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EhRho1, a RhoA-Like GTPase of *Entamoeba histolytica*, Is Modified by Clostridial Glucosylating Cytotoxins▽

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Clostridial glucosylating cytotoxins inactivate mammalian Rho GTPases by mono-O glucosylation of a conserved threonine residue located in the switch 1 region of the target protein. Here we report that EhRho1, a RhoA-like GTPase from the protozoan parasite *Entamoeba histolytica*, is glucosylated by clostridial cytotoxins. Recombinant glutathione *S*-transferase–EhRho1 and EhRho1 from cell lysate of *Entamoeba histolytica* were glucosylated by *Clostridium difficile* toxin B and *Clostridium novyi* alpha-toxin. In contrast, *Clostridium difficile* toxin A, which shares the same mammalian protein substrates with toxin B, did not modify EhRho1. Change of threonine 52 of EhRho1 to alanine prevented glucosylation by toxin B from *Clostridium difficile* and by alpha-toxin from *Clostridium novyi*, which suggests that the equivalent threonine residues are glucosylated in mammalian and *Entamoeba* Rho GTPases. Lethal toxin from *Clostridium sordellii* did not glucosylate EhRho1 but labeled several other substrate proteins in lysates from *Entamoeba histolytica* in the presence of UDP-[^{14}C]glucose.

The Rho GTPases form a subgroup of the Ras superfamily of low-molecular-mass GTP-binding proteins, which are ubiquitously expressed and conserved across various eukaryotic species, including yeast and humans. Prominent members of this GTPase subfamily are Rho, Rac, and Cdc42, which function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state (9, 31). The ratio of the two forms is regulated by numerous guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins. Guanine nucleotide dissociation inhibitors are another group of regulatory proteins. Guanine nucleotide dissociation inhibitors extract Rho GTPases from membranes, keep them in the cytosol, and block nucleotide exchange (27). In response to extracellular stimuli, Rho proteins control a large array of cellular processes, including organization of the cytoskeleton, motility, cell polarity, endocytosis, gene transcription, regulation of microtubule dynamics, and cell cycle progression (9).

Several bacterial protein toxins which target the cytoskeleton through modification of actin and low-molecular-mass GTPases involved in regulation of the actin cytoskeleton have been identified (2, 6). Besides their pathophysiological significance, these toxins are widely used as tools to study the mechanism of cellular pathways responsible for the maintenance and stability of the cytoskeleton network. Cytotoxins from *Clostridium* species inactivate Rho family GTPases by glucosylation (7, 17, 19, 24). Members of this toxin family are *Clostridium difficile* toxins A and B, *Clostridium sordellii* lethal and hemorrhagic toxins, and *Clostridium novyi* alpha-toxin (30). These toxins are single-chain proteins with molecular masses of 250 to 308 kDa (17). *C. difficile* toxins A and B glucosylate members of the Rho GTPase family (Rho, Rac, and Cdc42), whereas *C. sordellii* lethal toxin glucosylates Rac and Ras subfamily proteins (e.g., Ras, Ral, and Rap) but not RhoA (19, 20). *C. novyi* toxin, which shares the substrate specificity of toxin B, is an *O*-GlcNAc transferase (26). These toxins modify the highly conserved threonine 37 residue in RhoA (Thr35 of Rac or Cdc42) located in the switch 1 region of these GTPases (19). Mono-O glucosylation of this threonine residue has important functional consequences. First, it inhibits activation of Rho proteins by GEFs. Second, glucosylation of Rho proteins increases membrane binding. Third, it inhibits the interaction of GTPases with their effectors (17, 25). Moreover, recent studies indicate that glucosylation of Rho/Ras proteins blocks the active conformation of the GTPases and stabilizes their inactive forms (11, 28). Therefore, glucosylation of Rho GTPases block Rho-dependent signaling pathways (6).

Entamoeba histolytica is a parasite which is the causative agent of amoebiasis. Signal-induced rearrangement of the actin cytoskeleton plays a crucial role in parasitic motility and pathogenicity in terms of vesicle trafficking, contact-dependent cell killing, and phagocytosis (4, 5, 29). Previously, several low-molecular-mass GTPases, sharing significant sequence similarity with mammalian Rho and Rac proteins, were identified in *Entamoeba histolytica* (15, 22, 23). The roles of *Entamoeba* Rac orthologous EhRacA and EhRacG were established in phagocytosis, uroid formation, cytokinesis, and invasive behavior (10, 12, 15). EhPAK was identified as the effector of EhRacG, which also efficiently interacted with human Rac1 (HsRac1) (21). Recently, an *Entamoeba* GEF (EhGEF1) which stimulates the rate of nucleotide exchange of EhRacG (~20-fold) was identified (1). Functional studies with EhRho proteins are limited in this organism. Recent study shows that lysophosphatidic acid stimulation activated EhRhoA1 and induced rearrangement in filamentous actin, suggesting that Rho signaling modulates actomyosin-dependent motility in this organism (10).

