UDP-Glucose Deficiency in a Mutant Cell Line Protects against Glucosyltransferase Toxins from Clostridium difficile and Clostridium sordellii*

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We have previously isolated a fibroblast mutant cell with high resistance to the two Rho-modifying glucosyltransferase toxins A and B of Clostridium difficile. We demonstrate here a low level of UDP-glucose in the mutant, which explains its toxin resistance since: (i) to obtain a detectable toxin B-mediated Rho modification in lysates of mutant cells, addition of UDP-glucose was required, and it promoted the Rho modification dose-dependently; (ii) high pressure liquid chromatography analysis of nucleotide extracts of cells indicated that the level of UDP-glucose in the mutant (0.8 nmol/10^6 cells) was lower than in the wild type (3.7 nmol/10^6 cells); and (iii) sensitivity to toxin B was restored upon microinjection of UDP-glucose. Using the mutant as indicator cell we also found that the related Clostridium sordellii lethal toxin is a glucosyltransferase which requires UDP-glucose as a cofactor. Like toxin B it glucosylated 21-23-kDa proteins in cell lysates, but Rho was not a substrate for lethal toxin.

Antibiotic-associated diarrhea and its potentially fatal form pseudomembranous colitis are human diseases caused by the anaerobic intestinal pathogen Clostridium difficile (1). Only strains producing the enterotoxin (ToxA) and the cytotoxin (ToxB) are pathogenic (1). Both toxins disrupt the actin cytoskeleton, causing rounding of cultured cells (2, 3) and inhibition of cytokinesis (4). The cytoskeletal collapse is mediated via a modification of the small GTPases Rho, Rac, and Cdc42, members of the Rho subfamily of Ras-related proteins (5, 6). The molecular mode of action of LT is unknown. The difference in sensitivity as compared to the wild type cell is 10^6 for ToxB and 10^7 for ToxA (1). The mutant cell is resistant also to microinjected ToxB whereas wild type cells are sensitive. Thus, the ToxB resistance of the mutant cell cannot be explained by either a mutation in the cell surface receptor or in the endocytic (12) internalization process.

UDP-Glc and UDP-GlcUA were purchased from Boehringer Mannheim, Mannheim, Germany. UDP-Glc and UDP-GlcUA were purchased from Rohrer, Mannheim, Germany. Clostridium botulinum C3 exoenzyme (C3) was obtained from Upstate Biotechnology Inc., New York. All other reagents were of analytical grade and obtained from local commercial sources.

Cell Culture and Preparation of Lysates—Diploid Chinese hamster lung fibroblasts (Don cells; ATCC No. CCL 16 = wild type) and a mutant of this cell line (11) denoted as Dkα-Q (here referred to as the mutant) were cultivated in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 5 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml) in a humid atmosphere containing 5% CO2. After 48 h the confluent cells were rinsed, mechanically removed and washed twice with ice-cold Hanks' balanced salt solution.*

EXPERIMENTAL PROCEDURES

Materials—ToxB (4) and LT (10) were purified as described earlier. UDP-[14C]glucose (specific activity 318 mCi/mmol) and [32P]NAD (specific activity 800 Ci/mmol) were obtained from DuPont NEN, Dreieich, Germany. UDP-Glc and UDP-GlcUA were purchased from Boehringer Mannheim, Mannheim, Germany. Clostridium botulinum C3 exoenzyme (C3) was obtained from Upstate Biotechnology Inc., New York. All other reagents were of analytical grade and obtained from local commercial sources.

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C3-catalyzed [32P]ADP-ribosylation as described under "Experimental Procedures". Proteins were separated by 12.5% SDS-PAGE, and radiolabeled bands detected by PhosphorImager analysis.

Cell pellets (approximately 70 μl) were resuspended in 200 μl of lysis buffer (50 mM triethanolamine, 150 mM KCl, 2 mM MgCl2, 5 mM GDP, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, pH 7.8) and sonicated five times for 5 s. After centrifugation (14,000 x g, 3 min) the supernatant was used as postnuclear cell lysate. The amount of protein in lysates was determined by Bio-Rad Protein Assay using bovine serum albumin as a standard.

ADP-ribosylation Reaction—Cell lysates (50 μg of protein) were treated with C3 (10 μg/ml) and [32P]NAD (0.06 μCi) for 30 min at 37°C according to Just et al. (5). The reaction was terminated by addition of SDS-PAGE sample buffer according to Laemmli (13).

