Expression of Recombinant Proteins—Toxin fragments were expressed as glutathione S-transferase fusion proteins from E. coli expression vector pGEX-2T (GE Healthcare) as previously reported (5). Affinity chromatography with glutathione-Sepharose (GE Healthcare) was used for purification. Proteins were cleaved from glutathione S-transferase fusion protein with thrombin in TBP-150 elution buffer (thrombin 100 μg/ml, Tris-HCl 50 mM, KCl 150 mM, and β-mercaptoethanol 10 mM, pH 7.5) for at least 1 h at 4 °C. Thrombin was removed by binding to benzamidine-Sepharose (GE Healthcare). Recombinant GTP-binding proteins RhoA, Rac1, Cdc42, H-Ras, and RalA were prepared as glutathione S-transferase fusion proteins as described (15).

Glucosylation Reaction—Recombinant GTP-binding proteins (50 μg/ml) were incubated with the indicated concentrations of recombinant toxin fragments and 10 μM UDP-[14C]sugars in a buffer containing 50 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl2, 1 mM MnCl2, and 100 μg/ml bovine serum albumin for the indicated times at 30 °C. Total volume was 20 μl. Labeled proteins were analyzed by SDS-PAGE followed by phosphorimaging (Molecular Dynamics/GE Healthcare).

UDP-Sugar Hydrolase Reaction—UDP-sugar hydrolysis was measured as described (15). Toxin fragments and mutants were incubated with 20 μM UDP-[14C]sugar and 100 μM cor- responding unlabeled UDP-sugar in a buffer containing 50 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl2, 1 mM MnCl2, and 100 μg/ml bovine serum albumin, and 100 μM MnCl2. Total volume was 20 μl. Samples of 1 μl were taken at each time point and subjected to PEI (polyethyleneimine)-cellulose thin layer chromatography (Merck) with 0.2 mM LiCl as mobile phase to separate the hydrolyzed sugar from intact UDP-sugar. The plates were dried and analyzed by phosphorimaging analysis. Quantification was carried out with ImageQuant (Molecular Dynamics/GE Healthcare).
**TABLE 1**

Kinetic parameters of recombinant toxin B mutants for the glucosyltransferase reaction (A) and hydrolysis (B) of UDP-glucose

|             | K_m (μM) | k_cat (s⁻¹) | rel. k_cat/k_m | k_cat/K_m (μM⁻¹ s⁻¹) | rel. k_cat/K_m |
|-------------|----------|-------------|----------------|----------------------|---------------|
| (A) Transferase |          |             |                |                      |               |
| Wild-type   | 4.3 ± 0.7 | 10.9 ± 2.6  | 100            | 2.5 × 10⁷           | 100           |
| W520A       | 2.9 ± 1.3 | 0.013 ± 0.005 | 0.12 | 4.6 × 10⁷  | 0.18           |
| D270N       | 7.2 ± 0.5 | 0.02 ± 0.0008 | 0.18 | 2.7 × 10⁷  | 0.11           |
| D270A       | ND        | ND          | ND             | ND                   | ND            |
| R273A       | 6.0 ± 3.5 | 0.018 ± 0.001 | 0.17 | 3.0 × 10⁷  | 0.12           |
| N384A       | 16.1 ± 2.5 | 0.034 ± 0.008 | 0.3  | 2.1 × 10⁷  | 0.08           |
| Y284A       | 0.9 ± 0.8 | 0.004 ± 0.002 | 0.04 | 4.7 × 10⁷  | 0.18           |
| H17-C       | 6.6 ± 2.2 | 0.016 ± 0.01  | 0.15 | 2.5 × 10⁷  | 0.10           |
| (B) Hydrolase |          |             |                |                      |               |
| Wild-type   | 12.6 ± 2.5 | 274.5 ± 24.4 | 100           | 2.2 × 10⁷           | 100           |
| W520A       | 18.7 ± 3.8 | 0.60 ± 0.3      | 0.22 | 3.2 × 10⁷  | 0.15           |
| D270N       | 23.3 ± 1.5 | 0.24 ± 0.1      | 0.09 | 1.1 × 10⁷  | 0.05           |
| D270A       | ND        | ND          | ND             | ND                   | ND            |
| R273A       | ND        | ND          | ND             | ND                   | ND            |
| N384A       | 31.5 ± 5.7 | 0.73 ± 0.6      | 0.27 | 2.3 × 10⁷  | 0.11           |
| Y284A       | ND        | ND          | ND             | ND                   | ND            |
| H17-C       | 9.7 ± 1.5 | 246.8 ± 16.1  | 89.9 | 2.6 × 10⁷  | 116.9          |