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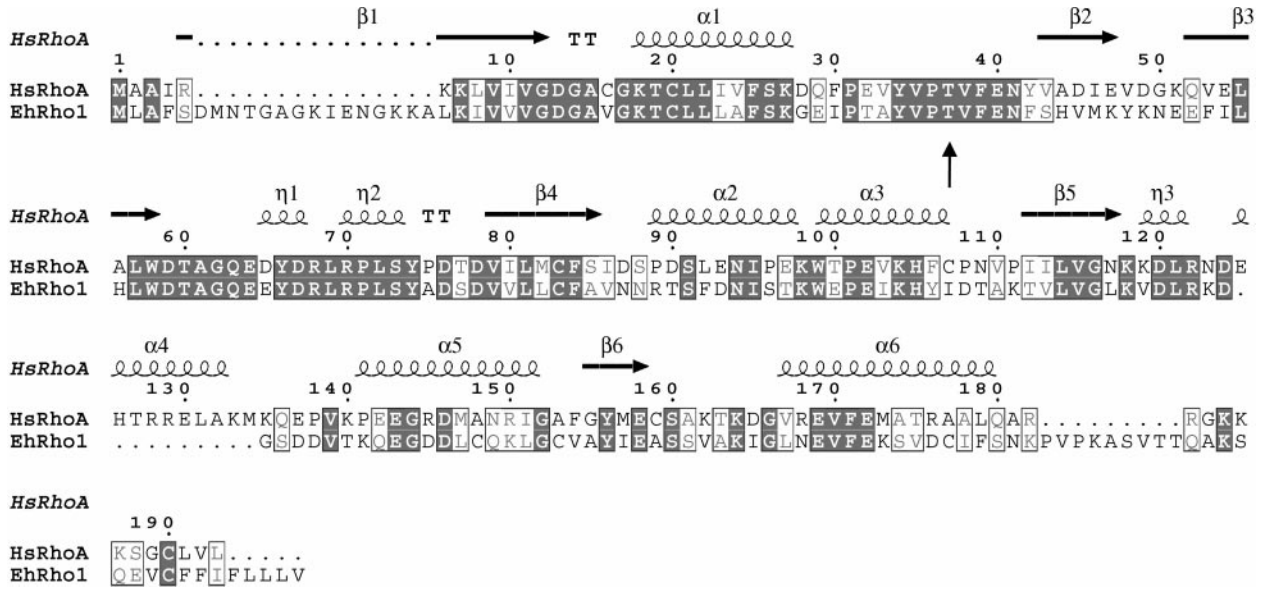

FIG. 1. Amino acid sequence alignment of EhRho1 and HsRhoA. The amino acid sequence of EhRho1 (accession no. XP_652646) was aligned with that of human RhoA (HsRhoA; accession no. AAM 21117) using ClustalW. The secondary structure of HsRhoA (PDB code 1ftn) was included above the alignment using ESPript. The arrow below the EhRho1 sequence indicates the threonine residue which is the target for glucosylation (Thr52 in EhRho1 is equivalent to Thr37 in HsRhoA).

We asked whether Rho GTPases from *Entamoeba histolytica* are also substrates of bacterial protein toxins. Previous studies demonstrate that EhRho1, the Rho-like GTPase from *Entamoeba histolytica*, is not a substrate of the ADP-ribosylating C3 exoenzyme of *Clostridium botulinum* (13). However, the expression of C3 in *Entamoeba histolytica* showed an inhibition of cytolytic activity and monolayer destruction (14). In the present study, we used clostridial glucosylating cytotoxins to modify the Rho proteins from *Entamoeba histolytica*. Our study shows that EhRho1 is a preferred substrate for glucosylation by *Clostridium difficile* toxin B and *Clostridium novyi* alpha-toxin in vitro, although the holotoxins do not affect the *Entamoeba histolytica* trophozoites in culture.

MATERIALS AND METHODS

Cell culture and maintenance. *Entamoeba histolytica* HM1:IMSS trophozoites were maintained in TYIS 33 medium (22) at 37°C. Cells were routinely subcultured for maintenance every 2 to 3 days, and trophozoites in the log phase of growth (1 to 2 days) were used in all experiments. *Clostridium difficile* toxins A and B (from strain VPI 10463) were prepared as described previously (18). *Clostridium sordellii* lethal toxin (strain 6018) was purified as reported for toxin B. *Clostridium novyi* alpha-toxin (strain type A 19402) was prepared as described recently (26). The N-terminal enzymatic domain of toxin B, covering residues 1 to 546, was purified as described previously (16), and the N-terminal domain of *Clostridium novyi* alpha-toxin (residues 1 to 551) was prepared as reported previously (8) and used as a glutathione *S*-transferase (GST) fusion toxin.

