Involvement of a Conserved Tryptophan Residue in the UDP-Glucose Binding of Large Clostridial Cytotoxin Glycosyltransferases*

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Large clostridial cytotoxins catalyze the glucosylation of Rho/Ras GTPases using UDP-glucose as a cosubstrate. By site-directed mutagenesis of Clostridium sordelli lethal toxin and Clostridium difficile toxin B fragments, we identified tryptophan 102, which is located in a conserved region within the catalytic domain of all clostridial cytotoxins, to be crucial for UDP-glucose binding. Exchange of Trp-102 with alanine decreased the glucosyltransferase activity by about 1,000-fold and blocked cytotoxic activity after microinjection. Replacement of Trp-102 by tyrosine caused a 100-fold reduction in enzyme activity, indicating a partial compensation of the tryptophan function by tyrosine. Decrease in glucosyltransferase and glycohydrolase activity was caused predominantly by an increase in the $K_m$ for UDP-glucose of these mutants. The data indicate that the conserved tryptophan residue is implicated in the binding of the cosubstrate UDP-glucose by large clostridial cytotoxins. Data bank searches revealed different groups of proteins sharing the recently identified DXD motif (Busch, C. Hofmann, F., Selzer, J., Munro, J., Jeckel, D., and Aktories, K. (1998) J. Biol. Chem. 273, 19566–19572) and a conserved region defined by a tryptophan residue equivalent to Trp-102 of C. sordelli lethal toxin. From our findings, we propose a novel family of glycosyltransferases which includes both prokaryotic and eukaryotic proteins.

The family of large clostridial cytotoxins consists of the toxins A and B of Clostridium difficile, the lethal and hemorrhagic toxins of Clostridium sordelli, and the $a$-toxin from Clostridium novyi (1, 2). C. difficile toxins A and B are responsible for symptoms in C. difficile-induced antibiotic-associated diarrhea and pseudomembranous colitis, whereas lethal toxin, hemorrhagic toxin, and $a$-toxin are virulence factors in gas gangrene (3, 4).

The toxins are glucosyltransferases that specifically modify and thereby inactivate small GTP-binding proteins of the Rho/Ras subfamily (5–8). Whereas the $a$-toxin from C. novyi catalyzes the transfer of an N-acetylglucosamine moiety from UDP-GlcNAc to its substrates (9), all other members of the toxin family share UDP-glucose as a cosubstrate. The modification occurs at amino acid Thr-37 in Rho or at Thr-35 in the other GTPases, resulting in inhibition of the interaction of the GTPases with their effectors (10, 11). Because Rho proteins are regulators of the actin cytoskeleton, their inactivation results in a breakdown of cytoskeletal structures within target cells (12). Furthermore, various other signal transduction processes controlled by the small GTPases are inhibited (13).

The large clostridial cytotoxins belong to the superfamily of glycosyltransferases that have been classified in different families depending on their sequence homologies (14). Some of these glycosyltransferases share a DXD motif also conserved among all large clostridial cytotoxins. This motif is located within the NH$_2$-terminal part of the toxins, which has been shown to be the catalytic domain of the toxins (15, 16). Several groups showed that the DXD motif is essential for the enzyme activity of the glycosyltransferases (17–20) and is probably involved in the binding of the cosubstrate UDP-glucose.

Recently, crystal structures of two glycosyltransferases of families 2 and 7, exhibiting the DXD motif, have been solved (21, 22), showing that the proteins share a similar topology of the catalytic center and that most of the amino acid residues involved in nucleotide recognition are conserved among all members of the respective families. Based on these findings, we compared thoroughly the sequences of glycosyltransferases showing sequence homology to the large clostridial cytotoxins in the region around the DXD motif and identified another homology region conserved in all of these proteins. Here we report on a tryptophan residue located in this second conserved region of many putative glycosyltransferases, including clostridial cytotoxins, which is likely to be implicated in the binding of the cosubstrate UDP-glucose.

