Ras, Rap, and Rac Small GTP-binding Proteins Are Targets for Clostridium sordellii Lethal Toxin Glucosylation∗

(Received for publication, August 2, 1995, and in revised form, January 16, 1996)

Michel R. Popoff, Esteban Chaves-Olarte§, Emmanuel Lemichez‡, Christoph von Eichel-Streiber†, Monica Thelestanė, Pierre Chardin**, Didier Cussac**, Bruno Antoны**, Philippe Chavriеr‡‡, Gilles Flataуt, Murielle Giry, Jean de Gunzburg§§, and Patrice Boquet††

From the 1 Institut Pasteur, Unité des Toxines Microbiennes, 75724 Paris, Cedex 15, France, the 2 Microbiology and Tumorbiology Center, Karolinska Institute, P. O. Box 280, S-171 77 Stockholm, Sweden, 3**CNRS, Institut de Pharmacie Moléculaire et Cellulaire, Sophia-Antipolis, 06560 Valbonne, France, 4INSERM, U452 Faculté de Médecine de Nice, 06107 Nice, Cedex 2, France, 5 Centre d’Immunologie de Marseille-Luminy, Case 906, 13286 Marseille Cedex, France, the 6 Institut für Medizinische Mikrobiologie und Hygiene Verfüngsgebäude für Forschung und Entwicklung, Obere Zahlbacher Strasse 63, J ohannes Gutenberg-Universität, 55101 Mainz, Federal Republic of Germany, and 7§§INSERM U248, 10 avenue de Verdun, 75010 Paris, France

Lethal toxin (LT) from Clostridium sordellii is one of the high molecular mass clostridial cytotoxins. On cultured cells, it causes a rounding of cell bodies and a disruption of actin stress fibers. We demonstrate that LT is a glucosyltransferase that uses UDP-Glc as a cofactor to covalently modify 21-kDa proteins both in vitro and in vivo. LT glucosylates Ras, Rap, and Rac. In Ras, threonine at position 35 was identified as the target amino acid glucosylated by LT. Other related members of the Ras GTPase superfamily, including RhoA, Cdc42, and Rab6, were not modified by LT. Incubation of serum-starved Swiss 3T3 cells with LT prevents the epidermal growth factor-induced phosphorylation of mitogen-activated protein kinases ERK1 and ERK2, indicating that growth factor-induced phosphorylation of mitogen-activated protein (MAP) kinases. The recombinant Y64W Ras mutant used for two major virulence factors inducing gas gangrene and hemorrhagic diarrhea in humans and animals (6). These C. sordellii toxins have some similarities to toxins A and B from C. difficile in terms of amino acid sequences and immunological epitopes (7). Despite these similarities, it seems that LT and toxins A and B affect different intracellular target proteins. LT causes morphological and cytoskeletal effects different from those elicited by the C. difficile toxins. The effects consist of the rounding of cell bodies with the reorganization of F-actin structures into numerous cell-surface filopodia and a loss of actin stress fibers (8, 9). In addition, we have recently shown that overexpression of RhoA, RhoB, or RhoC cDNA in HeLa cells protects these cells from the effects of toxins A and B, but not from those of LT (9). These observations clearly pointed out that Rho small GTP-binding proteins were the main substrate for the C. difficile toxins and that the targets of LT were distinct.

A mutant hamster fibroblast cell line has been described that is resistant to toxins A and B from C. difficile (10). This resistance was attributed to a low intracellular UDP-Glc content, and the fact that this mutant cell line was not intoxicated by LT indicated that LT is a glucosyltransferase (11).

In this paper, we report that LT modifies and inactivates p21ras by glucosylation of threonine 35. In addition, LT was also found to glucosylate Rap and Rac proteins. No activity was found on other Ras-related proteins including Ral, Rho, Cdc42, Arf1, and Rab.