Expression and purification of EhRho1. EhRho1 was picked from *Entamoeba* genomic DNA (23), and the full-length coding DNA (636 bp) was subcloned in pGEX-2TK. The recombinant plasmid was transformed in *Escherichia coli* BL21(DE3), GST fusion protein production in bacterial culture was induced with 250 μM IPTG (isopropyl-β-D-thiogalactopyranoside) at an optical density of 0.6, and the culture was incubated for a further 16 h at 25°C. Bacterial cells from 1.5 liters of culture were collected by centrifugation (6,000 × *g* at 4°C) and sonicated in 30 ml lysis buffer (25 mM Tris-HCl [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation (23,500 × *g* at 4°C), the lysate was incubated with preequilibrated GST-Sepharose 4B beads (Amersham Bioscience, Freiburg, Germany) for 1 h. Beads were washed with high-salt buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, and 5 mM $MgCl_2$). GST-EhRho1 was eluted in a buffer containing 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, and 5% glycerol in the presence of 10 mM reduced glutathione for 30 min at 4°C. The purity of the eluted protein was checked by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1).

Site-directed mutagenesis of EhRho1. A QuickChange kit (Stratagene, Heidelberg, Germany) in combination with *Pfu* Turbo DNA polymerase was used for the replacement of a single nucleotide in EhRho1–pGEX-2TK to prepare EhRho1T52A–pGEX-2TK. The following oligonucleotides were used: EhRhoT52A sense, CCG CTT ATG TCC CAG CAG TTT TTG AAA ATT TC; and EhRhoT52A antisense, GAA ATT TTC AAA AAC TGC TGG GAC ATA AGC GC. The corresponding plasmid was selected in *E. coli* XL1-Blue and the sequence confirmed by automated sequencing. The mutated recombinant protein was purified as described above for EhRho1.

Intrinsic GTPase assay. Equivalent amounts (1 μM) of GST-EhRho1 and GST-HsRhoA were loaded with [γ-^{32}P]GTP for 5 min at 37°C in loading buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, and 2 mM dithiothreitol). $MgCl_2$ (final concentration, 12 mM) and unlabeled GTP (final concentration, 2 mM) were added. GTP hydrolysis was carried out at 37°C. Proteins were collected by filtration through wet 0.45-μM nitrocellulose filter discs. The filters were washed three times with ice-cold buffer A (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, and 5 mM $MgCl_2$), and protein-bound [γ-^{32}P]GTP on the filters was quantified by liquid scintillation counting.

Intrinsic GTP-binding assay. Equivalent amounts (1 μM) of GST-EhRho1 and HsRhoA were loaded with [α-^{32}P]GTP (20 μM final concentration) as described above. After addition of unlabeled GTP (2 mM final concentration), the intrinsic GTP release was carried out at 30°C, and the bound radioactivity was measured by a filter binding assay as described above.

Preparation of cell lysate for in vitro glucosylation assay. A total of 3×10^5 *Entamoeba histolytica* trophozoites were harvested by chilling and then lysed in 100 μl extraction buffer, containing 25 mM HEPES-KOH (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 2% glycerol supplemented with complete protease inhibitor (Roche, Germany), 1 mM E-64 (Sigma, Germany), 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. Thirty to 40 μg of total cellular protein was used in glucosylation reactions.

In vitro glucosylation assay. In vitro glucosylation was performed with the holotoxins or with the N-terminal enzymatic domain of *Clostridium difficile* toxin B (residues 1 to 546), *Clostridium sordellii* lethal toxin (residues 1 to 546), and *Clostridium novyi* alpha-toxin (residues 1 to 551), which were purified as GST fusion proteins. A total of 1 to 2 μg purified GST-EhRho1 or GST-EhRho1T52A was diluted in 20 μl reaction buffer, containing 50 mM HEPES-KOH (pH 7.4),

100 mM KCl, 2 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1 mg/ml bovine serum albumin, and added with 10 μM UDP-[^{14}C]glucose or UDP-[^{14}C]GlcNAc as required for the corresponding enzyme. Reactions were started with the addition of recombinant toxins (final concentration, 7 to 10 ng/μl). The reaction mixture was incubated at 30°C for 30 min, stopped by SDS-PAGE sample buffer, and run on SDS-PAGE followed by PhosphorImager analysis (Molecular Dynamics). The labeled band intensity was measured by ImageQuant 5.2. Cold reactions were carried out with unlabeled cosubstrate. Changes and modifications in the reaction condition are mentioned in the figure legends.