**EXPERIMENTAL PROCEDURES**

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1 The abbreviations used are: B546, NH$_2$-terminal C. difficile toxin B fragment of amino acid residues 1–546; LT546, NH$_2$-terminal C. sordelli lethal toxin fragment of amino acid residues 1–546; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; $\Omega$, $\mu$mol(s).

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Large Clostridial Cytotoxins: Role of a Conserved Tryptophan

TATGGTACAGGATGTTG-3′/–CCACATCTCTGCTGACAT-AGATTAGTC-3′).

W102A.LT546: Primer pair S1W102A sense/antisense (5′-AAAAAT-TTACATTTGATTGGAGGACAA-3′/–TGTGCCCTCAATG-CTTACATTTT-3′).

W102Y.LT546: Primer pair S1W102Y sense/antisense (5′-AAAAATTTACATTTGATATTTGACAA-3′/–TTGCTCCATCAATATAAAGTTT-3′).

W102R.LT546: Primer pair S1W102R sense/antisense (5′-AAAAATTTCATTTGATATTTTATTTGTG-3′/–TGTGCCCTCAATGATTATT-3′).

GTG105A.LT546: Primer pair S1GTG105A sense/antisense (5′-TTTATAT-GGATGAGGACAAATAATAGATAC-3′/–GTATGATATTGTGTGTCATAC-3′).

D109A.LT546: Primer pair S1D109A sense/antisense (5′-GGAGGATCAAAATGCTACCGTATCAAC-3′/–GGTTAGATGCTGGAATATTATTTT-3′).

W102B.A546: Primer pair B1W102B sense/antisense (5′-AAAAATT-TTACATTTGTTGATATTGGAGGACAA-3′/–TGTGCCCTCAATGATTATT-3′).

Sequence—Sequencing of the mutated clones of LT546 and B546 was done with the Applied Biosystems, Inc. Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Weiterstadt, Germany) to check both for correct cloning and mutations caused by polymerization chain reaction amplification.

Expression of Recombinant Proteins—The recombinant GTP-binding proteins RhoA, Rac, Cdc42 and Ha-Ras were prepared from their fusion proteins as described (7, 8). The recombinant toxin fragments were expressed and purified as GST fusion proteins in accordance with the manufacturer’s instructions (see Fig. 2). GST fusion proteins from the Escherichia coli expression vector pGEX 2T were isolated by affinity chromatography with glutathione–Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) followed by removing GST fusion proteins with glutathione elution buffer (10 mM glutathione in 50 mM Tris-HCl, pH 8.0). Recombinant Rap1a was a gift from Dr. A. Wittinghofer (Dortmund, Germany).

Glucosylation Assay—Recombinant GTP-binding proteins (50–250 µg/ml) were incubated with recombinant toxin fragment LT546, B546, or mutated fragments at the indicated concentrations in a buffer containing 50 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl₂, 1 mM MnCl₂, 100 µg/ml bovine serum albumin, and the indicated concentrations of UDP-[3H]glucose and unlabeled UDP-glucose at 37 °C for the indicated periods. The total volume was 20 µl. Labeled proteins were analyzed by SDS-PAGE and subsequently by PhosphorImaging (Molecular Dynamics, Germany).

Glycohydrolase Assay—LT546 and the mutated fragments were incubated with [14C]-labeled UDP-glucose and unlabeled UDP-glucose at the indicated concentrations in a buffer containing 50 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl₂, 100 µM bovine serum albumin, 1 mM MnCl₂. The total volume was 5–10 µl.

Samples of 1.5 µl were taken out at each time point and subjected to thin layer chromatography with polyethylenimine cellulose plates (Merck, Germany) and 0.2 M LiCl as mobile phase in order to separate hydrolyzed glucose from UDP-glucose. The plates were dried and analyzed by PhosphorImaging.

Tryptophan Quenching Experiments—The decrease in intrinsic protein fluorescence of the wild-type fragment LT546 or the mutant W102Y.LT546 as a function of the UDP-glucose concentration was measured at 25 °C (25). The proteins were diluted in a buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 2.5 mM CaCl₂. The solutions were excited at 280 nm (3.5-mm band pass), and the fluorescence intensity was measured at 340 nm (3.5-mm band pass) in a Perkin Elmer LS50B luminescence spectrometer. The final concentrations of the fragments used in the experiments were 0.8 µM for LT546 and 1 µM for W102Y.LT546. The fluorescence decrease of tryptophan was used to correct for the inner filter effect. All data points were corrected additionally for dilution.