EXPERIMENTAL PROCEDURES

Materials

C. sordellii LT was obtained from culture supernatants of the pathogenic C. sordellii IP82 strain and purified to homogeneity as described previously (8). Recombinant Ha-Ras, RhoA, Rac1, Rap2, Ral, Rab6, and Cdc42 proteins were made either in baculovirus (Rac1, Rab6, and Arf1) or in Escherichia coli through either GST fusions (Ha-Ras, Rap1, RhoA, and Rac1) or a histidine-tagged fusion (His-Cdc42). The Ral-GST fusion could not be processed by thrombin to yield a 21-kDa protein due to the fact that Ral itself was proteolyzed. Ral was therefore tested for LT glucosylation as a 47-kDa fusion protein. The Rho protein, used in this study, could be fully ADP-ribosylated by exoenzyme C3 or glucosylated by C. difficile toxin B. Rac1 and Cdc42, used in this work, could be fully glucosylated by C. difficile toxin B. Rabbit polyclonal antibodies against fimbrin were a gift of Monique Arpin (Unité de Biologie des Membranes, Institut Pasteur, Paris). The monocular antibody MK12 (Zymed Laboratories, Inc., S. San Francisco, CA) was used for immunoblotting MAP kinases. The recombinant Y46W Ras mutant used for...
tryptophan fluorescence experiments was produced in E. coli and purified as described previously (12).

Methods

Glucosylation Reactions—Incorporation of LT-catalyzed [14C]Glc in the presence of cell lysates was performed as described by J. List et al. (3) in the case of C. difficile toxin B. Briefly, 10 μl of UDP-[14C]Glc in ethanol (0.2 μCi, 300 mCi/mmol; DuPont NEN, Les Ulis, France) was dried down under vacuum. Recombinant proteins (2 μg) dissolved in 15 μl of 50 mM triethanolamine HCl buffer (pH 7.5) containing 2 mM MgCl2, 150 mM KCl, 100 μM dithiothreitol, and 2 mM GDP were added to the cell lysate and incubated at 37°C. LT (2 μg/ml) was then added to start the reaction, as described by S. K. Just et al. (3). The reaction was terminated by adding 5 μl of 2 × SDS sample buffer, boiled, and electrophoresed on a 15% SDS-polyacrylamide gel. Under staining with Coomassie Blue followed by destaining, the gel was dried, and radioactivity was recorded and counted using a PhosphorImager system (Molecular Dynamics, Inc., Sunnyvale, CA). Glucosylation of HeLa cell lysates by LT was performed as follows. HeLa cell lysates (5 × 107) were homogenized by three cycles of freeze-thawing in 200 μl of 50 mM triethanolamine HCl buffer (pH 7.5) containing 100 μM dithiothreitol, 1 μM leupeptin, and 1 μg/ml pepstatin (glucosylation buffer). Cell lysates (20 μl) were added to 10 μl of dried UDP-[14C]Glc, and 2 μg/ml LT was added to start the enzymatic reaction. After 1 h at 37°C, further processing and imaging were done as described. In Vivo Glucosylation by LT of Small GTP-Binding Proteins in Rat Fibrobasts—Rat-1 fibroblasts (Rat-1-EJ-Rap2.31.A8) stably transfected with G12V Ras and Rap2 (13) were grown in 60-mm Petri dishes to a subconfluent density. LT was added to the cells at the indicated concentration in 5 ml of fresh medium containing 10% fetal calf serum. After 2 h, the cells were removed from the dishes with a rubber policeman and washed in 10 ml of PBS followed by centrifugation at low speed. Washing was repeated five times to remove residual LT, and finally, the cell pellets were resuspended in 50 μl of glucosylation buffer. Cells were then lysed by four cycles of freeze-thawing. After homogenization, the amount of protein in each lysate was estimated. Glucosylation of small GTP-binding proteins with LT, 40 μl of cell lysate was added to 15 μl of dried UDP-[14C]Glc with 5 μg/ml LT. This mixture was incubated for 1 h at 37°C. Then, 5 μl of each reaction was added to 10 μl of sample buffer, boiled, and electrophoresed on a 15% SDS-polyacrylamide gel. The gel was stained, destained, dried, and analyzed for radioactivity by autoradiography.

Localization of the Glucosylated Amino Acid in Ha-Ras—This experiment was performed by microsequencing the radiolabeled protein. The Ha-Ras protein (10 μg) was first radioactively glucosylated by LT with 40 μl of dried UDP-[14C]Glc (0.8 μCi) for 1 h (under reaction conditions as described above). Then, 10 μl unlabeled UDP-Glc was added; the reaction was further incubated for an additional 1 h at 37°C, and the proteins were separated on a 12.5% SDS-polyacrylamide gel. After migration and staining with Amido black, the band containing Ras was excised, and the gel was cut out of the gel and digested with 1 μg of trypsin in 200 μl of 100 mM Tris-HCl buffer (pH 8.8) containing 0.01% Tween 20. The reaction was incubated for 18 h at 35°C. The resulting peptides were separated by HPLC using a hydrophobic C8 column with a acetonitrile/trifluoroacetic acid gradient. Fractions eluted from the column were collected and counted for radioactivity.