Treatment of *E. histolytica* trophozoites with clostridial toxins. *Entamoeba histolytica* trophozoites were grown on coverslips at 37°C in 24-well plates (2.4 ml). *C. difficile* toxin B or *Clostridium novyi* alpha-toxin was treated at various concentrations ranging from 0.5 to 5 μg/ml for 12 to 24 h at 37°C, and cells were fixed with 3.7% formaldehyde–0.1% Triton X-100.

Staining for actin cytoskeleton and fluorescence microscopy. Formaldehyde-fixed untreated and toxin-treated *E. histolytica* cells were extensively washed with phosphate-buffered saline. Then the cells were incubated with rhodamine-conjugated phalloidin (1 U per coverslip) at room temperature for 1 h, washed again with phosphate-buffered saline, and used for fluorescence microscopy (bleaching preservative was Kaiser's glycerol gelatin from Merck) under 40× oil objective using an Axiocam camera (Carl Zeiss, Germany).

RESULTS

Purification of EhRho1 as a GST fusion recombinant protein. EhRho1 was identified as the closest homolog of eukaryotic Rho proteins in *Entamoeba histolytica* (22). Compared to the primary structure of HsRhoA, EhRho1 showed 48% identity, and predominant convergence of these two proteins lies in the N-terminal region (alignment shown in Fig. 1). EhRho1 was expressed as a GST fusion protein in *E. coli*. The soluble bacterial extract was affinity purified as described in Materials and Methods. The eluted protein comigrated with the 45-kDa molecular standard (Fig. 2A). Removal of the GST fusion using thrombin was performed, but the resultant protein was less stable. Hence, we used the stable GST-EhRho1 in most of our studies.

GTPase activity of EhRho1 is comparable with that of HsRhoA. To compare the biochemical properties of EhRho1 with human RhoA, we tested the intrinsic nucleotide binding and GTP hydrolysis of purified EhRho1 and HsRhoA using a filter binding assay as described in Materials and Methods. GST-EhRho1 showed [α-^{32}P]GTP binding comparable to that of HsRhoA. The GTPase activities of the two proteins, which were measured by hydrolysis of [γ-^{32}P]GTP, were similar (Fig. 2B).

EhRho1 is a substrate for *C. difficile* toxin B and *C. novyi* alpha-toxin but not for *C. difficile* toxin A. *Clostridium difficile* releases two major toxins, toxin A and toxin B, which inactivate the Rho family proteins (Rho, Rac, and Cdc42) by mono-O glucosylation at the conserved Thr37. Purified EhRho1 was subjected to glucosylation by these two toxins. GST-EhRho1 was a substrate for toxin B (Fig. 3), but toxin A was not able to label the *Entamoeba* GTPase in the presence of UDP-[^{14}C]glucose, although toxin A could modify HsRhoA (Fig. 3). In accordance with results obtained with recombinant EhRho1, toxin B glucosylated EhRho1 in the cell lysate from *Entamoeba histolytica.* Again toxin A could not label any protein in the cell lysate (Fig. 3). Thus, toxin A and toxin B share protein substrate specificity of mammalian Rho GTPases but not of Rho GTPases from *Entamoeba histolytica*. Next, we studied the glucosylation of EhRho1 by *Clostridium novyi* alpha-toxin, which is an *O*-GlcNAc transferase but exhibits the same protein substrate specificity as toxin B in mammalian cells. For these and the following in vitro studies, the recombinant catalytic domains of the toxins were used. Alpha-toxin modified GST-EhRho1 (Fig. 4A) and also EhRho1 in cell lysate (see Fig. 7B). The GlcNAc transferase from *Clostridium novyi* modified the same amino acid residue as toxin B, because the extent of radiolabeling decreased when EhRho1 was first preincubated with toxin B and then treated with alpha-toxin (Fig. 4B).