**Determination of Kinetic Parameters**—Initial rate data for the glucosyltransferase and glycohydrolase reactions were determined by varying the UDP-sugar concentrations from ~0.1 to 4 × K_m. For the glucosyltransferase reaction, the experiments were performed with a fixed Rac concentration of 14.3 μM. The Michaelis–Menten parameters were obtained by computer-assisted analysis of Lineweaver-Burk plots of initial velocities from at least three independent experiments with the curve-fitting function in Sigma plot (Systat Software, Inc.).

**Microinjection Studies**—For microinjection, embryonic bovine lung (EBL) cells were seeded on CELL Locate coverslips (Eppendorf) at ~10⁴ cells/dish and cultivated for 24 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in humidified 5% CO₂ at 37 °C. Microinjection was performed with Femtotips II and the microinjector 5242 and micromanipulator 5171 from Eppendorf.

**RESULTS**

**Amino Acid Residues Involved in Catalysis and Sugar Substrate Interaction**—The glucosyltransferase located in the N-terminal domain of *C. difficile* belongs to the GT-A family of glycosyltransferases (13). Fig. 1 shows the conserved catalytic core of the glucosyltransferase domain in white. In Fig. 1, B and C, several amino acid residues that are in close proximity to the substrate UDP-glucose are indicated (note that UDP-glucose was found to be cleaved in the crystal structure of the GT domain of toxin B). Whereas the roles of the DxD motif (Asp²⁸⁶ and Asp³⁸⁸) and of tryptophan 102 have been already biochemically analyzed (16), we studied the function of additional amino acids that are most likely involved in binding and/or interaction with the substrate UDP-glucose. In the following, the glucosyltransferase domains of *C. difficile* toxin B and of *C. sordellii* lethal toxin are referred to as toxin B and LT, respectively. As shown in Fig. 2, exchange of Asp²⁷⁰, which appears to be involved in binding of sugar hydroxyl groups to asparagine or alanine, completely blocked or reduced the glucosyltransferases activity by ~500-fold (Table 1A). Surprisingly there was no major change in the K_m value (7.2 μM for the D270N mutant compared with 4.3 μM wild-type enzyme). To answer the question whether the mutation prevents the cytotoxic effect of the toxin, we microinjected the recombinant enzyme domain of the toxin into EBL cells. Fig. 2f shows that the D270A mutant was no longer able to intoxicate cells. The mutation of tyrosine 284 to alanine even decreased k_cat from 10.9 (wild-type) to 0.004 s⁻¹ and also prevented the intoxication of EBL cells by microinjection (Fig. 2f). These findings indicate that the residues are directly involved in the catalytic transferase reaction. Reductions in enzyme activity were also observed for the alanine mutant of arginine 273. Here 0.16% residual activity remained.

To exclude that inhibition of Rho glucosylation was caused by blockage of the interaction of the GTPase with the enzyme, we studied the UDP-glucose hydrolyzing activity of these mutants (Fig. 2C and Table 1B). Similar to findings for the transferase reaction, D270A, R273A, and Y284A did not exhibit any hydrolyzing activity compared with the wild-type enzyme. D270N showed a 1000-fold loss in activity; k_cat dropped from 274.5 (wild-type) to 0.24 h⁻¹. Once again the mutation leads to only minor changes in the K_m values (22.3 μM in comparison with 12.6 μM for the wild-type enzyme). These findings are in line with the view that these residues are directly involved in the catalytic mechanism and/or in the binding of the sugar donor.

