A fragment of the N-terminal 546 amino acid residues of Clostridium sordellii lethal toxin possesses full enzyme activity and glucosylates Rho and Ras GTPases in vitro. Here we identified several amino acid residues in C. sordellii lethal toxin that are essential for the enzyme activity of the active toxin fragment. Exchange of aspartic acid at position 286 or 288 with alanine or asparagine decreased glucosyltransferase activity by about 5000-fold and completely blocked glucohydrolase activity. No enzyme activity was detected with the double mutant D286A/D288A. Whereas the wild-type fragment of C. sordellii lethal toxin was labeled by azido-UDP-glucose after UV irradiation, mutation of the DxD motif prevented radiolabeling. At high concentrations (10 mM) of manganese ions, the transferase activities of the D286A and D288A mutants but not that of wild-type fragment were increased by about 20-fold. The exchange of Asp270 and Arg277 reduced glucosyltransferase activity by about 200-fold and blocked glucohydrolase activity. The data indicate that the DxD motif, which is highly conserved in all large clostridial cytotoxins and also in a large number of glycosyltransferases, is functionally essential for the enzyme activity of the toxins and may participate in coordination of the divalent cation and/or in the binding of UDP-glucose.

The family of large clostridial cytotoxins comprises Clostridium difficile toxins A and B, the lethal toxin (LT)† and hemorrhagic toxin of Clostridium sordellii, and the α-toxin from Clostridium novyi (1, 2). Whereas C. difficile toxins A and B are the major virulence factors of the antibiotic-associated diarrhea and pseudomembranous colitis (3–6), the toxins from C. sordellii and C. novyi are implicated in gas gangrene syndrome (7, 8). All these toxins possess glucosyltransferase activity and modify small GTPases of the Rho family (2, 9–12). The toxins differ in cosubstrate and protein substrate specificity. Whereas α-toxin from C. novyi catalyzes the N-acetylglucosaminylolation by using UDP-GlcNAc as a cosubstrate (13), the other toxins use UDP-glucose and cause glucosylation of the protein targets. All Rho subfamily proteins including Rho, Rac, and Cdc42 are modified by C. difficile toxins A and B, the hemorrhagic toxin of C. sordellii, and the C. novyi α-toxin (9, 10, 13, 14). By contrast, the lethal toxin from C. sordellii glucosylates Rac and Cdc42 but not (or much less) Rho. In addition, Ras proteins (Ras, Rap, Ral) are modified by C. sordellii lethal toxin (11).

Toxin-induced glucosylation of low molecular mass GTPases occurs at Thr237 of Rho (Thr259 of all other GTPases) (10). This amino acid residue is conserved in all low molecular mass GTPases and involved in divalent cation and nucleotide binding. Moreover, Thr237/Thr237 is located in the effector region of the GTPases, and glucosylation appears to block the interaction of the GTPases with their effectors (15, 16). Thus, glucosylation of Rho proteins by large clostridial cytotoxins renders the GTPases biologically inactive (17).

The large clostridial cytotoxins possess molecular masses between 250 and 308 kDa and are, therefore, the biggest bacterial toxins known. It has been suggested that these toxins are constructed of three functional parts (18). The C terminus, which consists of a large region of repetitive oligopeptides, is assumed to be important for receptor binding to the eukaryotic target (19). A hydrophobic region almost in the middle of the toxins is believed to participate in membrane translocation, and finally, the N terminus harbors the enzyme activity. Deletion analysis performed with C. difficile toxin B, and with C. sordellii lethal toxin revealed that a fragment consisting of the N-terminal 546 amino acid residues possesses full glucosyltransferase activity and is able to induce the typical cytotoxic effects after microinjection (20, 21).