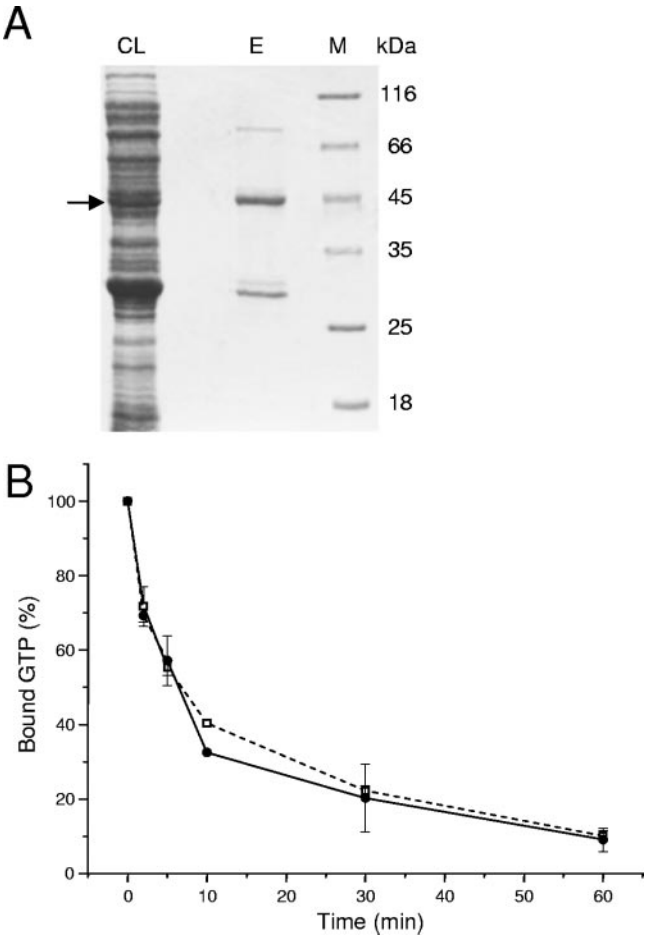

FIG. 2. Gel electrophoresis and biochemical activity of EhRho1. (A) Bacterial cell lysate (CL) expressing GST-EhRho1 (arrow) was loaded on glutathione-Sepharose matrix, and purified proteins were eluted in the presence of 10 mM reduced glutathione. A 12.5% SDS-PAGE was run and Coomassie-stained to check the purity of the eluate (E). M, molecular mass marker. (B) Equivalent amounts (1 μM final concentration) of GST-EhRho1 (●) and GST-HsRhoA (□) were loaded with [γ-^{32}P]GTP, and the intrinsic GTPase activity was measured by a filter binding assay. The remaining bound radioactivity on GST-EhRho1 and GST-HsRhoA is given as the percentage of the maximum. Shown are data from experiments performed in triplicate (± standard error of the mean) (n = 3).

EhRho1 is glucosylated at the conserved threonine residue. Toxin B-induced depolymerization of actin filaments in mammalian cells is due to the mono-O glucosylation of RhoA at Thr37 or Thr35 in Rac1 and Cdc42 (19). Also, Ras proteins, which are targets of lethal toxin from *Clostridium sordellii*, are modified at this conserved Thr35 residue, which is essential for the functions of the GTPases. EhRho1 shows sequence con-

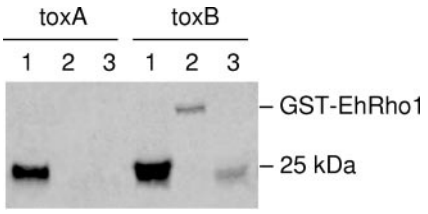

FIG. 3. EhRho1 is glucosylated by *Clostridium difficile* toxin B but not by *Clostridium difficile* toxin A. Affinity-purified GST-EhRho1 (1 μg, lane 2) and total lysate from *Entamoeba histolytica* trophozoites (30 μg of protein, lane 3) were incubated with toxin A (toxA) or toxin B (toxB) (final concentrations, 7 ng/μl of each of the holotoxins) in the presence of 10 μM UDP-[^{14}C]glucose for 30 min at 30°C in a total volume of 20 μl. Purified HsRhoA (lane1) was used as the control for glucosylation with each toxin. Radiolabeled proteins were analyzed by SDS-PAGE and phosphorimaging.

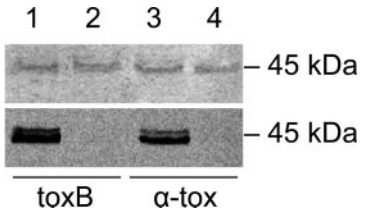

FIG. 5. EhRho1 is glucosylated at the conserved threonine residue. Purified GST-EhRho1 (lanes 1 and 3) and GST-EhRho1T52A (lanes 2 and 4) were used at the same concentration for glucosylation with *Clostridium difficile* toxin B (toxB) or *Clostridium novyi* alpha-toxin (α-tox) (recombinant catalytic domains were used; final concentrations, 10 ng/μl each). Labeled proteins were analyzed by SDS-PAGE (upper panel) and phosphorimaging analysis (lower panel). The double band may indicate partial degradation of proteins. Shown is a representative experiment repeated twice with similar results.

servation in the putative GTP-binding and effector binding sites compared with the mammalian RhoA. According to the sequence alignment shown in Fig. 1, Thr52 of EhRho1 was assumed to be the conserved target for glucosylation. Hence, site-directed mutagenesis was performed to construct EhRho1T52A. Both toxin B and alpha-toxin did not modify GST-EhRho1T52A (Fig. 5), suggesting that the covalent modification catalyzed by these toxins occurred at the same amino acid as that known for mammalian Rho protein substrates.