Kinetic Experiments—Initial rate data for the glucosyltransferase and glycohydrolase reaction were determined with regard to UDP-glucose binding by varying the UDP-glucose concentration from about 0.2 to 2 × 10⁻⁴ M. The glucosyltransferase reaction was performed at fixed GST concentrations of 12.5 µM. The kinetic values were obtained by analysis of Lineweaver-Burk plots of initial velocities from three independent experiments.

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.

Electropermeabilization Experiments and Measurement of Transepithelial Resistance—Caco-2 cells (Cell Line Service, Heidelberg, Germany) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were subcultured every week and seeded on filter inserts for cell culture (12-mm diameter, Falcon) at a density of approximately 4 × 10⁴ cells × cm⁻² for electropermeabilization experiments and determination of transepithelial resistance. Caco-2 cells on filter inserts, cultured in 12-well plates, were transfected once with Hanks’ balanced salt solution and equilibrated at 37 °C for 30 min in HBSS containing a 10 mM concentration of the toxin fragment to be inserted into cells in the apical and basolateral reservoir. Before precision discharge a pulse generator (Gene Pulser II, Bio-Rad) with a capacitance extender was used. According to the initial transepithelial electrical resistance of the filter, the parallel shunt resistor of the pulse controller was adjusted to 400 Ω for filters with 200–400 cm² or to 600 Ω for filters with 400–600 cm² in parallel to the resistance of cell monolayer. After the pulse (200 V/cm; 10 microfarads; 1.2 ms), the filter insert was transferred back into 12-well plates and incubated in Hanks’ balanced salt solution at 37 °C. The transepithelial electrical resistance was measured after the indicated times.

RESULTS

Identification of Proteins with Sequence Homology to Members of the Glucosyltransferase Family of Large Clostridial Cytotoxins—We have recently shown the importance of a highly conserved region (DXD motif) in active fragments of large clostridial cytotoxins for their glucosyltransferase activities (17). Besides the DXD motif, which we found to be essential for enzyme activity, this entire region contains 16 additional residues conserved among all large clostridial cytotoxins (Fig. 1A). At least two of these additional residues appear to be important for enzyme activity (17).

When this whole region, which we refer to as the extended DXD motif, is used to search protein data bases, several further sequences can be identified, all of which share at least six residues of this motif with the toxicodion toxins. Fig. 1A shows an alignment of some of these proteins in the mentioned section. Furthermore, another short amino acid stretch located NH₂-terminal of the DXD motif is conserved in all sequences (Fig. 1B). From these findings, we set out to elucidate the significance of this second homology region for the enzyme activity of large clostridial cytotoxins.

Effects of Site-directed Mutagenesis on Enzyme Activity of Toxin Fragments—Alanine mutants of all conserved amino acid residues in the region of amino acids 96–109 of the enzymatically active NH₂-terminal fragment of C. sordellii lethal toxin (LT546) were constructed. The mutants were expressed as GST fusion proteins and purified as described. Fig. 2A shows a SDS-PAGE analysis of the purified fusion proteins. Next, we tested the glucosyltransferase activity of these mutants in a glucosyltransferase assay with the substrate GST-Rac. We found that all mutations (K96A, H99A, G105A, and D109A) lead to a decrease in enzyme activity, although the conservative muta-
tion W102Y.LT546 resulted in an enzyme activity higher than that of the alanine mutant, the arginine mutant only showed a residual activity (Fig. 4A).

To exclude that the reduced activity was caused by an impaired binding only to the substrate GST-Rac, we determined the substrate specificity of the mutant W102A.LT546. As shown in Fig. 5, we found that W102A.LT546 was capable of modifying all subtypes of the wild-type fragment, even though to a considerably lesser extent. When we compared LT546 and the mutant in time courses of the modification of the GST-Rac or Ras-protein, respectively, we observed an approximately 1,000-fold reduction of enzyme activity of the mutant W102A.LT546 for both protein substrates (Fig. 3).