RESULTS

Disruption of Actin Stress Fibers and Formation of Filopodia Induced in HeLa Cells by LT—The cytopathic effect of C. sordellii LT consists of the rounding of cell bodies and profound alteration of F-actin-containing structures (8, 9). After a 3-h incubation with 2 μg/ml LT, HeLa cells became round, displaying actin rearrangements and cell surface filopodia, and exhibited a loss of actin stress fibers (Fig. 1). Using a polyclonal rabbit antibody that reacts against all known isoforms of the actin-bundling protein filamin/plastin (14), we observed that filamin/plastin was present in LT-induced filopodia (Fig. 1).

LT Catalyzes the UDP-Glc-dependent Glucosylation of 21–23-kDa Proteins in HeLa Cell Lysates—Incubation of HeLa cell lysates with LT in the presence of UDP-[14C]Glc followed by gel electrophoresis of the reaction products showed that the toxin induced labeling of proteins in the range of 21–23 kDa (Fig. 2). This reaction could be displaced by adding an excess of nonradioactive UDP-Glc, but not UDP-glucuronic acid (Fig. 2). No modification of proteins by LT was found with [14C]Glc alone (data not shown).

LT Glucosylates 21-kDa Proteins in Vivo—To demonstrate that small GTP-binding proteins were glucosylated by LT in vivo, Rat-1-EJ-Rap2.31.A8 fibroblasts were incubated with increasing amounts of LT (from 0.005 to 5 μg/ml). The highest concentration of toxin caused the characteristic cytopathogenic effect of LT in 100% of the cells within 1 h. All cells were then lysed, and the lysates were glucosylated with LT a second time, now in vitro with radioactive UDP-Glc. If LT acts from inside the cell, there should be an inverse correlation between the LT dose used for routine procedures in H21 medium supplemented with 10% fetal calf serum. When the cells reached confluency, they were serum-starved overnight in 0.1% fetal calf serum. After 3 h of incubation with LT (17 μg/ml) in serum-free medium (the activity of LT was monitored by the cytopathogenic effect on cells), EGF was added (or not) at a 50 ng/ml final concentration for 5 min. Cells were then scraped into polyacrylamide gel electrophoresis sample buffer, and 30 μg of total protein, for each experiment, was electrophoresed on a 12.5% SDS-polyacrylamide gel. The gel was blotted onto nitrocellulose and incubated with a monoclonal antibody directed against MAP kinases (anti-ERK1 and anti-ERK2). Immune complexes were detected by horseradish peroxidase-conjugated secondary antibody followed by the ECL kit (Amersham International). Fluorescence Measurements—LT-catalyzed glucosylation of Y64W Ras-GDP was performed in 50 mM triethanolamine HCl buffer (pH 7.5) containing 140 mM KCl, 1 mM MgCl2, and 0.1 μM dithiothreitol. Y64W Ras-GDP (50 μM) was incubated with 100 μM UDP-Glc and 2.5 μg/ml LT at 37°C for 2 h. Control experiments were performed in the absence of LT.

Guanine nucleotide exchange and GTP hydrolysis of glucosylated versus unmodified Y64W Ras (0.5 μM) were measured at 37°C in 50 mM Hepes (pH 7.5), 1 mM MgCl2, and 1 mM dithiothreitol by monitoring tryptophan fluorescence at 340 nm upon excitation at 292 or 300 nm (12). When needed, 2 μM EDTA was added to reduce free magnesium to 0.05 μM above.

Cell Microinjections—Diploid Chinese hamster lung fibroblasts (Don cells; ATCC CCL16, Don-wt (where "wt" indicates wild-type)) and the C. difficile toxin A- and B-resistant mutant of this cell line, CdA-Q (10, 11), referred to here as Don-Q (a UDP-Glc-deficient mutant of these cells), were grown on 13-mm slides for 48 h. Semiconfluent wild-type and mutant cells were microinjected (Eppendorf microinjector) with the indicated concentrations of LT, UDP-Glc, or anti-LT antibodies with fluorescein isothiocyanate-labeled dextran (Sigma) in calcium-free PBS. Approximately 100 cells were microinjected in each experiment. The cultures were further incubated for 30 min at 37°C and fixed with 3.7% paraformaldehyde for 10 min. Cells were visualized by phase-contrast and fluorescence microscopy.