ToxB-induced Rho Modification—Confluent cells (wild type or mutant) were treated with ToxB at concentrations and times indicated in the figures. Cells were mechanically removed and lysates were prepared. The ToxB-induced Rho modification is known to prevent subsequent ADP-ribosylation by C3 (5). The C3-catalyzed ADP-ribosylation reaction is Rho specific and was performed as described above. For in vitro treatment cell lysates were incubated 1 h at 37°C with ToxB (2 μg/ml unless otherwise stated) without or with the indicated concentrations of UDP-Glc. After C3-catalyzed ADP-ribosylation the proteins were separated by 12.5% SDS-PAGE (13). Radiolabeled bands were detected and quantified using a PhosphorImager SF (Molecular Dynamics). The percentage of modification was calculated as follows: (|C – X|/X) × 100, where C = counts of the control and X = counts of the test sample.

Quantification of UDP-Glc by HPLC Analysis—UDP-glucose was quantified according to Grubb et al. (14). Briefly, confluent cell monolayers in two 75-cm² culture flasks were washed twice with ice-cold phosphate-buffered saline and carefully dried. The nucleotide pool was extracted with 4 ml of 80% methanol per flask (1 h at 4°C). The methanol was evaporated under vacuum and the pellet resuspended in 500 μl of water (Milli Q). 100 μl of this preparation was loaded on a Bondesil C18 column (Varian) and eluted as follows: 0–25 min 100% buffer A (5 mM tetrabutylammonium phosphate, 80 mM ammonium acetate, 1.26% acetonitrile, pH 7.16); 25–45 min 0–75% buffer B (5 mM tetrabutylammonium phosphate, 80 mM ammonium acetate, 25% acetonitrile, pH 7.16); 45–60 min 75–0% buffer B. The wavelength for detection was 254 nm. UDP-Glc was added to the samples as an internal standard (final concentration 100 μM) to define its retention time. The peak area corresponding to 100 μM of similarly chromatographed UDP-Glc was subtracted from the peak areas of the samples containing the internal standard. The resulting value was used to calculate the amount of endogenous UDP-Glc according to a standard curve. In parallel flasks the number of cells was counted. The amount of UDP-Glc is reported as nanomole per 10⁶ cells.

Alternatively, the difference in amount of UDP-Glc between wild type and mutant cells was estimated by enzymatic conversion of endogenous UDP-Glc to UDP-GlcUA (15) followed by determination of the area of the UDP-GlcUA peak generated by ion exchange HPLC (16). Briefly, protein-free extracts of cells were prepared by addition of 2 volumes of 10% trichloroacetic acid to pellets of cells. After incubation (30 min, 4°C) and centrifugation, the trichloroacetic acid was extracted twice with ether. 30 μl of the preparation were added to 70 μl of reaction buffer (0.27 mM glycine, 1.6 mM NAD, 4.3 mM EDTA; pH 8.7) and the reaction initiated by addition of UDP-Glc-dehydrogenase (2 μl, 65 units/ml). After incubation for 1 h at 22°C, when the reaction was completed (monitored by the formation of NADH at 340 nm), the whole mixture (100 μl) was loaded onto an anion exchange column (QMA MemSep, Millipore) and eluted for 50 min with a linear gradient of K2HPO4 (0.1–0.5 M; pH 4.5). The eluting nucleotides were detected at 254 nm and the corresponding areas calculated.

Microinjection—Cells were grown on 13-mm slides for 48 h. Semi-confluent wild type and mutant cells were treated with ToxB (12 ng/ml) until 100% of the wild type cells were affected (1 h). Then the mutant cells were microinjected (Eppendorf microinjector) with the indicated concentrations of UDP-Glc or UDP-GlcUA in phosphate-buffered saline with 2% FITC-dextran (Sigma) to allow localization of microinjected cells. Approximately 100 cells were microinjected per experiment. The cultures were further incubated for 30 min at 37°C and fixed with 3.7% paraformaldehyde (10 min at 22°C). Cell morphology was visualized by phase-contrast and fluorescence microscopy and the fraction of ToxB-affected cells calculated as percent of the microinjected (fluorescent) cells. All experiments were performed three times, and three randomly selected fields were counted for each experiment.

Effect of LT on Wild Type and Mutant Cells—Wild type and mutant cells growing in 96-well microtiter plates were treated with LT (625 ng/ml) and the percentage of rounded cells scored visually. To demonstrate the effect of LT on the wild type and mutant actin cytoskeleton, cells grown in 8-well microslides were treated with LT (625 ng/ml) for 8 h at 37°C. Then cells were fixed with 3.7% paraformaldehyde (10 min at 22°C), permeabilized with 0.5% Triton X-100 (10 min at 22°C), stained with 0.5 μg/ml FITC-phalloidin (30 min at 37°C), and visualized by fluorescence microscopy.