To confirm the significance of Trp-102 for the glucosyltrans-

![Figure 1](http://www.jbc.org/)  
**FIG. 1.** Alignments of the large clostridial cytotoxins and related proteins. Panel A, alignment of the region around the DXXD motif. Panel B, alignment of the second homology region including the conserved tryptophan. LT, C. sordelli 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 Z23277); Toxin A, C. difficile strain 10463 cytotoxin A (GenBank M30307); Alpha-tox, C. novyi alpha-toxin (GenBank Z48636); EHEC, E. coli enterohemorrhagic strain O157:H7 toxin A (sptrembl o82916); Chlam., C. trachomatis CT166 (sptrembl o84168). Yeast proteins: Ochlp, Saccharomyces cerevisiae och1 mannose transferase (GenBank D11095); Hoclp, C. albicans putative glycosyltransferase Hoc1 precursor (pir s7094); YDB6, Schizosaccharomyces pombe hypothetical protein (EMBL s07986); Surlp, S. cerevisiae hypothetical protein surlp (GenBank M96648). Cps proteins: bacterial capsular polysaccharide synthesis proteins; C. diff., C. difficile strain VPI 10463; C. trachomatis, C. trachomatis CT166 (sptrembl o84168).

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**Panel A**

![Figure 2](http://www.jbc.org/)  
**FIG. 2.** Panel A, purified recombinant lethal toxin fragment mutants. The NH2-terminal toxin mutants were constructed as GST fusion proteins, expressed in E. coli, and purified by affinity chromatography. Lethal toxin fragment mutants as fusion proteins were wild-type LT546, K96A.LT546, H99A.LT546, W102Y.LT546, W102A.LT546, W102R.LT546, G105A.LT546, and D109.LT546 (approximately 1 μg of protein was loaded on each lane). Panel B, NH2-terminal toxin B fragment mutants as GST fusion proteins B546 and W102AB546 (approximately 0.5 μg was loaded on each lane). Panel C, protease digest of toxin fragment and mutants by trypsin. Lethal toxin fragment LT546 mutant W102A.LT546 (each 20 μg) were incubated with 180 μg of trypsin in a buffer containing 10 mM glutathione and 50 mM Tris-HCl, pH 8.0, in a total volume of 26 μl. At the indicated time points, aliquots of 7 μg of protein were taken out and analyzed by SDS-PAGE.

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**Panel B**

![Figure 3](http://www.jbc.org/)  
**FIG. 3.** The results of the glucosyltransferase reaction with the substrate GST-Rac and the mutants K96A.LT546, H99A.LT546, W102Y.LT546, W102A.LT546, W102R.LT546, G105A.LT546, and D109.LT546 (each 20 μg) were incubated with 180 μg of trypsin in a buffer containing 10 mM glutathione and 50 mM Tris-HCl, pH 8.0, in a total volume of 26 μl. At the indicated time points, aliquots of 7 μg of protein were taken out and analyzed by SDS-PAGE.

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**Panel C**

![Figure 4](http://www.jbc.org/)  
**FIG. 4.** Kinetic Studies of Trp-102 Mutants—The effects of replacing Trp-102 with tyrosine or alanine on enzyme activity of LT546 were evaluated by comparing the relative specific activities for the glucosyltransferase and the glycohydrolase reaction. Panel A, LT546 mutant W102A.LT546 was found to be much less active than the wild-type fragment in a glucosylation assay (Fig. 4B). Kinetic Studies of Trp-102 Mutants—The effects of replacing Trp-102 with tyrosine or alanine on enzyme activity of LT546 were evaluated by comparing the relative specific activities for the glucosyltransferase and the glycohydrolase reaction (kcat and km/Km) of the wild-type and mutant proteins. Furthermore, we also compared the Michaelis constant (Km) of the fragments toward UDP-glucose.

