Localiation of the Glucosyltransferase Activity of Clostridium difficile Toxin B to the N-terminal Part of the Holotoxin*

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Clostridium difficile toxin B that is one of the largest cytotoxins (270 kDa) known acts on Rho subfamily proteins by monoglucosylation (Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) Nature 375, 500–503). By deletion analysis we identified the enzyme and cytotoxic activity of the toxin to be located at the N terminus of the holotoxin. A 63-kDa fragment of toxin B covering the first 546 amino acid residues glucosylated Rho, Rac, and Cdc42, but not Ras, by using UDP-glucose as a cosubstrate. As known for the holotoxin, glucosylation by the toxin fragment was favored with the GDP-bound form of the low molecular mass GTPases. Microinjection of the toxin fragment into NIH-3T3 cells induced rounding up of cells and redistribution of the actin cytoskeleton. In contrast, a toxin fragment encompassing the first 516 amino acid residues was at least 1000-fold less active than toxin fragment 1–546 and cytotoxicity inactive. The data give direct evidence for location of the enzyme activity of C. difficile toxin B at the N-terminal part of 546 amino acid residues and indicate a functionally and/or structurally important role of the region from amino acid residues 516 through 546 for enzyme and cytotoxic activities.

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The abbreviations used are: ToxB, C. difficile toxin B; GST, glutathione S-transferase; CDB1, N-terminal C. difficile toxin B fragment of amino acid residues 1–900; CDB1–546, N-terminal C. difficile toxin B fragment of amino acid residues 1–546; CDB1–516, N-terminal C. difficile toxin B fragment of amino acid residues 1–516; CDB2–468, N-terminal C. difficile toxin B fragment of amino acid residues 1–468; CDB2, C. difficile toxin B fragment of amino acid residues 901–1750; CDB3, C-terminal C. difficile toxin B fragment of amino acid residues 1751–2366; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

small GTPases. Because Rho proteins are regulators of the actin cytoskeleton (6–8), glucosylation results in destruction of the cytoskeleton (3) and in inhibition of various signal transduction processes that are controlled by these GTPases (9).

Recently, the genes for C. difficile toxins were cloned and sequenced to show that they encode proteins of 308 and 270 kDa (10–12). From these data it has been proposed that the primary structures of these large toxins are characterized by three domains: the C-terminal part with repetitive oligopeptides suggested to be involved in receptor binding via glycosyljugates. The middle part has a rather small hydrophobic region and is tentatively assumed to be involved in membrane translocation (10, 12). Finally, it was speculated that the N terminus of the toxins including a putative nucleotide binding site bears the biological activity. This hypothesis was deduced from preliminary mutational studies showing that deletion of the C-terminal part or deletion of the middle part of ToxB reduced cytotoxicity but did not completely block the cytotoxic effects (13). However, no direct evidence for the location of cytotoxic activity at the N terminus has been presented. Because it is now clear that C. difficile toxins A and B are monoglucosyltransferases, we were prompted to localize their enzymatic activity to specific parts of the toxin molecule. Here we report that N-terminal fragments of ToxB that are expressed in Escherichia coli possess glucosyltransferase activity capable of modifying specifically small GTPases and cause potent cytotoxic effects after microinjection into culture cells.

EXPERIMENTAL PROCEDURES

Materials—UDP-[14C]hexoses were obtained from DuPont NEN (Dreieich, Germany). PCR primers were from Pharmacia (Freiburg, Germany). All other reagents were of analytical grade and purchased from commercial sources.

Purification of C. difficile ToxB—A dialysis bag containing 900 ml of 0.9% NaCl in a total volume of 4 liters of brain heart infusion was inoculated with 100 ml of an overnight brain heart infusion culture of C. difficile strain VPI 10463. After incubation under microaerophilic conditions at 37 °C for 72 h, the culture was centrifuged at 8000 × g for 20 min in a Sorvall GSA rotor. Ammonium sulfate was slowly added to the cleared culture filtrate to a final concentration of 70%. After 3 h of mixing at 4 °C, the suspension was centrifuged at 8000 × g for 30 min at 4 °C. The precipitate formed was dissolved in 15 ml of 0.05 M Tris-HCl buffer (pH 7.5) and dialyzed overnight at 4 °C against 1 liter of the Tris buffer. The dialyzed sample was cleared by centrifugation (8000 × g for 30 min at 4 °C) and directly applied to a MonoQ column (HR 10/10, Pharmacia, Freiburg, Germany) previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). Proteins were eluted by using a 0.05 to 0.7 M NaCl gradient. ToxB eluted at about 0.5 M NaCl as a single protein peak (>90% pure) and was identified by cytotoxicity.2