Recently, it has been reported that various families of glycosyltransferases, which exhibit high sequence homology within the same family but no overt similarity between different families, share a common small motif consisting of DxD, which is conserved in the bacterial toxins. Therefore, we studied whether this short motif has any functional relevance and is involved in enzyme activity of the toxins. Here we report that the exchange of aspartic acid residues 286 and 288, which form the DxD motif in C. sordellii lethal toxin, decreases the glucosyltransferase activity several thousand-fold and blocks the glucohydrolase activity, indicating an essential role in catalysis. Moreover, we identified additional amino acid residues located in close vicinity of the DxD motif, which are of functional importance and conserved in all large clostridial cytotoxins.
Motif Essential for Glucosyltransferase Activity

Experimental Procedures

Materials—14C-labeled UDP-hexoses were obtained from DuPont NEN Life Science Products (Deireich, Germany). Polymerase chain reaction primers were from MWG Biotech (Ebersberg, Germany). Other reagents were of analytical grade and purchased from commercial sources.

Polymerase Chain Reaction Amplification—Amplification of the C. difficile toxin B fragment CDB1 and C. sordellii CS1 and construction of C-terminal truncated fragments B546 and LT546 were performed as described previously (20, 21).

Site-directed Mutagenesis of Toxin Fragments LT546 and B546—The QuikChange Kit™ (Stratagene) was used for mutating one or two nucleotides in the pGEZT-LT546 construct or in the pGEZT-B546 construct, respectively. Procedures were carried out according to the manufacturer’s instructions. Primers were constructed as follows: D270ALT546, primer pair S1D270Asen/anti (5'-CTGCTGTTGACATCTTAGCAGGTATACAACC-3' / 5'-CTGCTGTTGACATCTTAGCAGGTATACAACC-3'); R273ALT546, primer pair S1R273Asen/anti (5'-CTGCTGTTGACATCTTAGCAGGTATACAACC-3' / 5'-CTGCTGTTGACATCTTAGCAGGTATACAACC-3'); approximately 1 μg of DNA was loaded on lanes 2, 4, and 5).

Sequencing—Sequencing of CS1 and CDB1, their truncated derivatives LT546 and B546, and the mutated clones was done with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) to check both for correct cloning and mutations due to polymerase chain reaction amplification. Sequencing was performed at least twice with overlapping DNA fragments.

Expression of Recombinant Proteins—The recombinant GTP-binding proteins RhoA, Rac1, Cdc42, Ha-Ras, and Ral were prepared from their fusion proteins as described (10, 11). The recombinant toxin fragments

P291ALT546, primer pair S1G291Asen/anti (5'-GGTTGTATACCTGGTAAGATGTTAACATCTTACCAGGTA-3' / 5'-GGTTGTATACCTGGTAAGATGTTAACATCTTACCAGGTA-3'); D286ALT546, primer pair S1D286Asen/anti (5'-CTGGTAAGATGTTAACATCTTACCAGGTA-3' / 5'-CTGGTAAGATGTTAACATCTTACCAGGTA-3'); D288ALT546, primer pair S1D288Asen/anti (5'-CTGCTGTTGACATCTTAGCAGGTATACAACC-3' / 5'-CTGCTGTTGACATCTTAGCAGGTATACAACC-3'); D286ALT546, primer pair S1D286Asen/anti (5'-CTGGTAAGATGTTAACATCTTACCAGGTA-3' / 5'-CTGGTAAGATGTTAACATCTTACCAGGTA-3').

Fig. 1. Alignment of residues 263–298 of the large clostridial cytoxins. LT, C. sordellii strain 6018 lethal toxin (GenBank™ X82638); Toxin B, C. difficile strain VPI 10463 cytotoxin B (GenBank™ X53138); Toxin BF, C. difficile strain 1470 variant cytotoxin B (GenBank™ D22771); Toxin A, C. difficile strain VPI 10463 cytotoxin A (GenBank™ M30307); α-Toxin, C. novyi strain VPI 10463 cytotoxin B (GenBank™ Z48636); OchIp, S. cerevisiae Och1p mannosyltransferase (GenBank™ D11905); SurIp, S. cerevisiae Sur1p (GenBank™ M96648); Mnn1p, S. cerevisiae Mnn1p mannosyltransferase (GenBank™ L23753).