Glucosylation Reaction—16 μl of UDP-[14C]Glucose dissolved in ethanol were dried under vacuum and 10 μl of lysate (10 mg of protein/ml) was added (final UDP-[14C]Glucose concentration: 100 μM unless otherwise stated). These mixtures were incubated with ToxB (6 μg/ml) or LT (5 μg/ml) for 1 h at 37°C and the reaction terminated by heating at 95°C in sample buffer (Laemmli). Proteins were separated by 12.5% SDS-
RESULTS AND DISCUSSION

ToxB-mediated Modification of Rho in Whole Cells—The modification of Rho by ToxB has been monitored as a reduction of the C3-catalyzed ADP-ribosylation of Rho in several cell lines (5). Initial experiments were conducted to verify that this modification occurs also in the Don wild type cells, and to clarify how Rho in the mutant cell responds to ToxB. In wild type cells treated with ToxB until 100% of the cells were rounded, Rho was modified, as it could no longer be ADP-ribosylated by C3 (Fig. 1, lanes 1 and 2). Mutant cells treated at the same conditions showed neither CPE nor any modification of Rho (Fig. 1, lanes 3 and 4). However, when the ToxB exposure of mutant cells was extended to 16 h, 100% of the cells were rounded and a strong modification of Rho was observed (Fig. 1, lanes 5 and 6). A modification of Rho in the mutant cells was observed also after shorter times with still higher amounts of ToxB, or with lower amounts of toxin if the exposure was prolonged until all cells were rounded (data not shown). These experiments indicated that (i) Rho of the mutant cell could indeed serve as a substrate for the ToxB-mediated modification (as well as for C3-catalyzed ADP-ribosylation), and (ii) ToxB-mediated modification of Rho correlated with the appearance of a CPE also in the mutant cell. Thus, it appears unlikely that the ToxB resistance of this cell is due to mutation of either Rho or some signaling component downstream Rho.

ToxB-mediated Modification of Rho in Cell Lysates—Cell lysates were treated with ToxB followed by C3-catalyzed ADP-ribosylation as described previously (5). A modification of Rho was detected in lysates from the wild type but not from the mutant (Fig. 2A). ToxB was recently reported to be a glucosyltransferase requiring UDP-Glc as a cofactor (7). When 100 μM UDP-Glc was added to the lysates, Rho of both cell types was strongly modified by ToxB (Fig. 2B). The effect of decreasing...
the amount of UDP-Glc in this reaction was quantified using a given concentration of ToxB (Fig. 3A). At low amounts of added UDP-Glc there was a clear difference in the Rho modification of the respective cell lysates. However, with UDP-Glc concentrations above 75 nM, Rho modification in the mutant cell lysates was strongly increased, reaching at 750 nM the levels seen in the wild type (Fig. 3B).

These results suggested that the mutant cell may have less UDP-Glc available for glucosylation than the wild type. However, the data are also consistent with the possibility that the mutant cell could contain components acting as competitive inhibitors of either Rho or UDP-Glc. The first possibility was assessed by titrating the toxin. At each toxin dose the Rho modification was identical in lysates from both cell types (Fig. 4A). This excludes competitive interference with Rho or with the toxin by some factor present only in the mutant. The second possibility was assessed by directly labeling the substrates with decreasing amounts of UDP-[14C]Glc. If a competitive inhibitor of UDP-Glc was present in mutant cell lysates, the substrates should be less labeled than substrates in the wild type at low concentrations of UDP-[14C]Glc. In contrast they were more labeled, ruling out this possibility (Fig. 4B). On the other hand the observed result could be expected if the mutant has a lower level of UDP-Glc, since this would imply less competition from endogenous UDP-Glc with the added UDP-[14C]Glc.

Cellular Levels of UDP-Glc—The levels of UDP-Glc in wild type and mutant cells were measured in extracts of nucleotides from both cell types according to Grubb et al. (14). The position of the UDP-Glc peak in chromatograms from reverse phase HPLC was identified by adding an internal standard of UDP-Glc, and the latter subsequently separated and quantified by ion exchange HPLC (see "Experimental Procedures"). After subtraction of the endogenous levels of UDP-GlcUA (which were identical in wild type and mutant cells, accounting for 20% of the area in the wild type) the areas of the enzymatically generated UDP-GlcUA were compared. The position of UDP-GlcUA was determined using a purified standard which eluted at 27.6 min.