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**Panel D**

![Figure 5](http://www.jbc.org/)  
**FIG. 5.** The glucosyltransferase reaction, the kinetic parameters were determined by varying the UDP-glucose concentrations from 0.2 to 2 mM at a fixed GST-Rac concentration of 5 μM. The values obtained by Lineweaver-Burk plots are summarized in Table I. Kinetic Studies of Trp-102 Mutants—The effects of replacing Trp-102 with tyrosine or alanine on enzyme activity of LT546 were evaluated by comparing the relative specific activities for the glucosyltransferase and the glycohydrolase reaction (kcat and km/Km) of the wild-type and mutant proteins. Furthermore, we also compared the Michaelis constant (Km) of the fragments toward UDP-glucose.

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**Panel E**

![Figure 6](http://www.jbc.org/)  
**FIG. 6.** Conservative substitution of Trp-102 with tyrosine resulted in a 20-fold increase in Km, whereas kcat was reduced 4-fold. The mutant was about 100-fold less efficient (9 × 103-fold lower kcat/Km) compared with the wild-type fragment. Glycohydrolase activity was less influenced by the replacement with tyrosine. As can be seen in Table I, the activity (kcat) was un-

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**Panel F**

![Figure 7](http://www.jbc.org/)  
**FIG. 7.** The results of the glycohydrolase reaction, the kinetic parameters were determined by varying the UDP-glucose concentrations from 0.2 to 2 mM at a fixed GST-Rac concentration of 5 μM. The values obtained by Lineweaver-Burk plots are summarized in Table I. Kinetic Studies of Trp-102 Mutants—The effects of replacing Trp-102 with tyrosine or alanine on enzyme activity of LT546 were evaluated by comparing the relative specific activities for the glucosyltransferase and the glycohydrolase reaction (kcat and km/Km) of the wild-type and mutant proteins. Furthermore, we also compared the Michaelis constant (Km) of the fragments toward UDP-glucose.
changed, whereas the binding affinity was reduced about 10-fold, resulting in a 10-fold less efficient enzyme.

On the other hand, the replacement of Trp-102 with alanine resulted in an enzyme that was altered much more dramatically in its kinetic properties. W102A.LT546 showed no detectable glycohydrolase activity (Fig. 6). Moreover, the $K_m$ value for the glucosyltransferase reaction was reduced more than 200-fold, whereas the specificity constant $k_{cat}$ was reduced 16-fold. Reflecting these alterations, the mutant W102A.LT546 was more than 3,500-fold less efficient ($2.7 \times 10^{-4}$) than the wild-type toxin fragment.

Fluorescence Studies—To determine the dissociation constants for UDP-glucose binding independently of enzyme activity, we measured the decrease in intrinsic protein fluorescence caused by quenching of the tryptophan fluorescence (excitation at 280 nm, emission at 340 nm) as a function of UDP-glucose concentration. The values recorded at the fluorescence maximum at 340 nm were corrected for dilution and the inner filter effect.

We tested the wild-type toxin fragment and observed a quenching of about 20% at a concentration of 23 mM UDP-glucose (Fig. 7). A dissociation constant ($K_d$) of 6 mM was determined for this interaction. Similar results were obtained for UDP-mannose, a nucleotide sugar that inhibits the glucosylation reaction with UDP-glucose (not shown). GDP-mannose is neither a cosubstrate for the glucosylation reaction of the lethal toxin, nor does it inhibit the glucosylation reaction. When we tested this nucleotide sugar with the wild-type fragment, we observed a much weaker quenching effect (less than 10%). The quenching curve of the mutant W102Y.LT546 was superimposable on that of the wild-type fragment with GDP-mannose (Fig. 7). This was also found for higher concentrations of the respective nucleotide sugars (not shown). Because of the low quenching effect, no dissociation constant could be determined.