Fig. 2. A, purified recombinant toxin fragment mutants. The N-terminal toxin mutants were constructed as glutathione S-transferase fusion proteins, expressed in E. coli, and purified by affinity chromatography. Toxin fragment mutants are shown as fusion proteins, D282ALT546 (lane 1), D288ALT546 (lane 2), LT546 (lane 3), D286ALT546 (lane 4), D288ALT546 (lane 5), D286ALT546 (lane 6), D283ALT546 (lane 7), D286ALT546 (lane 8), D288ALT546 (lane 9), D288ALT546 (lane 10), D288ALT546 (lane 11), B546 (lane 12), D286ALT546 (lane 13), D288ALT546 (lane 14), LT 546 (lane 15); approximately 1 μg of protein was loaded on lanes 10 and 12; 2 μg was loaded on lanes 7–9, 11, 13, and 14; 3 μg was loaded on lanes 1, 3, 6, and 15; 4 μg was loaded on lanes 2, 4, and 5. B, protease digest of toxin fragment and mutants by trypsin. Toxin fragment and mutants (each 20 μg) were incubated with 60 ng of trypsin in a buffer containing 10 μl glutathione and 50 μl Tris-HCl (pH 8.0) in a total volume of 45 μl. At the indicated time points, aliquots of 3 μl of protein were taken and analyzed by SDS-PAGE.
were expressed and purified as glutathione S-transferase fusion proteins in accordance with the manufacturer’s instructions (see Fig. 2). Glutathione S-transferase fusion proteins from the Escherichia coli expression vector pGEX2T were isolated by affinity chromatography with glutathione-Sepharose (Amersham Pharmacia Biotech, Germany) followed by removing glutathione S-transferase fusion proteins with glutathione elution buffer (10 mM glutathione in 50 mM Tris-HCl, pH 8.0). Recombinant Rap1a was a gift of Dr. A. Wittinghofer (MPJ Dortmund, Germany).

Glucosylation Assay—Recombinant GTP-binding proteins (50 μg/ml) were incubated with recombinant toxin fragment LT546 or mutated fragments of LT at the indicated concentrations in a buffer containing 50 mM Hapes (pH 7.5), 100 mM KCl, 2 mM MgCl₂, 1 mM MnCl₂, 100 μg/ml bovine serum albumin, and 10 μM ¹⁴C-UDP-glucose for 30 min at 37 °C or for the indicated periods. The total volume was 20 μl. Labeled proteins were analyzed by SDS-PAGE and subsequently by PhosphorImager analysis (Molecular Dynamics, Inc.). Quantitative data are given as arbitrary units as means ± S.E. (n = 3).

Glucosylhydrolase Assay—LT546 and the mutated fragments (at the indicated concentrations) were incubated with 20 μM ¹⁴C-labeled UDP-glucose and 100 μM unlabeled UDP-glucose in a buffer containing 50 mM Hapes (pH 7.5), 100 mM KCl, 2 mM MgCl₂, 100 μM bovine serum albumin, 100 μM MnCl₂. The total volume was 10 μl. Samples of 1.5 μl were taken at each time point and subjected to thin layer chromatography with PE-cellulose plates (Merck, catalog number 1.05579, Darmstadt, Germany) and 0.2 x LiCl as mobile phase in order to separate hydrolyzed glucose from UDP-glucose. The plates were dried and analyzed by PhosphorImager analysis. Quantitative data are given as pmol per time indicated as means ± S.E. (n = 3).

Photoaffinity Labeling—Indicated proteins (2 μg) were incubated on ice, in the presence of 50 μM [β-³²P]5N3-UDP Glc, reactions were allowed to equilibrate for 30 s, followed by UV irradiation with a hand-held UV lamp (254/366 nm) with the glass face removed at a distance of 3 cm for 3 min. For competition experiments, 1 mM of unlabeled UDP-glucose was added to the reaction mixture just before the addition of [β-³²P]5N3-UDP Glc. Reactions were terminated by the addition of 5 μl of sample buffer. Thereafter, radiolabeled proteins were analyzed by SDS-PAGE and PhosphorImager analysis.

Microinjection Studies—For microinjection studies, HeLa cells were grown for 24 h in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 4 mM glutamine/penicillin/streptomycin and plated on Cellocate (Eppendorf, Germany) coverslips at about 105 cells/dish at 37 °C and 5% CO₂. Microinjection was performed with the Microinjector 5242 and Micromanipulator 5171 from Eppendorf.