GDP-EhRho1 is the preferred substrate for glucosylation. Previous studies have shown that RhoA when bound to GDP is preferentially glucosylated compared to the GTP-bound state (19). When recombinant EhRho1 was preloaded with GDP, a higher incorporation of [^{14}C]glucose was induced by toxin B than with the GTP-bound form of GST-EhRho1, which was poorly glucosylated (Fig. 6).

EhRho1 is a specific target of toxin B in the cell lysate of *Entamoeba histolytica*. In higher eukaryotic organisms, toxin B specifically blocks the function of RhoA/Rac1 proteins, whereas lethal toxin from *C. sordellii* targets Rac and Ras proteins but not RhoA. When the cellular lysate of *Entamoeba histolytica* was treated with toxin B or alpha-toxin in the presence of ^{14}C-labeled cosubstrates, only a single band, which was most likely EhRho1, was detected (Fig. 7B). When the lysate was pretreated with toxin B and unlabeled UDP-glucose, no labeling with alpha-toxin could be detected in the presence of ^{14}C-labeled cosubstrates (Fig. 7B). This result showed that alpha-toxin and toxin B share the same substrate and acceptor amino acid specificity towards the amoebic proteins. Lethal

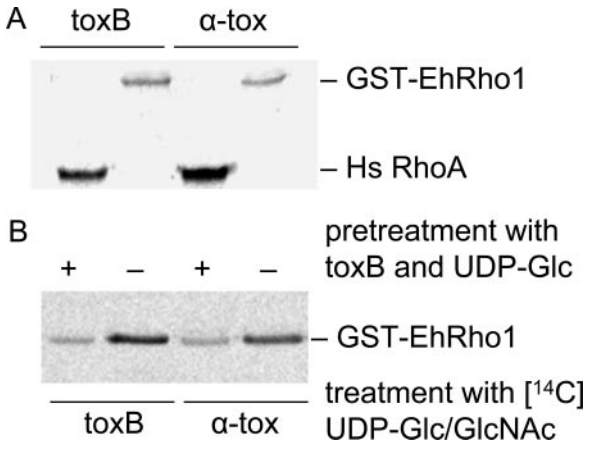

FIG. 4. *Clostridium novyi* alpha-toxin modified EhRho1 similarly to toxin B. (A) Purified GST-EhRho1 or HsRhoA (each 1 μg) was incubated with *Clostridium difficile* toxin B (toxB) or *Clostridium novyi* alpha-toxin (α-tox) (final concentrations, 7 ng/μl of each of the catalytic domains) in the presence of UDP-[^{14}C] glucose (for toxin B) or UDP-[^{14}C]GlcNAc (for alpha-toxin) in a 20-μl reaction volume. The samples were subjected to SDS-PAGE followed by autoradiographic analysis. (B) GST-EhRho1 (2 μg) was preincubated with or without *Clostridium difficile* toxin B and UDP-glucose (10 μM) for 20 min at 30°C in a 20-μl reaction volume. The preincubated samples were subjected to a second modification by toxin B (toxB) and alpha-toxin (α-tox) (final concentrations, 10 ng/μl each) with radiolabeled cosubstrates for another 20 min at 30°C. Labeled proteins were analyzed by SDS-PAGE and phosphorimaging. Pretreatment with toxin B or alpha-toxin reduced subsequent second glucosylation, indicating the same target sites.

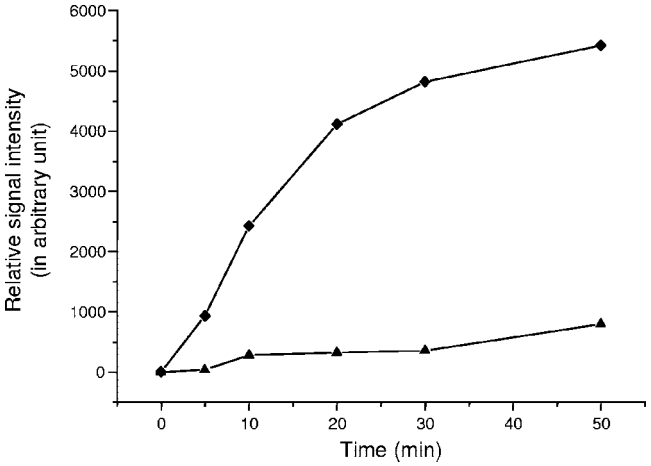

FIG. 6. GDP-EhRho1 is more efficiently modified by *Clostridium difficile* toxin B than GTP-EhRho1. GST-EhRho1 was preloaded with either GDP (◆) or GTPγS (▲), and glucosylation reactions were started with the addition of toxin B (final concentration, 10 ng/μl of the recombinant catalytic domain). At the indicated time points, labeled proteins were analyzed by SDS-PAGE and phosphorimaging. Relative band intensities were quantified with ImageQuant 5.2 and were plotted as a function of time. Shown is a representative experiment performed twice with similar results.