Effects of Lethal Toxin Mutants on Cultured Cells—To characterize further the mutants W102Y.LT546 and W102A.LT546, we tested their biological activity by microinjection into cultured cells.
As shown in Fig. 8, we found the tyrosine mutant to elicit the same morphological effects as the wild-type fragment after 30 min when applied in a high concentration of 800 nM, whereas the alanine mutant showed no effect even after more than 4 h of treatment. To obtain more quantitative data, we measured the effect of LT546 and the mutants W102A.LT546 and W102Y.LT546 on transepithelial resistance of Caco-2 cells. For this purpose, the fragments were introduced in the cells by electropermeabilization. As can be seen in Fig. 9, treatment with the wild-type toxin fragment LT546 led to an 80% reduction of transepithelial resistance after 450 min, whereas only a 20% decrease was observed with the tyrosine mutant of Trp-102. The mutant W102A.LT546 was without effect on the integrity of the monolayer.

**DISCUSSION**

The large clostridial cytotoxins belong to the superfamily of glycosyltransferases that use nucleotide sugars as cosubstrates. However, their homology to glycosyltransferase families as established by Campbell et al. (14) is restricted to the small peptide motif DxD, which can be found in several otherwise unrelated families of glycosyltransferases. This motif has recently been shown to be crucial for enzyme activity of different types of glycosyltransferases (17–20). Elucidation of crystal structures of two glycosyltransferases has revealed the involvement of the aspartates of the DxD motif in the binding of the nucleotide sugar (21, 22).

Based on the DxD motif and its conserved surrounding, the large clostridial cytotoxins appear to be related to some recently described putative glycosyltransferases. Among these proteins are a number of hypothetical enzymes that are part of capsular polysaccharide synthesis clusters in bacteria, such as Streptococcus pneumoniae cap8J and a related sequence in the C. difficile genome (23, 24). Furthermore, a group of yeast proteins related to the mannosyltransferase Och1p and a family of closely related proteins of E. coli EPEC and EHEC strains...
and a number of hypothetical proteins of *Chlamydia trachomatis* serotype D are homologous to large clostridial cytotoxins. All of these proteins share at least an extended form of the DXD motif and another short peptide motif containing a conserved tryptophan residue with the toxins (Fig. 1).

Investigating the latter homology region by site-directed mutagenesis of an enzymatically active NH₂-terminal fragment of *C. sordellii* lethal toxin, we observed only a small reduction (up to 50%) of enzyme activity with the alanine mutants of Lys-96, His-99, Gly-105, and Asp-109 (not shown). This decrease in activity might be caused by minor alterations of the tertiary structure, whereas a direct involvement of these residues in the catalytic process or substrate binding seems unlikely.

Substitution of Trp-102, however, was found to have a major impact on enzyme activity of both the lethal toxin and the toxin B fragment. We observed a decrease in enzyme activity for all constructed mutants of this residue (Figs. 4 and 5). Because both glucosyltransferase and glycohydrolase activity were affected, an involvement of Trp-102 in the binding of the cosubstrate or the catalytic reaction was assumed. In line with this hypothesis, a similar decrease in glucosyltransferase activity was observed with two different protein substrates (Fig. 3).

Kinetic studies of different mutants of Trp-102 provided further insight into the role of this residue (Table I). As for the glycohydrolase reaction, we found about a 10-fold increase in the *Km* for the tyrosine mutant compared with the wild-type fragment, whereas *kcat* was slightly increased. From these results, a direct involvement of Trp-102 in the catalytic process could be excluded when *kcat* is taken as a measure for enzyme activity. The decrease in activity is rather caused by a reduction in binding affinity as the *Km* value is increased. The arginine and the alanine mutants showed no detectable glycohydrolase activity in our system, presumably reflecting the lower sensitivity of the glycohydrolase compared with the glucosyltransferase assay.