**RESULTS**

Effects of Alteration of the DXD Motif of C. sordellii Lethal Toxin on Glucosyltransferase and Glucosylhydrolase Activities—It has been reported recently that the amino acid motif DXD is crucial for the activity of the yeast mannosyltransferase Mnn1p (26). This motif is also conserved among the large clostridial cytotoxins in a region of high homology of the toxins (Fig. 1). To elucidate the significance of this motif for the enzymatic activity of the toxins, we constructed various mu-

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**FIG. 3.** A and B, protein substrate specificity of glucosylation by LT546, D286ALT546, and D288ALT546. Recombinant Rho, Rac, Cdc42, Ras, Rap, and Rap (each 1 μg) were glucosylated by LT546 (1 nM each in panel A and 100 nM each in panel B), D286ALT546 and D288ALT546 (1 nM each in panel A and 1 μM each in panel B) in the presence of UDP-¹⁴C-glucose (10 μM) for 30 min. Then labeled proteins were analyzed by SDS-PAGE and PhosphorImager analysis (shown). C, protein substrate specificity of glucosylation by B546, D286AB546, and D288AB546. Recombinant Rac (1 μg) was glucosylated by B546 (1 nM), D286AB546 (1 nM or 1 μM), or D288AB546 (1 nM or 1 μM) in the presence of UDP-¹⁴C-glucose for 30 min. Then labeled proteins were analyzed by SDS-PAGE and PhosphorImager analysis (shown).

**FIG. 4.** Glucosyltransferase and glucosylhydrolase activities of LT546 mutants. A, time course of the glucosylation of Ras by LT546 and the mutants. Ras (1 μg) was incubated with LT546 (1 nM, ■), or toxin fragment mutants D286ALT546 (1 μM, ◆), D288ALT546 (1 μM, ▲), and D286A/D288ALT546 (3 μM, ▼) in the presence of UDP-¹⁴C-glucose (10 μM) for the indicated times. Then labeled proteins were analyzed by SDS-PAGE and PhosphorImager analysis. B, time course of the glucosylhydrolase activity by LT546 and mutants. LT546 (100 nM, ■), D286ALT546 (1 μM, ◆), D286A/D288ALT546 (3 μM, ▼) were incubated with 20 μM UDP-¹⁴C-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 PhosphorImager analysis.
tants of the enzymatically active N-terminal fragment LT546 of C. sordellii lethal toxin and expressed them in E. coli as glutathione S-transferase fusion proteins. The conserved amino acid residues were changed to alanine or asparagine, respectively, and Fig. 2A shows the SDS-PAGE analysis of the proteins.

At first, we tested the single mutants D286ALT546 and D288ALT546 and the double mutant D286A/D288ALT546. In contrast to the active wild-type fragment, the mutants did not glucosylate any of the substrates of LT when applied at a concentration of 1 nM (Fig. 3A). Increasing the concentration of the wild-type fragment up to 100 nM resulted in a significant modification of Rac1, Rap1a, Ras, and Cdc42, whereas RhoA was much less labeled. At the same concentration (100 nM), the mutants caused only a faint labeling of Ras, Rap1a, Ral, and Rac1 but not of RhoA or Cdc42 (Fig. 3B). This decrease in activity of the mutant proteins was most likely not caused by major changes in the overall structure of the protein, because the susceptibility of the toxins toward trypsin treatment was not changed (Fig. 2B). To get more quantitative data, the time courses of glucosylation of Ras by these mutants were examined. As shown in Fig. 4A, the initial activities of the mutants D286ALT546 and D288ALT546 were reduced by more than 5000-fold with respect to the activity of the wild-type fragment, while the double mutant D286A/D288ALT546 was completely inactive. Similar inhibition was observed when the aspartic acid residues were changed to asparagine (not shown). To exclude the possibility that the loss of transferase activity was caused by an inhibition of the interaction of small GTPases with the transferase, we determined the UDP-glucosylhydrolyase activity of the mutants in the absence of a protein substrate. As shown in Figs. 4B, 8B, and 8C, no glucosylhydrolyase activity of the mutants was detected. A similar inhibition of glucosyltransferase activity was obtained with the related C. difficile toxin B in the presence of Rac1 protein as substrate when the aspartic acid residues of the DXD motif of toxin B were changed to alanine (Fig. 3C).