Downloaded from http://www.jbc.org/ by guest on July 18, 2018
of a 23-kDa protein was observed in control cells. Two minor bands of 21 and 25 kDa glucosylated by LT were also noticed in control lysates (Fig. 3). Fig. 3 also demonstrates that a clear decrease to a total absence of labeling of these bands was observed when the cells had been preincubated in vivo with increasing concentrations of LT prior to the in vitro radioactive LT glucosylation. Assuming that LT reacts with small G-proteins, in accordance with its homology to C. difficile toxin B (15), this dose-dependent activity of LT suggests that the toxin exerts its action from within the cell.

LT Glucosylates Ras, Rap, and Rac Small GTP-binding Proteins in Vitro—Specificity of LT was studied by incubating UDP-[14C]Glc and LT with different members of the p21ras superfamily of small GTP-binding proteins. As shown in Fig. 4, Ha-Ras, Rap2, and Rac1 were substrates for LT-catalyzed glucosylation. In contrast, RhoA, Cdc42, and Rab6 were not modified in vitro by LT. Since Ral was only available as a GST fusion protein, we tested a possible influence of the fusion with GST by adding a Rac-GST construct to the series. As evidenced by Fig. 4, Rac1-GST was a substrate for LT glucosylation, whereas Ral-GST was not modified by the toxin. This suggests that Ral is not modified by LT. Finally, no incorporation of glucose catalyzed by LT could be found on Arf1 (data not shown).

LT Glucosylates Threonine 35 of Ha-Ras—To identify the acceptor amino acid glucosylated by LT, Ha-Ras protein was modified by LT in the presence of UDP-[14C]Glc, electrophoresed on SDS-polyacrylamide gel, and digested with trypsin, and the resulting peptides were separated as described under “Experimental Procedures.” As shown in Fig. 5, 47 fractions were obtained. The radioactivity was exclusively associated with fractions 39 and 40 (Fig. 5A). As shown in Fig. 5 (B and C), repurification of fraction 39 or 40 gave rise to a major peptide (peptide D for fraction 39 and peptide E for fraction 40) containing the radioactivity and several other small peptides. Peptides D and E were microsequenced and gave exactly the same
amino acid sequence. Each cycle of Edman degradation was collected and counted for radioactivity. We found the following unambiguous sequence for these peptides: SALTIQLIQN-HFVDEYDPTIEDSYR. Cycle 19 corresponding to a threonine gave a very small signal. The small amount of threonine detected in position 19 may be the consequence of the LT-catalyzed glucosylation of most of the Ras molecules present in the reaction. A decrease in or absence of threonine 37 of RhoA in automated amino acid sequencing, after glucosylation by toxin A or B, has been already reported (3, 4). The amino acid sequence found for both peptides D and E corresponds exactly to a sequence found in the Ha-Ras protein between amino acids 17 and 41 (16). Radioactivity was associated first with cycle 19 and decreased thereafter (Fig. 5E). The rise in radioactivity at cycle 19 establishes threonine 35 (of the Ha-Ras molecule) as the unique amino acid glucosylated by LT.

Inhibition of EGF-induced Phosphorylation of MAP Kinases in Swiss 3T3 Cells by LT—In serum-starved Swiss 3T3 cells, the mitogenic signaling pathway involving tyrosine phosphorylation of growth factor receptors such as the EGF receptor and the subsequent Ras-dependent activation of MAP kinase phosphorylation is reduced to a basal level (17). After incubation with EGF, Ras-dependent activation of MAP kinases ERK1 and ERK2 can be followed by a shift in electrophoretic mobility resulting from phosphorylation (18). If the toxin blocks Ras activity, serum-starved Swiss 3T3 cells incubated with LT before the addition of EGF should not activate MAP kinases. As shown in Fig. 6, serum-starved Swiss 3T3 cells incubated with EGF had MAP kinases shifted toward higher molecular mass compared with MAP kinases of cells not incubated with EGF. In contrast, when serum-starved Swiss 3T3 cells were incubated with LT, prior to incubation with EGF, the growth factor was not able to induce a shift in electrophoretic mobility of the MAP kinases (Fig. 6).