Restored ToxB Sensitivity after Microinjection of UDP-Glc—Cell membranes are not readily permeable to UDP-Glc, and its extracellular addition to ToxB-treated mutant cells indeed did not alter their response to the toxin (data not shown). To elucidate if the low level of UDP-Glc is relevant for the in vivo ToxB resistance of the mutant, UDP-Glc was microinjected. Cells were pretreated for 1 h with a ToxB amount that did not
alter the morphology of the mutant, but induced 100% CPE in the wild type. Most mutant cells microinjected with 100 mM UDP-Glc developed a CPE characteristic for ToxB within 30 min, while neighboring cells remained unaffected (Fig. 6, A, 1–2, and B). Microinjection of 50 mM UDP-Glc caused a cell rounding in about 50% of injected cells whereas 10 mM was without effect (Fig. 6B). The specificity of the effect for UDP-Glc was confirmed by microinjecting 100 mM UDP-GlcUA, which did not cause any CPE in toxin-treated cells (Fig. 6A, 3 and 4). Neither nucleotide induced any CPE in cells not pre-treated with the toxin (data not shown).

The normal intracellular concentration of UDP-Glc is reported to be approximately 100 μM (18), and dilution of microinjected compounds is calculated to be 1/100. Thus microinjection of 50 mM UDP-Glc should restore the cellular level to the order of magnitude reported to be physiological. This amount

**Fig. 6. Effect of microinjection of UDP-Glc into mutant cells treated with ToxB.** Panel A, semiconfluent mutant cells were incubated at 37 °C with 12 ng/ml ToxB. After 1 h, when similarly treated wild type cells had developed a complete CPE, the mutant cells were microinjected with 100 mM UDP-Glc (1 and 2) or 100 mM UDP-GlcUA (3 and 4) in buffer containing 2% FITC-dextran to localize microinjected cells. The cells were further incubated at 37 °C for 30 min, then fixed and photographed as described under “Experimental Procedures.” Panel B, mutant cells were treated with ToxB as described above and then microinjected with 100, 50, or 10 mM UDP-Glc in buffer containing 2% FITC-dextran. Approximately 100 cells were microinjected per experiment. The percentage of affected cells was calculated as described under “Experimental Procedures.” Mean values S.D. of three experiments are presented.
The ED$_{50}$ was only 5 times higher than an amount which had no effect (10 mM), and half the amount which gave almost full effect, showing that the concentrations used were in the physiological range of cellular UDP-Glc concentration. This experiment also demonstrated that a 5 times difference in UDP-Glc concentration (from 10 to 50 mM) could in fact be a limiting factor for toxin B-mediated CPE. In conclusion, the ToxB-resistance of the mutant cell was abolished by raising its cytosolic level of UDP-Glc.

LT from *C. sordellii* is a glucosyltransferase—The *C. sordellii* LT is immunologically and genetically related to ToxB (9, 10, 19). Interestingly the mutant cell was highly resistant also to LT (Fig. 7A). The actin stress fibers in the wild type collapsed after LT treatment while those of the mutant cell remained intact (Fig. 7B). The lowest amount of LT inducing 100% CPE in the wild type in 24 h was 12.5 ng/ml, while it was not possible to induce a rounding of the mutant cells even with the highest tested concentration of LT (12.5 μg/ml). However, mu-
Concluding Remarks—Since the mutant cell line shows similar growth characteristics as the wild type (11) it is apparently adapted to this level of UDP-Glc. At least four proteins are consistently more abundant in the mutant than in the wild type (22). These proteins have been recently identified as belonging to the class of glucose-regulated stress proteins and are genetically co-regulated (23, 24). They have functions as chaperones in the endoplasmic reticulum (25). Thus they may help the cell to fold its proteins correctly despite the considerable reduction of UDP-Glc, a molecule reported to be important for the control of protein folding in the endoplasmic reticulum (26).

Proteins of this class can also interact with the actin cytoskeleton, which could thereby become stabilized when these proteins are up-regulated (27). That might explain the slightly reduced sensitivity of the mutant to toxins which disrupt the actin cytoskeleton independently of UDP-Glc. The mutation causing the UDP-Glc deficiency has not yet been defined. Whether the stress proteins are constantly overproduced because of the low level of UDP-Glc (24, 28), or are genetically up-regulated, with a possible over-consumption of UDP-Glc in the mutant cell as a consequence (29), remains to be seen. In either case this cell seems to be a promising tool for basic studies of endoplasmic reticulum chaperones and glycosylation reactions.

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