**Photoaffinity Labeling of DXD Mutants**—To test whether the DXD motif mutant proteins, which exhibited blocked or largely reduced enzyme activity, were still able to interact with the cosubstrate UDP-glucose, we performed photoaffinity labeling experiments with azido-UDP-glucose ([β-32P]5N3-UDP-glucose). Fig. 5 shows that the wild-type toxin fragment was labeled by [β-32P]5N3-UDP-glucose after UV irradiation. The addition of unlabeled UDP-glucose blocked the radiolabeling. By contrast, the single mutants D286ALT546 and D288ALT546 and the double mutant D286A/D288ALT546 were not labeled, suggesting that the respective aspartic acid residues are important for UDP-glucose binding or for photoaffinity labeling with azido-UDP-glucose.

**Effects of Manganese Ions on the Activity of DXD Mutants of C. sordellii Lethal Toxin**—Like various eukaryotic glycosyltransferases (22, 23) the glucosyltransferase activity of C. sordellii lethal toxin depends on manganese ions (11). Therefore, we studied whether alteration of the DXD motif affects the Mn2⁺ dependence of the glucosyltransferase activity of LT. As reported recently for the holotoxin, the active wild-type fragment of LT exhibited maximal activity at 1 mM Mn2⁺ (Fig. 6). The maximal increase in activity by the addition of manganese ions was by about 40% under the conditions used. Further increase in the concentration of the divalent cation > 1 mM reduced the enzyme activity. The single exchange of the aspartic acid res-
idues of the DXD motif of LT changed the Mn$^{2+}$ dependence. As shown in Fig. 6, the enzyme activity of the D288ALT546 mutant was affected at higher concentrations of Mn$^{2+}$ than the wild-type fragment. Whereas at 10 mM Mn$^{2+}$ the enzyme activity of the wild-type LT fragment was decreased, the enzyme activity of the mutant was increased by about 20-fold. Similar findings were obtained with the D286ALT546 mutant (not shown). By contrast, the double mutant was not affected by Mn$^{2+}$, not even at a high concentration of the divalent cation (not shown).

Microinjection Studies with DXD Mutants of C. sordellii Lethal Toxin—In order to further characterize the mutant fragments, we tested their biological activity by microinjection studies. For this purpose, the wild-type toxin fragment or the mutant toxins were injected into HeLa cells. As reported recently for C. difficile toxin B, microinjection of the wild-type fragment LT546 induced the typical morphological changes observed after treatment of cells with the holotoxin (Fig. 7A). Changes occurred 30 min after treatment of cells. In contrast to these findings, no change in cell morphology was observed, even 4 h after microinjection, when the single mutant D288ALT546 (Fig. 7C) or D286ALT546 (not shown) or the double mutant protein D286AD288ALT546 (Fig. 7D) was applied.

Effects of Mutations in the Surroundings of the DXD Motif—We also mutated various other amino acid residues that are conserved in all clostridial toxins and are located in the region of the DXD motif (Fig. 1). We tested the mutants K278ALT546, G281ALT546, G282ALT546, and P291ALT546 for transferase activity with Ras as a substrate. As can be seen in Fig. 8, all of these mutants exhibited similar properties with respect to both glucosyltransferase and glucohydrolase activity as compared with the wild-type toxin fragment, indicating no essential roles in catalysis. Moreover, these mutants were fully active after microinjection into culture cells (shown for K278ALT546; Fig. 7B) and could be labeled by $\beta^{32}$P$\gamma$-UDP-glucose (shown for G282ALT546; Fig. 5). By contrast, exchange of amino acid residues Tyr$^{284}$, Asp$^{270}$, or Arg$^{273}$ to alanine decreased the enzyme activity, whereas the transferase and glycohydrolase activity of the Tyr$^{284}$ mutant was reduced by 40- and 50-fold, respectively (Fig. 9). The glucosyltrans-