LT Acts in the Cytosol by Glucosylation—To further substantiate the notion that LT reaches the cytosol and acts by glucosylation of small GTP-binding proteins, a series of microinjection experiments was performed. Don-wt cells were incubated with LT in medium containing nonimmune rabbit serum. The expected characteristic cytopathogenic effect was observed in the whole cell population (Fig. 7A). When rabbit anti-LT antibodies were added to the medium, the same amount of LT as used in Fig. 7A did not affect the cells (Fig. 7B). Drugs blocking the endocytic pathway acidification (bafilomycin A1, chloroquine, or monensin), known to prevent many bacterial toxins from penetration into the cytosol (19), blocked the activity of LT on cells (data not shown). When Don-wt cells in medium containing anti-LT antibodies were microinjected with LT, they rapidly exhibited the cytopathogenic effect characteristic of LT (Fig. 7, C and D). Successful microinjection was monitored by a yellow-green fluorescence of fluorescein-labeled dextran added to the solutions microinjected (see “Experimental Procedures”). This showed that LT can exert its activity from the cytosol.

To demonstrate that the activity of LT is mediated through glucosylation (of G-proteins), we took advantage of a mutant Don cell (Don-Q). This cell has a low content of UDP-Glc, which renders it resistant to the glucosylating toxins A and B from C. difficile and also to LT (11). Don-Q cells were incubated with LT, followed by microinjection of UDP-Glc into some of them (those lighting up under fluorescence microscopy). As shown in Fig. 7 (E and F), only cells that were microinjected with UDP-Glc exhibited the characteristic cytopathogenic effect of the toxin, suggesting that the toxin and the cofactor act at the same side of the cell membrane. The specificity of the effect was confirmed by microinjecting, instead of UDP-Glc, UDP-Gal or UDP-GlcUA (100 mM) into cells similarly treated with LT. Neither of the additionally used activated sugars promoted any cytopathogenic effect. Finally, none of the three UDP-sugars used in this study had any effect if the cells were not pretreated with toxin (data not shown). Knowing that our rabbit anti-LT serum neutralized the toxin, we microinjected Don-wt cells with this serum and then incubated them with LT added to the medium. As shown in Fig. 7 (G and H), microinjection of anti-LT antibodies protected against LT, clearly indicating that the neutralizing antibody and the toxin meet each other in the

**Fig. 4. Glucosylation of recombinant Ras-related GTPases by LT.** Ha-Ras, Rap2, Rac1, Cdc42, RhoA, Rab6, Ral-GST, and Rac-GST (10 μg/assay) were incubated with LT and UDP-[14C]Glc. A, PhosphorImager picture; B, Coomassie Blue staining of the gel.
cytosol. Accordingly, cells not injected exhibited the cytopathogenic effect typical of LT (Fig. 7, G and H), as did cells microinjected with nonimmune rabbit serum (data not shown). The experiments shown in Fig. 7, together with those presented in Fig. 3, strongly suggest that LT acts from the cytosol by glucosylating small GTP-binding proteins using UDP-Glc as a cofactor.

LT Glucosylation of Ras Enhances the GTP Dissociation Rate and Reduces GTP Hydrolysis of the GTP-binding Protein—The effects of LT glucosylation on the intrinsic properties of Ras was studied using the Y64WRas mutant. This mutant has the same intrinsic biochemical properties as wild-type Ras, but its activation-deactivation cycle can be followed in real time by monitoring changes in the fluorescence of tryptophan 64 (12). In Fig. 8A, Y64WRas-GDP, glucosylated or not, was first activated by the addition of GTP. After several minutes, the protein was converted again to the GDP-bound form by addition of a large excess of GDP. This experiment was performed at a low magnesium concentration in order to favor the dissociation of the bound nucleotide (the rate-limiting step of nucleotide exchange) and to prevent GTP hydrolysis. Similar fluorescence changes were observed for the nonglucosylated and glucosylated Ras.

**Fig. 5.** Localization of LT-catalyzed 14C-glucosylated Ha-Ras by microsequencing. A, separation by HPLC of the peptides generated by trypsin and radioactivity of each fraction (on a 15-μl aliquot). B and C, purification by HPLC of fractions 39 and 40. Radioactivity associated with each peptide was counted on 50-μl aliquots. D, radioactivity associated with each Edman degradation cycle (each Edman cycle of peptides D and E was combined and counted).

**Fig. 6.** EGF-induced mobility shift of MAP kinases in cells pretreated with LT. Serum-starved Swiss 3T3 cells were treated with EGF and LT as shown. Cells were lysed, and ~30 μg of total protein/experiment was electrophoresed, blotted, and stained with the monoclonal antibody MK12 (ERK1, 44 kDa (p44); ERK2, 42 kDa (p42)).