PCR Amplification—C. difficile strain VPI 10463 was used as the source for preparation of chromosomal DNA as described previously (14). Amplification of the toxin fragments CDB1, CDB2, and CDB3 was performed by PCR with the GeneAmp PCR System 2400 from Perkin-
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Elmer using the primer pairs CDB1/CDB1N (5'-AGATCTAGACTGTTAGATTAAGAAAAC-3'/-5'-GGATCAGATTTTATTACGCT-3') for CDB1, CDB2/CDB2N (5'-AGATCTAGAGTAAAGAAGGAGTGAAGAAGA-3'/-5'-GGATCCTACTATCCTATGAAAGA-3') for CDB2, and CDB3/CDEIN (5'-AGATCTAGCTGCAGACTTGCTAGAAGA-3'/-5'-GGATCCTACTATCCTATGAAAGA-3') for CDB3, respectively.

Amplification was done with 300 nmol of primers, 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 BglBI/EcoRI for CDB1–546, NheI/EcoRI for CDB1–516, and AflII/SmaI for CDB1–468. Religation of the resulting truncated fragments was performed after treatment with DNA polymerase I, large (Klenov) fragment.

**Sequence**—Sequencing of CDB1 and all its truncated derivatives, CDB2, and CDB3 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 PCR amplification. Sequencing was performed at least twice with overlapping DNA fragments. Cycle sequencing revealed nucleotide exchanges in CDB1 at three positions, two of which resulted in amino acid exchanges (M substituted by I at position 1; Y substituted by C at position 55). These amino acid exchanges did not result in changes of enzyme activity.

**Expression of Recombinant Proteins**—The recombinant GTP-binding proteins RhoA, Rac, Cdc42, and Ha-Ras were prepared from their fusion proteins as described previously (4). The recombinant toxin fragments were expressed and purified as GST fusion proteins in accordance with the manufacturer’s instructions. GST fusion proteins from the E. coli expression vector pGEX-2T were isolated by affinity chromatography with glutathione-Sepharose (Pharmacia, Germany) followed by cleavage 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.

**Microinjection Studies**—For microinjection studies, NIH-3T3 cells were grown for 24 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 4 mM glutamine/penicillin/streptomycin and plated on Celllocate (Eppendorf, Germany) overlayers at about 10⁴ cells/dish. Microinjection was performed with a Microinjector 5242 and Micromanipulator 5171 from Eppendorf. Actin cytoskeleton staining was performed with rhodamine-phalloidin after fixation of cells with paraformaldehyde (4%) plus Triton X-100 (0.2%).

**Glucosylation Reaction**—Rat brain tissue lysate (1 mg/ml) or recombinant GTP-binding proteins (30 μg/ml) were incubated with either ToxB (1 μg/ml) or recombinant toxin fragments (1–25 μg/ml) in a buffer containing 50 mM Heps (pH 7.5), 100 mM KCl, 2 mM MgCl₂, 100 μM bovine serum albumin for 30 min at 37 °C. The total volume was 20 μl. For sequential glucosylation, GST-RhoA (1 μg) was glucosylated with either ToxB or CDB1 in the presence of unlabeled UDP-glucose (1.5 mM) for 30 min at 37 °C and washed three times. Thereafter, a second glucosylation in the presence of UDP-[14C]glucose (10 μM) and CDB1 (10 μg/ml) or ToxB (10 μg/ml) was performed for 10 min at 37 °C. Labeled proteins were analyzed by SDS-PAGE and subsequently by phosphorimaging (Molecular Dynamics, Inc.). For studies on the nucleotide dependence of glucosylation, RhoA was incubated for 15 min at room temperature in a buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 2.5 mM Ca²⁺, 5 mM EDTA, 2.5 mM GDP or 2.5 mM GTP. Thereafter, MgCl₂ was added to give 7 mM. Then, nucleotide-loaded Rho was glucosylated as described above.