![Fig. 7 Microinjection into HeLa cells. HeLa cells were microinjected without (E) and with LT546 (A), K278ALT546 (B), D288ALT546 (C), and D286A/D288A (D). Photographs were taken 30 min (panels A, B, and E) or 4 h (panels C and D) after microinjection.](image1)

![Fig. 8 Glucosyltransferase and glucohydrolase activities of G281ALT546, G282ALT546, K278ALT546, P291ALT546, D286ALT546, D288ALT546, D286NL546, and D288NL546. A, glucosyltransferase activity. Ras (1 µg) was glucosylated by LT546 (A1), G281ALT546 (A2), G282ALT546 (A3), K278ALT546 (A4), and P291ALT546 (A5; each 1 nM) and by D286ALT546 (A6) and D288ALT546 (A7, each 1 µM) for 15 min. Signal intensity of Ras glucosylation of LT546 was set as 1. Data are given as means ± S.E. (n = 3). B and C, glucohydrolase activity. LT546 (B1, C8), G281ALT546 (B2), G282ALT546 (B3), K278ALT546 (B4), and P291ALT546 (B5; each 100 nM) and D286ALT546 (B6), D288ALT546 (B7), D286NL546 (C9), and D288NL546 (C10; each 1 µM) were incubated with 20 µM UDP-$^{[34]}$Cglucose and 100 µM UDP-glucose for 20 min and analyzed as described. Signal intensity of Ras glucosylation by LT546 was set as 1. Data are given as means ± S.E. (n = 3).](image2)
ferase activities of the D270ALT546 and R273ALT546 mutants were reduced by at least 200-fold (Fig. 9), and no glucosydrolase activity was detected (Fig. 9C). Whereas microinjection of the Y284ALT546 mutant induced the typical cytotoxicity, the D270ALT546 and R273ALT546 mutants exhibited variable results. Thus, we cannot exclude the possibility that these mutant proteins possess a small cytotoxic activity dependent on the amount of toxin microinjected.

**DISCUSSION**

Recently, we and others reported that the N terminus of C. difficile toxin B and of the lethal toxin from C. sordellii harbors the glucosyltransferase activity of the toxins (20, 21, 24). Here we continued the structure-function analysis of this family of large clostridial cytotoxins and report the demonstration that a small motif formed by two aspartic acid residues (D motif) is essential for glucosyltransferase activity by at least 5000-fold. Microinjection studies corroborated that these mutant proteins had lost their cytotoxic activity supports their essential role. Interestingly, at high concentrations of Mn2+ ions, the double mutants exhibited no activity of the D270ALT546 and R273ALT546 (1 μM, □) were incubated with 20 μM UDP-[3H]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 PhosphorImager analysis. Data are given in pmol of hydrolyzed UDP-glucose as means ± S.E. (n = 3).

**FIG. 9.** Glucosyltransferase and glucosydrolase activities of D270ALT546, R273ALT546, and Y284ALT546. A, time course of the glucosylation of Ras by LT546 and R273ALT546. Ras (1 μg) was incubated with LT546 (1 nM, ■) or toxin fragment mutant R273ALT546 (100 nM, □) in the presence of UDP-[14C]glucose (10 μM) for the indicated times. Then labeled proteins were analyzed by SDS-PAGE and PhosphorImager analysis. Data given are arbitrary units as means ± S.E. (n = 3). B, time course of the glucosylation of Ras by LT546, Y284ALT546, and D270ALT546. Ras (1 μg) was incubated with LT546 (1 nM, ■) or toxin fragment mutants Y284ALT546 (10 nM, ○) and D270ALT546 (100 nM, □) in the presence of UDP-[3H]glucose (10 μM) for the indicated times. Then labeled proteins were analyzed by SDS-PAGE and PhosphorImager analysis. Data given are arbitrary units as means ± S.E. (n = 3). C, time course of glucosydrolase activity of LT546, R273ALT546, Y284ALT546, and D270ALT546. LT546 (200 nM, ■), Y284ALT546 (1 μM, ○), R273ALT546 (1 μM, □) and D270ALT546 (1 μM, ▲) were incubated with 20 μM UDP-[3H]glucose and 100 μM UDP-glucose in a total volume of 20 μL.