*C. sordellii* Lethal Toxin Glucosylates Ras
glucosylated forms of Ras (Fig. 8A). Indeed, binding of GTP in place of GDP induced a decrease in fluorescence, and conversely, binding of GDP in place of GTP induced an increase in fluorescence. Upon GTP addition, the time course of the fluorescence decrease was similar for the two forms of Ras, indicating that glucosylation did not greatly modify the GDP dissociation rate. In contrast, the increase in fluorescence by GDP addition was four times faster for glucosylated Ras than for unmodified Ras (Fig. 8A). This result demonstrates that glucosylation weakened GTP binding in the nucleotide site of Ras by accelerating its dissociation rate. Similar effects of glucosylation were observed for the dissociation rate of GTP\_S either at low (1 \( \mu \)M) or high (1 mM) magnesium concentration (data not shown).

The effect of glucosylation on GTP hydrolysis by Ras is shown in Fig. 8B. Y64W Ras-GDP was incubated with GTP at 1 mM magnesium. Activation was triggered by the addition of 2 mM EDTA, which reduced the free magnesium concentration to <1 \( \mu \)M. The first instantaneous fluorescence decrease reflected the dissociation of magnesium from Y64W Ras-GDP, whereas the slower fluorescence decrease reflected (as in Fig. 8A) the exchange of GTP for GDP. After completion of GDP/GTP exchange, magnesium was added back to the reaction (1 mM free magnesium). Due to the intrinsic GTPase activity of the protein, the fluorescence of the unmodified form of Ras slowly increased toward the level of fluorescence initially observed for Ras-GDP (Fig. 8B). In the case of the glucosylated form of Ras, much slower kinetics of GTPase activity was observed. Indeed, upon glucosylation of threonine 35, Ras had a four times slower intrinsic GTPase activity (Fig. 8B).

Glucosylation of Y64W Ras by LT slightly modified the fluorescence of the protein. As compared with unmodified Y64W Ras, LT-glucosylated Y64W Ras exhibited, on one hand, a larger absolute fluorescence level and, on the other hand, a smaller fluorescence change upon GDP/GTP exchange or GTP hydrolysis (Fig. 8, A and B). Therefore, we looked for a fluorescence signal that could correlate with the glucosylation of the protein. When Y64W Ras-GDP was incubated with LT and
UDP-Glc, fluorescence was enhanced by 2% within 2 h (Fig. 8C). Since this signal required both LT and UDP-Glc, it certainly reflects the time course of UDP-Glc incorporation.

**DISCUSSION**

Toxins A and B from *C. difficile* have been shown to covalently modify and thereby inactivate the small GTP-binding protein Rho, resulting in the disruption of F-actin structures (20, 21). In vitro and in vivo evidence indicates that toxins A and B modify RhoA by UDP-Glc-dependent glucosylation of threonine 37 (3, 4). In addition to RhoA, toxins A and B from *C. difficile* also modify in vitro Rac1 and Cdc42 (3, 4), two other proteins of the Rho subfamily involved in the control of membrane ruffling and filopodia formation, respectively (22–24). Also, it has recently been reported that the α-toxin from *C. novyi* is a glycosyltransferase that acts on the cytoskeleton through modification of Rho. However, in this case, UDP-Glc was not the cofactor required for modification. We report here that LT, like toxins A and B from *C. difficile*, is also a glucosyltransferase that uses UDP-Glc to modify small GTP-binding proteins. However, the substrate specificity of LT is different from that of toxins A and B. LT glucosylates Ras, Rap2, and Rac1 in vitro. LT had no effect on Rho or on Cdc42, two of the main substrates for *C. difficile* toxins A and B.

The effects induced by LT on the HeLa cell actin cytoskeleton are obviously different from those elicited by toxins A and B from *C. difficile*. The LT effects consist of the disruption of actin stress fibers and the formation of filopodia containing F-actin and fimbrin/plastin (9). Glycosyltransferase activity of both C. difficile and *C. novyi* toxins is directed toward GTP-binding proteins of the Rho subfamily. With *C. sordellii* LT, we have the first toxin that mainly acts on the Ras subfamily of GTPases. The specific effect of LT on the HeLa cell actin cytoskeleton is fundamentally different from that observed with toxin A or B from *C. difficile* (9). Since both toxin B (or A) and LT are able to glucosylate Rac (3, 4), the specific activity of LT on the cytoskeleton cannot be attributed to Rac modification alone. Instead, we believe that the combination of the modified GTPases causes LT to induce its cytopathogenic effect. We would like to stress that since the physiological function of Rap is still unknown, Rap modification by LT could be a key event in LT-induced cytoskeletal disruption.