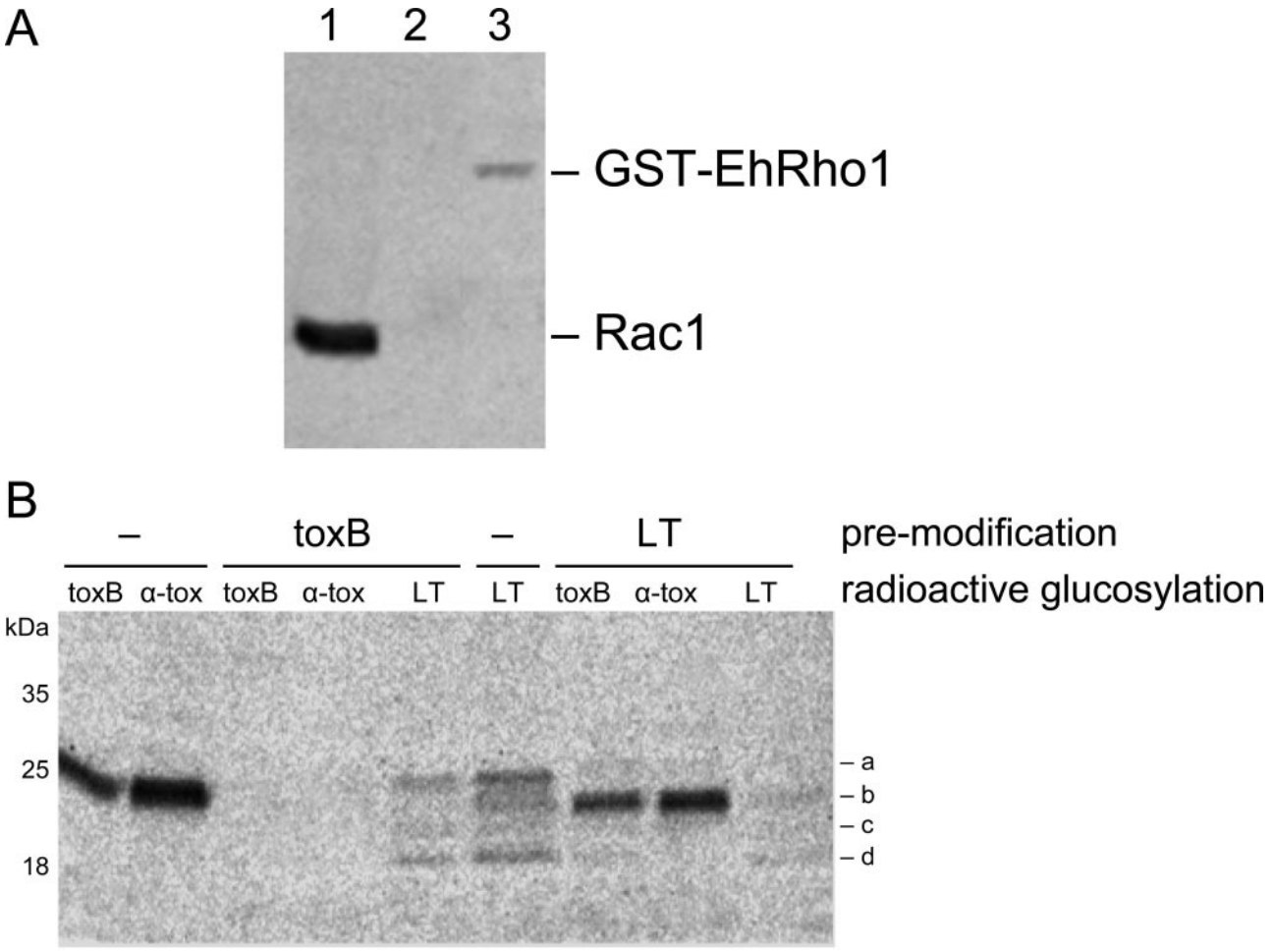

FIG. 7. *Clostridium sordellii* lethal toxin glucosylates other proteins from *Entamoeba histolytica* but not EhRho1. (A) Purified GST-EhRho1 (1 μg) was used for glucosylation with *Clostridium sordellii* lethal toxin (lane 2). The modifications of GST-EhRho1 with *Clostridium difficile* toxin B (lane 3) and HsRac1 (1 μg) with *Clostridium sordellii* lethal toxin (lane 1) were performed as controls as described in Materials and Methods. Final concentrations of the catalytic domains of the toxins were 7 ng/μl. (B) *Entamoeba histolytica* trophozoite cell lysates were preincubated with either *Clostridium difficile* toxin B (toxB) or *Clostridium sordellii* lethal toxin (LT) (concentrations of the catalytic domains of the toxins were 10 ng/μl) in the presence of unlabeled UDP-glucose for 20 min at 30°C. This was followed by a second incubation in the presence of radiolabeled cosubstrates with toxin B (toxB), alpha-toxin (α-tox), and lethal toxin (LT) for another 20 min under the same conditions. All samples were separated by SDS-PAGE, and the bands were detected by phosphorimaging (a, b, c, and d indicate proteins labeled by the various toxins).

toxin did not show any activity toward purified GST-EhRho1 (Fig. 7A, lane 2). However, when the cell lysate from *Entamoeba histolytica* was treated with lethal toxin, several labeled proteins with molecular masses of 20 to 30 kDa were detected (Fig. 7B, protein bands a, c, and d). These results indicated the presence of other Ras/Rac proteins, which were targets for lethal toxin. Interestingly, the specificity of lethal toxin does not overlap with the substrate specificity of toxin B or alpha-toxin (Fig. 7B).

Toxin B and alpha-toxin could not intoxicate *Entamoeba histolytica* trophozoites in culture. Rho GTPases are involved in the organization of the actin cytoskeleton, and treatment of cultured mammalian cells with the clostridial glucosylating cytotoxins induces a redistribution of actin filaments, leading to morphological alterations and finally to rounding up of cells (18). In contrast, microscopic studies of *Entamoeba histolytica* trophozoites treated with toxin B or alpha-toxin did not show such characteristic morphological changes, even at a comparably high concentration (5 μg/ml) of holotoxins (data not shown). We could not detect any significant changes in the morphology of actin structures of intoxicated cells compared to untreated cells (data not shown). Moreover, when *Entamoeba histolytica* trophozoites were treated with toxin B and alpha-toxin at high concentrations (5 μg/ml) and cell lysates were prepared after 12 h, radiolabeling of proteins in the lysates was not reduced compared to naïve cells (data not shown). These results suggest that the holotoxins are not able to enter *Entamoeba histolytica* trophozoites in culture.