**RESULTS**

To identify functional domains of the C. difficile toxin B, we first split the structural gene encoding the toxin B into three parts (CDB1, CDB2, CDB3) of almost similar length and engineered these fragments into an E. coli expression vector as described under “Experimental Procedures” (see also Fig. 1). As shown in Fig. 2A, the N-terminal part of ToxB (fragment CDB1) was able to glucosylate Rho subtype proteins in lysates of rat brain tissue as efficiently as the holotoxin. Deduced from previous studies, the upper band is most likely Rho while the lower band is Rac and Cdc42 (15). In contrast, the C-terminal part (CDB3) and the middle fragment (CDB2) of ToxB were without activity. To study whether the N-terminal fragment of ToxB modifies the same amino acid as the holotoxin, we first treated recombinant GST-RhoA with the holotoxin or with fragment CDB1 in the presence of unlabeled UDP-glucose. Thereafter, the GST-RhoA beads were washed and a second glucosylation was initiated by addition of either holotoxin or CDB1 plus labeled UDP-[14C]glucose. As shown in Fig. 2B, pretreatment of Rho with the holotoxin or with CDB1 prevented the second glucosylation reaction indicating that both the holotoxin and the toxin fragment modified the same amino acid residue (e.g. threonine 37 of Rho).

Recently, it has been proposed that the region from amino acid residue 651 through 683 plays a pivotal role in cytotoxicity...
of ToxB (13). This region was suggested to be involved in nucleotide binding. Therefore, we studied the effects of deletion of this region on enzyme activity of fragment CDB1 of ToxB. We engineered a toxin fragment encompassing the first 546 amino acid residues but missing the proposed nucleotide-binding region (see Figs. 1 and 3). This toxin fragment (CDB1–546) was still able to glucosylate Rho proteins (Fig. 4). It was even more active than the holotoxin. At present we cannot conclude that the deletion activates ToxB because the difference in activity was rather small and the holotoxin and the toxin fragment were prepared by completely different procedures. When the active toxin fragment CDB1–546 was further deleted by 30 amino acid residues at the C terminus on enzyme activity of fragment CDB1–546. Recombinant Rho, Rac, Cdc42, and Ras (each 40 μg of protein/ml) were glucosylated by ToxB (1 μg/ml) and CDB1–546 (10 μg/ml) in the presence of UDP-[14C]glucose for 30 min. Then, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (shown). B, cosubstrate specificity of glucosylation by ToxB and CDB1–546. Rat brain tissue lysates (1 mg/ml) were incubated with ToxB and CDB1–546, respectively, in the presence of UDP-[14C]glucose (UDP-Glc), UDP-[14C]galactose (UDP-Gal), and UDP-[14C]N-acetylglucosamine (UDP-GlcNAc) for 30 min. Thereafter, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (shown).

Next we tested the biological activity of the toxin fragments by microinjection studies. For this purpose, the CDB1 fragment of C. difficile ToxB was microinjected into NIH-3T3 cells. About 30 min after microinjection into NIH-3T3 cells, morphological changes occurred. The cells rounded up in a manner typical for the action of C. difficile ToxB (Fig. 6). As shown by the rhodamine-phalloidin staining of the actin cytoskeleton (Fig. 6), the toxin fragment caused destruction of the actin microfilaments as observed after treatment of cells with the holotoxin. These cytotoxic effects were not detected after microinjection of

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![Fig. 3](image_url)

**Fig. 3. Purified recombinant toxin fragments.** The N-terminal toxin fragments CDB1–546, CDB1–516, and CDB1–468 were constructed as GST fusion proteins, expressed in E. coli, and purified by affinity chromatography and subsequent thrombin cleavage. GST fusion proteins of CDB1–546 (lane 1), CDB1–516 (lane 2), CDB1–468 (lane 3); toxin fragments after thrombin cleavage of the fusion proteins, CDB1–546 (lane 4), CDB1–516 (lane 5), CDB1–468 (lane 6, upper band; lower band represents GST) (5 μg of protein were loaded on lanes 1–4 and lane 6; 2.5 μg of protein were loaded on lane 5).

![Fig. 4](image_url)

**Fig. 4. Glucosylation of Rac1 (A) or RhoA (B) by the N-terminal toxin fragments CDB1–546, CDB1–516, and CDB1–468.** A, time course of the glucosylation of Rac by N-terminal toxin B fragments. Rac (40 μg/ml) was incubated with ToxB (270 ng/ml = 1 nM, △) or purified N-terminal toxin fragments CDB1–546 (32 μg/ml = 0.5 μM, ○; 63.4 ng/ml = 1 nM, ◇), or CDB1–516 (30 μg/ml = 0.5 μM, ▽) in the presence of UDP-[14C]glucose (10 μM) for the indicated times. Then, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (given as the glucosylation in percent of maximal labeling which was 0.7 mol/mol). B, Rho protein (40 μg/ml) was incubated with N-terminal toxin fragments CDB1–546 (1.5 μM, lane 1), CDB1–516 (1.5 μM, lane 2), or CDB1–468 CDB1–468 (1.5 μM, lane 3) for 60 min. Thereafter, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (shown).