In a second set of mutations we studied the consequences of the change of asparagine 139, serine 269, serine 518, and tryptophan 520 to the respective alanine mutants (Fig. 2B). With the exception of W520A, all other mutants, e.g. N139A, S269A, and S518A, only exhibited reduction in transferase activity of up to 10-fold (Fig. 2E). Similar results were obtained in the glycohydrolase reaction (Fig. 2, D and F). These findings indicate that these residues are most likely not directly involved in binding of the protein substrate or the sugar donor and do not participate.

**CLOSTRIDIUM DIFFICILE GLUCOSYLTRANSFERASE TOXIN B**

**FIGURE 2.** Glucosyltransferase (A and B) and glycohydrolase activity (C and D) of mutants of the catalytic domain of toxin B, A and B, and time course of glycosylation of RhoA (1 μg) by the glucosyltransferase domain of toxin B and mutants D270N, D270A, N384A, R273A, Y284A, W520A, S518A, N139A, and S269A in the denoted concentrations in the presence of UDP-[3H]glucose (10 μM) for the indicated times. After separation by SDPAGE, radiolabeled proteins were detected by phosphorimaging and quantified by ImageQuant. C and D, time course of glycohydrolase activity of the catalytic domain of wild-type toxin B and of the mutants mentioned in panels A and B. The toxins were incubated with radiolabeled substrate UDP-[3H]glucose at 30 °C. After the indicated time points the radioactive components were separated by PEI-cellulose thin layer chromatography, detected by phosphorimaging, and quantified by ImageQuant. E, relative glucosyltransferase activity of the toxin B mutants S269A, N139A, E515Q, E515A, and S518A. The glucosyltransferase domain of wild-type toxin B and of the mutants (each 1 nm) were investigated with RhoA (1 μg) at 30 °C for 5 min. In the initial phase, the transfer reaction was stopped and proteins separated by SD-PAGE and analyzed by autoradiography. The signal intensities of Rho glucosylation were set in relation to wild-type toxin B. Data are given as means ± S.D. (n > 3), F, relative glycohydrolase activity of toxin B mutants. The glucosyltransferase domains of wild-type toxin B and mutants (each 100 nm) were incubated with UDP-[3H]glucose for 10 min. After separation by PEI-cellulose thin layer chromatography, radiolabeled compounds were analyzed by autoradiography and quantified by ImageQuant. The percentage of hydrolyzed sugar substrate was set in relation to wild-type toxin. All data are given as means ± S.D. (n > 3). G–K, microinjection into EBL cells. EBL cells marked with an arrow were injected with control buffer (E) or with buffer containing 900 nm recombinant toxin B enzymatic domain (F), or the mutants D270A (G), Y284A (H), or W520A (K). Photographs were taken 2 h after microinjection.
in the catalytic reaction. By contrast, W520A exhibited >800-fold reduction in enzyme activity. The \( k_{\text{cat}} \) dropped from 10.9 (wild-type) to 0.013 s\(^{-1}\). Also, the glucohydrolase activity was strongly reduced (Table 1B). Owing to the strong reduction in \textit{in vitro} activity also, microinjection experiments with EBL cells were accomplished. We could not see any morphological cytopathic effects after injection of the recombinant W520A mutant (Fig. 2K).

In addition, we studied the role of glutamate 515, which appears to be involved in the coordination of the manganese ion, having a distance to Mn\(^{2+}\) of only 2.2 Å (Fig. 1, B and C). Surprisingly, change of glutamate to glutamine had nearly no consequences for glucosyltransferase and glucohydrolase activity (Fig. 2, E and F). In addition, a change to alanine did not cause a major drop in enzyme activity. This supports the notion that the second aspartate residue from the DXD motif has the main function in coordination of manganese ion.