In Swiss 3T3 cells, stimulation of Ras activates membrane ruffling and actin stress fiber organization by Rac- and Rho-dependent mechanisms (22). Since LT inactivates both Ras and Rac, this may in turn inhibit Rho, resulting in the collapse of actin stress fibers. It is interesting to note that filopodia induced by LT markedly resemble those generated by microinjection of the activated form of Cdc42 into Swiss 3T3 cells (23, 24). It is tempting to speculate that LT is responsible for the formation of filopodia by indirectly activating Cdc42, which in turn is a consequence of a toxin-induced Ras inactivation. An alternative hypothesis to explain the formation of filopodia due to LT could be that Cdc42 is already in an active state, but formation of filopodia is masked by an inactive Ras, leading to a dominant phenotype of membrane ruffling and actin stress fibers. Inactivation of Ras and Rac would therefore allow observation of the Cdc42 phenotype.

LT inactivates Ras by glucosylation of threonine 35, which corresponds to threonine 37 of Rho (25), the residue modified by toxins A and B (3, 4). In addition, our data strongly suggest that LT acts on the cytosol and glucosylates small 21-kDa molecules in vivo, resulting in the inactivation of Ras, since serum-starved Swiss 3T3 cells intoxicated with LT have no Ras-dependent induced MAP kinase phosphorylation (see Fig. 6).

At the present time, we do not understand the nature of the substrate specificity of LT for Ras, Rap, and Rac. It seems reasonable that amino acid sequences apart from the threonine 35 acceptor site of glucosylation enable LT to specifically recognize the various small G-proteins.

LT glucosylation of Ras at threonine 35 induced a small but significant decrease in the K_{eff} of GDP, most likely due to a higher affinity of glucosylated Ras for magnesium. Such a difference in magnesium affinity has not been observed for the T35A mutant of ras (26). Apart from this small difference, the...
Thr-35 glucosylated form of Ras in the GTP-bound form has properties very similar to those of the T35A mutant: a 4-fold increase in the GTP K\textsubscript{off} and a four to five times slower rate of GTP hydrolysis (26). It is thus extremely likely that the Thr-35 glucosylation of Ras, as the T35A mutant of Ras, has a much decreased affinity for the Raf Ras-binding domain (27). The T35A mutant of Ras has a 200-fold reduced affinity for the Raf Ras-binding domain (27) and represents the mutation that has the most drastic effect on the Ras/Ras-binding domain interaction (27). Thr35nine 35 contacts both magnesium and γ-phosphate in the GTP-bound form and a water molecule that also makes a hydrogen bond with aspartic acid 38 in the Rap/Raf Ras-binding domain complex (28). Thr35nine 35 is conserved in all of the small G-proteins and is an essential residue of the switch I region (29). Thus, the modification of threonine 35 either by mutation (T35A) or by glucosylation would result in the inability of Ras to interact with its effector (27). Even the conservative T35S mutation greatly decreases (∼20-fold) the transforming potential of an oncogenic Ras, pointing to the importance of this residue in switching to the active conformation and/or interacting with the Raf effector (30).

It is remarkable that four out of five members of the group of these large clostridial cytotoxins (toxins A and B, LT, and α-toxin) have glucosyltransferase activities on small GTP-binding proteins. Recently, we have found that C. sordellii hemorhagic toxin, the fifth member of this large clostridial cytotoxin group, is also a glucosyltransferase.\(^3\)

Taking into account that LT is the first toxin that inactivates the Ras small GTP-binding protein, it should soon become a powerful laboratory reagent to explore cellular signaling pathways stimulated by this molecule.

Acknowledgments—We thank Jacques d’Alayer (Institut Pasteur), who performed microsequencing of the LT-modified form of Ha-Ras; Keith Iretton (Institut Pasteur) for stimulating discussions; Bruno Goud (Institut Curie, Paris) for the gift of Rab6; Pierre Vignais and Alexandra Fuchs (Centre Biologie Moleculaire et Structurale, Grenoble, France) for the gift of Rac1; and Martina Schmitd (Institut für Pharmakologie, Universität GH Essen, Essen, Germany) for the gift of Arfl. We specially thank M. Weidmann for critically reading the manuscript.