![Fig. 5](image_url)

**Fig. 5. A, protein substrate specificity of glucosylation by ToxB and CDB1–546.** Recombinant Rho, Rac, Cdc42, and Ras (each 40 μg of protein/ml) were glucosylated by ToxB (1 μg/ml) and CDB1–546 (10 μg/ml) in the presence of UDP-[14C]glucose for 30 min. Then, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (shown). B, cosubstrate specificity of glucosylation by ToxB and CDB1–546. Rat brain tissue lysates (1 mg/ml) were incubated with ToxB and CDB1–546, respectively, in the presence of UDP-[14C]glucose (UDP-Glc), UDP-[14C]galactose (UDP-Gal), and UDP-[14C]N-acetylglucosamine (UDP-GlcNAc) for 30 min. Thereafter, labeled proteins were analyzed by SDS-PAGE and phosphorimaging (shown).
the C-terminal part (CD3) or the middle part (CD2) of the toxin. Moreover, the same changes in cell morphology and destruction of the actin cytoskeleton were observed with the 63-kDa fragment CDB1–546 of ToxB (not shown) indicating that after microinjection this small N-terminal toxin fragment encompasses the cytotoxicity of the 270-kDa holotoxin.

**DISCUSSION**

Here we present direct evidence that the N-terminal part (amino acid residues 1–546) of *C. difficile* toxin B bears the enzyme activity of the holotoxin. Only in the presence of the N-terminal fragment, but not with the middle or C-terminal fragment of the toxin, were Rho subfamily proteins glucosylated. Glucosylation of Rho proteins by N-terminal toxin fragments exhibits the same biochemical characterization as glucosylation catalyzed by the holotoxin. First, the protein substrate specificity of the constructed N-terminal fragments (CDB1, CDB1–546) and of the holotoxin are the same showing modification of Rho subfamily proteins (Rho, Rac, Cdc42) but not of Ras. Second, modification of Rho by the toxin fragments occurs most likely at Thr-37 of Rho, because pretreatment with the holotoxin inhibits subsequent modification by fragment CDB1 and, conversely, glucosylation by CDB1 blocks subsequent modification by the holotoxin. Third, the GDP-bound form of the GTPase is the preferred substrate for glucosylation. The latter finding has been recently explained by conformational changes of Rho occurring upon GDP/GTP-binding. As deduced from the structure of Ras (16), in its GDP bound form, the hydroxyl group of Thr-37 is likely to be directed to the solvent and, therefore, accessible for glucosylation by the toxin. Finally, the cosubstrate of the toxin fragments CDB1 or CDB1–546 is UDP-glucose but not other activated monosaccharides. Thus, the N-terminal fragments of the *C. difficile* toxin B share the same enzymatic properties as the holotoxin. Further evidence for location of the biological activity of ToxB in the N-terminal part of the molecule is provided by microinjection studies. We show that the N-terminal part of the protein is responsible for the morphological changes caused by the holotoxin. These findings are also in line with recent reports that glucosylated Rho proteins *per se* are sufficient to induce cytotoxic effects (3).

Studies from past years indicate that bacterial toxins acting inside cells are constructed of three major domains (17–19), a cell surface binding site, a domain involved in membrane translocation, and a third part responsible for the biological activity. Accordingly, it has been suggested that the C terminus of *C. difficile* toxins, which is characterized by several repeating sequences, is involved in binding to the eukaryotic target cells (10, 12). The middle part of the toxins containing a hydrophobic region with putative membrane spanning regions was tentatively implicated in membrane translocation (20), and the N-terminal part was suggested to harbor the cytotoxic activity (13, 20). Our findings unequivocally locate the biological activity of the *C. difficile* toxins to the N terminus. Moreover, our studies largely extend our knowledge about the structure-activity relationship of ToxB. It has been proposed that a putative nucleotide binding region (amino acid residues 651–683) located in the N-terminal part of the toxin is essential for cytotoxicity (13). A change of His-653 to Glu reduced the cytotoxic action at least 100-fold (13). Here we show that this region is not essential for the glucosylation of Rho subfamily proteins and for induction of cytotoxic effects, because fragment CDB1–546 (amino acid residues 1–546), showing the same enzymatic and cytotoxic activities as the holotoxin, does not contain this region. Moreover, several cysteine residues highly conserved in *C. difficile* toxins have been suggested to be important for the cytotoxic action of the holotoxin. All these conserved cysteine residues were absent in the fragment CDB1–546, apparently without affecting its glucosyltransferase and cytotoxic activities. Thus, these cysteine residues are not crucial for the en-