**Potential Nucleophilic Amino Acids Involved in Catalysis**—Although the molecular reaction mechanism of inverting glucosyltransferases is reasonably well understood, this is not the case for retaining glucosyltransferases. Because double displacement is feasible, we tried to identify nucleophiles that might be involved in this type of reaction. To this end, the role of glutamate 472 was analyzed and the kinetics of the E472A and E472R mutants studied. As shown in Fig. 3, E472A exhibited glucosyltransferase and glucohydrolase activity comparable to that of the wild-type. The glucosyltransferase activity of E472R was reduced. However, the glucohydrolase activity was not changed (Fig. 3C), indicating that glutamate 472 is not essential for enzyme activity. Another potential nucleophile is asparagine 384, which is located in the loop between strands B9 and B10 on the \( \beta \)-face of the sugar moiety. The carbonyl group of Asn\(^{384}\) is in 3.5 Å distance and well directed to the anomeric C-atom of the glucose (Fig. 1B). Change of asparagine 384 to alanine decreased the \( k_{\text{cat}} \) from 10.9 (wild-type) to 0.034 s\(^{-1}\), meaning a 300-fold reduction in catalytic activity (Table 1). We then changed the nucleophilicity of this asparagine by a change to aspartic acid. No major effect was observed with an \( \sim \)50-fold reduction in enzyme activity (Fig. 3B). Moreover, we tried to identify a stable intermediate with this mutant. However, we
were not able to detect labeling of the enzyme in the presence of UDP-[14C]glucose (not shown). These data rather support the hypothesis that the glucosylation of Rho GTPases by toxin B follows a SNi-like mechanism (13).

**RhoA-Toxin B Interaction**—The catalytic domain of toxin B can be characterized by a “front side,” which harbors the catalytic cleft where UDP-glucose binds, and by a “back side” of the protein, which is not directly involved in the interaction with the sugar donor. The front side should be involved in protein substrate recognition. So far, our understanding of the interaction of Rho GTPases with the catalytic domain of toxin B was based only on in silico docking modeling. Suggesting that hydrogen and ion bondings are involved in enzyme-protein substrate interaction, we changed serine, threonine, aspartagine, and several charged residues that are located in this protein area. A further criterion for amino acid exchange was the fact that the residues were apparently not directly involved in UDP-glucose interaction. Moreover, we studied the glucosylation of RhoA, Rac, and Cdc42 with the mutants and compared the results with the glucosylation of small GTPases by the catalytic domain of lethal toxin, which additionally modifies Ras subfamily proteins but not RhoA. Fig. 4 gives an overview of these studies. From 20 single amino acid mutants studied, five mutant proteins exhibited gross changes in Rho GTPase glucosylation compared with the wild-type catalytic domain. Essential amino acid residues for the modification of Rho GTPases were glutamate 449, arginine 455, aspartate 461, lysine 463, and glutamate 472. The mutant toxin B proteins R455E, D461R, K463E, and E472R exhibited a dramatic loss in modification of all Rho GTPases (Fig. 4, s–v) whereas the glucohydrolase activity was much less affected (Fig. 5), indicating that these residues were involved in Rho GTPase recognition. Mutant E449K strongly reduced glucosylation of RhoA and Cdc42, whereas modification of Rac was minorly reduced (Fig. 4, r). Also, the glucohydrolase activity of this mutant was hardly affected (Fig. 5). Glutamate 449 is located in the middle of helix α17 of toxin B. This helix, covering residues Gly444 through Lys452 of toxin B, exhibits major differences in amino acid sequence in toxin B and LT, although the entire catalytic domain of both toxins is ∼90% similar. Therefore, we exchanged several amino acid residues and turned helix α17 of toxin B in three steps into that of LT (Fig. 6, located in the middle of helix α17 of toxin B. This helix, covering residues Gly444 through Lys452 of toxin B, exhibits major differences in amino acid sequence in toxin B and LT, although the entire catalytic domain of both toxins is ∼90% similar. Therefore, we exchanged several amino acid residues and turned helix α17 of toxin B in three steps into that of LT (Fig. 6,
A–C, and, vice versa, we turned this region of LT into that of toxin B (Fig. 6, D–F). Fig. 7 shows that the glucohydrolase activities of all these chimeras were not changed. Accordingly, the $k_{\text{cat}}$ and $K_m$ values of hydrolysis by toxin B-H17-C were unchanged (Table 1B). When helix $\alpha 17$ in toxin B was changed to that of LT, modification of RhoA decreased (Fig. 8A). The $k_{\text{cat}}$ for transferase activity was reduced ~700-fold (Table 1A). A modification of Ras and Ral, which are substrates of LT, was never observed even at high concentrations of 1 $\mu$m of the chimeric toxin B (Fig. 8B). On the other hand, change of the respective amino acid residues in LT to that of toxin B caused reduction of modification of Ras by LT (Fig. 8C, upper panel). When the complete helix $\alpha 17$ of toxin B was introduced into LT, a slight but significant modification of RhoA was detectable that was not observed with wild-type LT (Fig. 8C, lower panel).