**Motif Essential for Glucosyltransferase Activity**

It has been suggested that the DXXD motif participates in the coordination of the divalent cation by glycosyltransferases (26). It has been shown that the activity of C. sordellii lethal toxin depends on the presence of Mn2+ ions (11). Moreover, recent studies in our laboratory indicate that the activities of all large clostridial cytotoxins depend on Mn2+ ions.2 Divalent cations (mostly manganese ions) are suggested to be important for the binding of the nucleotide-sugar that serves in all glycosyltransferases like LPS synthases. Of all of the glycosyltransferases containing the DXD motif, those showing most similarity to the clostridial toxins in the region surrounding the motif were members of the family related to the Och1p α-1,6-mannosyltransferase (Fig. 1).

The finding that the site of the acetylation of the glucosyltransferase activity of the toxins is involved in divalent cation binding is an important finding for the binding of the nucleotide-sugar that serves in all glycosyltransferases as the cosubstrate (25). The notion that the DXXD motif is somehow involved in the UDP-glucose binding of LT is supported by the photoaffinity studies with azido-UDP-glucose. The wild-type fragment of LT was radiolabeled in the presence of [β-32P]5N3-UDP-glucose after UV irradiation. The incorporation of azido-UDP-glucose was specific by the criterion of competitive inhibition induced by excess unlabeled UDP-glucose. By contrast, mutants of the DXD motif were not photoaffinity-labeled in the presence of the azido-UDP-glucose derivative. Interestingly, at high concentrations of Mn2+ ions, the activity of the single DXD mutants was largely increased (about 20-fold), whereas the double mutants exhibited no activity even in the presence of 10 mM Mn2+. If the DXD motif is indeed involved in divalent cation binding, it is feasible that the presence of one aspartic acid residue of the DXD motif

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2 H. Genth, K. Aktories, F. Hofmann, C. Busch, and I. Just, manuscript in preparation.
allows binding of Mn$^{2+}$ and UDP-glucose, albeit with lower affinity, explaining the increase in activity with high concentrations of manganese ions. However, it should be noted that, even at high concentrations of Mn$^{2+}$, the activities of the single DXD mutants were reduced by several hundred-fold as compared with the wild-type LT fragment. Moreover, we did not observe photoaffinity labeling of 5-azido-UDP-glucose with the single DXD mutants even at high Mn$^{2+}$ concentrations (not shown).

In addition to the DXD motif, we changed several other amino acid residues that are located in the same region and are highly conserved within the family of large clostridial cytotoxins. Exchange of the glycine doublet (Gly$_{281}$/Gly$_{282}$), which was highly conserved within the family of large clostridial cytotoxins, did not change the enzyme activity of the mutant toxin. Unaltered activity was also observed with the mutant K278ALT546. However, we observed a 50-fold reduction with the mutant Y284ALT546 and an about 200-fold reduction in activity with the D270ALT546 and R273ALT546 mutants. However, all of these mutations did not show as large a decrease in activity as that observed with the DXD motif mutants, suggesting that these amino acid residues play not a direct role in catalysis. Nevertheless, those residues that showed the most effect after exchange apart from the DXD motif are also conserved in the Och1p family.

The full enzyme activity and cytotoxicity (after microinjection) of C. difficile toxin B and of C. sordellii lethal toxin depend on the presence of the N-terminal 546 amino acid residues of the holotoxins. The amino acid sequences of these major fragments are about 75% identical, showing no major changes in the similarity of amino acid sequences in the area around the DXD motif. Most distantly related in their primary structure are C. novyi α-toxin and C. sordellii lethal toxin with only 35% amino acid sequence identity at the N terminus (amino acids 1–546 of LT; amino acids 1–551 of α-toxin). However, a stretch of 30 amino acid residues between amino acid residues 264 and 294 is about 73% identical. Part of this highly conserved region is the DXD motif. Thus, from the data reported in this paper, we conclude that this region is essential for enzyme activity and most likely part of or near the active site of the glycosylating toxins.

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