**TABLE I**

**Nucleotide dependency of the glucosylation of Rho A by ToxB or toxin fragments CDB1 and CDB1–546**

| Experiment | Incorporation of [14C]glucose | arbitrary phosphorimaging units |
|------------|------------------------------|---------------------------------|
|            | ToxB | CDB1 | CDB1–546 |
| **Experiment I** |      |      |          |
| GDP        | 8.2  | 9.8  | ND*      |
| GTP        | 3.9  | 3.7  | ND       |
| **Experiment II** |      |      |          |
| GDP        | 14.5 | ND   | 32.2     |
| GTP        | 5.8  | ND   | 15.3     |

* ND, not determined.

**FIG. 6. Microinjection of toxin fragment CDB1 into NIH-3T3 cells.** NIH-3T3 cells were microinjected with toxin fragment CDB1 (10 μg/ml) or with the supernatant of the *E. coli* culture. After 3 h, cells were fixed, and the actin cytoskeleton was stained by rhodamine-phalloidin (shown; A, control; B, CDB1).
zyme activity of ToxB, but may play a role in endocytosis or translocation of the toxin into the cytosol. However, a toxin fragment encompassing amino acid residues 1–516 did show at least 1000-fold reduced glucosyltransferase activity. Thus, the amino acid residues 517 through 546 appear to be essential for enzyme activity and/or structure of ToxB. Taken together, we show for the first time that the N-terminal part (546 amino acids) of the C. difficile ToxB harbors the enzyme and cytotoxic activities of the 270-kDa holotoxin. This finding will facilitate the precise structure-function analysis of the family of large cytotoxins including C. difficile toxins A and B, the lethal and hemorrhagic toxins of Clostridium sordellii, and the α-toxin of Clostridium novyi.

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REFERENCES
1. Kelly, C. P., Pothoulakis, C., and LaMont, J. T. (1994) N. Engl. J. Med. 330, 257–262
2. Thielmann, N. M. and Guerrant, R. L. (1995) in Bacterial Toxins and Virulence Factors in Disease (Moss, J., Iglewski, B., Vaughan, M., and Tu, A. T., eds) pp. 327–366, Marcel Dekker, New York
3. Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) Nature 375, 500–503
4. Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) J. Biol. Chem. 270, 13932–13936
5. Aktories, K., and Just, I. (1995) Trends Cell Biol. 5, 441–443
6. Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990) J. Cell Biol. 111, 1001–1007
7. Hall, A. (1994) Annu. Rev. Cell Biol. 10, 31–54
8. Chardin, P., Boquet, P., Madura, P., Popoff, M. R., Rubin, E. J., and Gill, D. M. (1989) EMBO J. 8, 1087–1092
9. Preppens, U., Just, I., von Eichel-Streiber, C., and Aktories, K. (1996) J. Biol. Chem. 271, 7324–7329
10. Dove, C. H., Wang, S. Z., Price, S. B., Phelps, C. J., Lyerly, D. M., Wilkins, T. D., and Johnson, J. L. (1990) Infect. Immun. 58, 480–488
11. Barroso, L. A., Wang, S.-Z., Phelps, C. J., Johnson, J. L., and Wilkins, T. D. (1990) Nucleic Acids Res. 18, 4904
12. Eichel-Streiber, C., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J., and Sauerborn, M. (1992) Mol. Gen. Genet. 233, 260–268
13. Barroso, L. A., Moncrief, J. S., Lyerly, D. M., and Wilkins, T. D. (1994) Microb. Pathog. 16, 297–303
14. Wren, B. W., and Tabaqchali, S. (1987) J. Clin. Microbiol. 25, 2402–2404
15. Just, I., Selzer, J., Hofmann, F., Green, G. A., and Aktories, K. (1996) J. Biol. Chem. 271, 10149–10153
16. Pai, E. F., Kalsch, W., Krengel, U., Holmes, K. C., John, J., and Wittinghofer, A. (1989) Nature 341, 209–214
17. Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M. G., Kantardjieff, K. A., Collier, R. J., and Eisenberg, D. (1992) Nature 357, 216–222
18. Montecucco, C., Papini, E., and Schiavo, G. (1994) FEBS Lett. 346, 92–98
19. Sixma, T. K., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, B. A. M., Witholt, B., and Hol, W. G. J. (1991) Nature 351, 371–377
20. Von Eichel-Streiber, C., Heringdorf, D. M. Z., Habermann, E., and Sartingen, S. (1995) Mol. Microbiol. 17, 313–321