**DISCUSSION**

Deduced from the crystal structure of the catalytic domain of toxin B, we identified several amino acid residues involved in catalysis of the glucosyltransferase reaction and/or binding of sugar substrate or protein substrate. The role of the well known conserved DXD motif and tryptophan 102 of toxin B have been

**FIGURE 5.** Glucohydrolase activity of the glucosyltransferase domains of toxin B mutants E449K, E472R, D461R, K463E, and E455R. Recombinant wild-type and mutant toxin B proteins (each 100 nM) were incubated with UDP-[14C]glucose at 30 °C for the indicated times. After separation of the components by PEI-cellulose thin layer chromatography, radioactive components were analyzed and quantified as described under “Experimental Procedures.”

**FIGURE 6.** Illustration of the helix $\alpha 17$ constructs of the glucosyltransferase domains of toxin B and LT. In the glucosyltransferase domain of toxin B the amino acids of helix $\alpha 17$ were successively replaced (starting from the N-terminal end) by amino acids of LT (B H17-A (A), B H17-B (B), B H17-C (C)). In the catalytic domain of LT the same amino acid residues were replaced by the corresponding amino acids of toxin B but in counter direction (LT H17-A (D), LT H17-B (E), LT H17-C (F)). G, for orientation purposes of helix $\alpha 17$, the SWISS model of the catalytic domain of LT is depicted. Helix $\alpha 17$ is shown in red with amino acids in ball and stick representation (D–F). UDP and glucose (red) are shown as balls and sticks.
and aspartic acid (e.g. Asp286) residues bonded to the 3-OH group of the donor sugar as GT-A triad, which is conserved in many enzymes of this family of glycosyltransferases. Also known in other glycosyltransferases and glycosidases is bidentate hydrogen bonding of carbonyl groups with the vicinal hydroxyl groups of an active site sugar in which an aspartic acid residue is involved (19–21). Here the acidic residues are involved in the distortion of the sugar ring toward the intermediate or transition state conformation. Secondly, a negative potential is achieved in the catalytic site stabilizing oxocarbenium ion-like structures. In toxin B this might be achieved by Asp286, which bonds to the 4′- and 6′-OH group of the glucose. These results confirm that catalysis is enabled by the distinct interplay of a network of conserved residues in the catalytic site that serve to stabilize the transition states.

The very strong reduction in enzyme activity of the Y284A mutant cannot be explained by an impaired sugar substrate interaction via the 2′-hydroxyl group of the ribose, because of the distance of 3.3 Å from the hydroxyl group of Tyr284 to the 2′-hydroxyl group of the ribose. Secondly, the $k_{\text{cat}}$ value for UDP-glucose is not dramatically changed, whereas the $K_{m}$ is reduced >2000-fold (Table 1). We re-evaluated possible interactions of Tyr284 by means of the crystal structure of toxin B (13) and determined a distance between Tyr284 and Asp286 of 2.76 Å. Asp286 is part of the DXD motif, and therefore it is feasible that Tyr284 has a pivotal role in positioning this important residue for Mn$^{2+}$-dependent catalysis. A role of Mn$^{2+}$ in catalysis has been suggested for inverting GT-A glycosyltransferases, where it acts as an acid catalyst and appears to be involved in conformational changes of the so-called “flexible loop” that might be involved in product release (22, 23). A function of the DXD motif and of the divalent cation not only in substrate binding but also in catalysis of retaining enzymes has to be determined.