With respect to the glucosyltransferase reaction of the tyrosine mutant, both the catalytic activity and the binding affinity to UDP-glucose were reduced. The alanine mutant showed an even more impaired efficiency, mainly because the *Km* for UDP-

![Fig. 9. Effects of LT546 and mutants on transepithelial resistance of Caco-2 cells.](image)

![Fig. 10. Schematic representation of glycosyltransferases of family 2, 7, and of the proteins related to large clostridial cytotoxins.](image)
glucose was increased about 200-fold. This low in vitro activity of the alanine mutant is reflected by its lack of cytotoxic activity when introduced into cells by microinjection or electroporation. The tyrosine mutant, however, showed a clearly detectable, albeit reduced, cytotoxic activity (Figs. 8 and 9). The decrease in $k_{cat}$ of the latter mutant observed with the glycosyltransferase reaction is contradictory to the findings with the glycohydrolase reaction. However, it is conceivable that the complex interactions in the three-component glycosyltransferase reaction are more sensitive to alterations in the enzyme’s catalytic center than the glycohydrolase reaction. In contrast to recent findings, we found the glycohydrolase activity to be similar to the glycosyltransferase activity as the respective $k_{cat}$ values were similar. This can be explained, however, by our using the relatively bad substrate GST-Rac for the determination of enzyme activity. From the experimental data shown in Fig. 3, turnover rates of the modification of GST-Rac or Ras were determined to be approximately 260 h$^{-1}$ or 17,700 h$^{-1}$, respectively.

In conclusion, the results of the kinetic studies give evidence that the reduction in enzyme activity of Trp-102 mutants is mainly the result of a decrease in binding affinity for the cosubstrate UDP-glucose. These findings were corroborated further by tryptophan quenching experiments. We observed a quenching of about 20% with the cosubstrate UDP-glucose or the competitive inhibitor UDP-mannose, whereas the quenching effects of all other nucleotide diphosphate sugar used such as GDP-mannose (Fig. 7), UDP-GlcNAc, or GDP-glucose (not shown) were considerably lower. Because the quenching effect of UDP-glucose on the mutant protein W102Y.AT546 was reduced significantly, we conclude that the specific binding affinity of the mutant for the cosubstrate was decreased. Because of the low quenching effect we were unable to determine the $K_d$ for the mutant accurately. The overall low quenching of tryptophan fluorescence observed in our experiments implies that not all of the five tryptophan residues in the toxin fragment are involved in the interaction with the cosubstrate.

Concerning the mode of the interaction of Trp-102 with the cosubstrate, it should be noted that the replacing Trp-102 with tyrosine (Fig. 1B) leads us to hypothesize that Trp-102 is involved in a hydrophobic interaction with the cosubstrate UDP-glucose.

Recently, two crystal structures of glycosyltransferases containing the DXD motif have been solved. These are the family 2 glycosyltransferase spsA from B. subtilis and the bovine β4-galactosyltransferase of family 7 (21, 22). Although the homology of the amino acid sequences of these proteins is restricted to the DXD motif, they exhibit a similar fold of the catalytic core (26). Interestingly, both crystal structures comprise an aromatic residue that is involved in the stacking of the uracil ring of the cosubstrate in the catalytic fold. As found for the Trp-102 analog in the cytotoxin-related proteins, this conserved aromatic residue is located NH$_2$-terminally of the DXD motif, and there is no strictly defined distance between this residue and the motif (Fig. 10). Therefore, it is tempting to speculate that Trp-102 represents the analogous residue to the mentioned conserved aromatic residues.

Furthermore, all glycosyltransferase family 2 members share several other conserved amino acids, all of which appear to be located within the nucleotide binding domain as determined by the crystal structures. This binding domain is most often located within the NH$_2$-terminal half of the transferases, with the DXD motif representing its COOH-terminal end (Fig. 10). As shown in Fig. 10, this structure can also be found in the proteins related to large clostridial cytotoxins.

In conclusion, the large clostridial cytotoxins and the glycosyltransferase family 2 might share a similar architecture of their glycosyltransferase domain, with the nucleotide binding region located NH$_2$-terminally of the region that binds to the sugar molecule and the acceptor.

In this study, we have identified a conserved tryptophan residue that is essential for enzyme activity of large clostridial cytotoxins. A small group of proteins shares both the small motif in the vicinity of the tryptophan residue and an extended DXD motif with the toxins. Given the significance of those two motifs, it is conceivable that these proteins are also structurally related to large clostridial cytotoxins. Thus, we propose a family of large clostridial cytotoxin-related proteins.

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