\(^3\) C. von Eichel-Streiber, P. Boquet, M. Sauerborn, and M. Thelestam, manuscript in preparation.

REFERENCES

1. Bette, P., Oksche, A., Mauler, F., von Eichel-Streiber, C., Popoff, M. R., and Habermann, E. (1991) Toxicon 29, 877–887
2. Lydtr, D. M., Krivan, H. C., and Wilkins, T. D. (1988) Clin. Microbiol. Rev. 1, 1–18
3. Just, I., Selzer, T., 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. (1996) J. Biol. Chem. 270, 13932–13936
5. Hall, A. (1994) Annu. Rev. Cell Biol. 10, 31–34
6. Arscelerautone, S. N., Panabokke, R. G., and Wijesundera, S. (1969) J. Med. Microbiol. 2, 37–53
7. Martinez, R. D., and Wilkins, T. D. (1992) J. Med. Microbiol. 36, 30–32
8. Popoff, M. R. (1987) Infect. Immun. 55, 35–43
9. GirY, M., Popoff, M. R., von Eichel-Streiber, C., and Boquet, P. (1995) Infect. Immun. 63, 4063–4071
10. Florin, I. (1991) Microb. Pathog. 11, 337–346
11. Chaves-Diarte, E., Florin, I., Boquet, P., Popoff, M., von Eichel-Streiber, C., and Thelestam, M. (1996) J. Biol. Chem. 271, 6925–6932
12. Antonyon, B., Chardirn, P., Roux, M., and Chabrè, M. (1991) Biochemistry 30, 8287–8295
13. Jimenez, B., Pizon, V., Lerosey, I., Beranger, F., Tavitian, A., and de Gunzburg, J. (1991) Int. J. Cancer 49, 471–479
14. Britscher, A., and Weber, K. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6849–6853
15. Green, G. A., Schué, V., and Montelé, H. (1995) Gene (Amst.) 161, 57–61
16. Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827
17. Cobb, M. H., Boulton, T. G., and Robbins, D. T. (1991) Cell Regul. 2, 965–978
18. de Vries-Smits, A. M. M., Burgering, B. M. T., Leeners, S. J., Marshall, C. J., and Bos, J. L. (1992) Nature 357, 602–604
19. Sandvig, K., Dubinina, E., Garred, O., Prydz, K., Kadiv, J. V., Hansen, S. H., and van Deurs, B. (1992) Biochem. Soc. Trans. 20, 724–727
20. Just, I., Fritz, G., Aktories, K., Giry, M., Popoff, M. R., Boquet, P., Hegenbarth, S., and von Eichel-Streiber, C. (1994) J. Biol. Chem. 269, 10706–10712
21. Just, I., Selzer, J., von Eichel-Streiber, C., and Aktories, K. (1995) J. Clin. Invest. 95, 1026–1031
22. Ridley, A. J., Paterson, H. F., O’hlinston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
23. Nobes, C. D., and Hall, A. (1995) Cell 81, 53–62
24. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995) Mol. Cell. Biol. 15, 1942–1952
25. Madaule, P., and Axel, R. (1985) Cell 41, 31–40
26. John, J., Rendsland, H., Schlütting, I., Vetter, I., Borsao, G. D., Goody, R. S., and Wittinghofer, A. (1993) J. Biol. Chem. 268, 923–929
27. Herrmann, C., Martin, G. A., and Wittinghofer, A. (1995) J. Biol. Chem. 270, 2901–2905
28. Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) Nature 375, 554–560
29. Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., and Wittinghofer, A. (1990) EMBO J. 9, 2351–2359
30. White, M. A., Nicolette, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M., and Wigler, M. H. (1995) Cell 80, 533–541
Ras, Rap, and Rac Small GTP-binding Proteins Are Targets for Clostridium sordellii Lethal Toxin Glucosylation

Michel R. Popoff, Esteban Chaves-Olarte, Emmanuel Lemichez, Christoph von Eichel-Streiber, Monica Thelestam, Pierre Chardin, Didier Cussac, Bruno Antonny, Philippe Chavrier, Gilles Flatau, Murielle Giry, Jean de Gunzburg and Patrice Boquet

J. Biol. Chem. 1996, 271:10217-10224.
doi: 10.1074/jbc.271.17.10217

Access the most updated version of this article at http://www.jbc.org/content/271/17/10217

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 11 of which can be accessed free at http://www.jbc.org/content/271/17/10217.full.html#ref-list-1