Change of tryptophan 520 to alanine largely impaired enzyme activity and again prevented cellular toxic effects. This residue binds to the oxygen of β-phosphate of UDP-glucose

characterized in detail previously (16, 17), and the functions of these residues were confirmed by crystal structure analysis (13). Here we present a biochemical analysis of the functional roles of several additional amino acid residues that have been suggested to be involved in catalysis and/or in sugar substrate bind-
and is located on the putative flexible loop (amino acids 510–523) (23). This hypothesis is underlined by the fact that an alanine mutant shows almost no change in $K_m$ value, whereas the turnover of the enzyme ($k_{cat}$) was reduced by $\sim 800$-fold (Table 1A). Additionally, the importance of this region in enzyme activity explains previous findings that rather small deletions of the C terminus of the catalytic domain cause loss of enzyme activity (5).

The molecular mechanism of retaining glucosyltransferases is not clear. A plausible explanation would be a double displacement mechanism, which, however, depends on the presence of a nucleophilic amino acid close to the anomeric carbon of the donor substrate. As possible candidates for a nucleophilic attack of the anomeric carbon of UDP-glucose, we studied glutamate 472 and asparagine 384. At a position very similar to Glu472 in toxin B, many GT-A glycosyltransferases possess conserved nucleophilic residues. For example, Glu472 could be equivalent to the nucleophilic amino acid aspartate 190 in LgtC (24), glutamate 317 in $\alpha$3GalT (25), or aspartate 162 in glycogenin (26). However, changes of Glu472 in toxin B did not cause a major drop in enzyme activity, indicating no essential role in the catalytic reaction. The same was the case for Glu449. Glu449 is located in helix 17 of toxin B, which is a lysine in the case of $C. sordellii$ lethal toxin. Change of this residue to lysine reduced RhoA and Cdc42 glucosylation but the mutant protein was still able to modify Rac. Modification of Ras or Ral, which are main substrates for lethal toxin, could not be achieved by the point mutant. This led to the question whether additional residues around Glu449 are required for the substrate specificity of lethal toxin. Therefore, helix 17 chimeras of toxin B and of lethal toxin were constructed. On both sides exchange of helix 17 resulted in a strong reduction of glucosylation of their natural substrates (Fig. 8). Strikingly, lethal toxin with the whole helix 17 exchanged to toxin B was able to modify RhoA to a certain extent (Fig. 8C). The data indicate that helix 17 is essential for substrate recognition, but this region of LT introduced into toxin B is not sufficient for modification of Ras. Thus, Ras needs additional residues for recognition by the clostridial glucosylating toxins. Moreover, modification of Rac was decreased in mutant toxins, indicating

![FIGURE 9. RhoA-toxin B interaction model.](image-url)
that exchange of helix α17 of toxin B by the equivalent residues of LT may cause additional effects in the structure of toxin B. Accordingly, cell intoxication induced by microinjection of toxin B that harbored helix α17 from LT was slower than with the catalytic domain of wild-type toxin B (not shown).

Deduced from the above-mentioned findings and recent crystal structure analysis of the catalytic domain of toxin B, we suggest a first model for the interaction of RhoA with toxin B (Fig. 9). This model is based on the docking programs Hex and FTDOCK in consideration of amino acids Arg455, Asp461, Lys463, and Glu472 as well as helix α17, which are all important for Rho GTPase interaction with toxin B but have no influence on glucohydrolase activity. This model is also compatible with amino acid residues Thr37 and Ser73 of RhoA, which have been shown to be important for interaction with the catalytic domain of wild-type toxin B (not shown).

In line with this hypothesis, we observed recently by using the chimeras Ras124RhoA136Ras and Rac124Rho136Rac that the insert region has no effect on the substrate specificity of toxin B and LT, respectively (not shown).

Taken together, we identified amino acids in the glucosyltransferase domain of toxin B that are essential for enzyme activity and substrate recognition. These findings are likely to be important for the understanding of the activity and specificity of other members of the family of clostridial glucosylating toxins.

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