DISCUSSION

Rho family proteins play a crucial role in regulation of the actin cytoskeleton network (9). By inhibiting the Rho signaling cascade, clostridial glucosylating cytotoxins cause depolymerization of the actin cytoskeleton of target cells (6). These toxins monoglucosylate Rho proteins at a conserved threonine resi-

due (Thr37 in Rho and Thr35 in Rac, Ras, or Cdc42), which is located in the switch 1 region of the GTPases (17). The covalent modification of Rho proteins prevents the active conformation of the GTPase and results in uncoupling from its effectors and regulators.

Entamoeba histolytica, a human pathogen, is a unicellular eukaryotic organism which represents a complex signaling network. Although several Ras superfamily proteins, including the RhoA-like GTPase EhRho1, have been identified in this organism, specific functions and the regulation of Rho proteins are not well established (22, 23). EhRho1 shares high sequence similarity with mammalian RhoA in its N-terminal region, suggesting an important role in regulation of the actin cytoskeleton and in other Rho-dependent cellular functions of *Entamoeba histolytica* (10). In the present study, we purified recombinant EhRho1 and studied its intrinsic GTPase activity in comparison with HsRhoA. The data obtained suggest that EhRho1 has biochemical properties similar to those of mammalian RhoA. A major result of the present study is the finding that the *Entamoeba* RhoA-like GTPase EhRho1 is glucosylated by clostridial cytotoxins. Toxin B and alpha-toxin glucosylated the recombinant protein expressed in *E. coli* as well as the native EhRho1 in the cell lysate from *Entamoeba histolytica*. Our studies suggest that toxin B and alpha-toxin act on EhRho1 in a very similar manner as on mammalian RhoA. EhRho1 was glucosylated in the switch 1 region at the same conserved threonine residue as that known for mammalian RhoA (note that Thr52 in EhRho1 is equivalent to Thr37 of RhoA) (19). The toxin-catalyzed glucosylation of EhRho1 was more efficient with the GDP-bound form than with the GTP form of the GTPase. This finding suggests a very similar three-dimensional structure of the switch 1 domains of EhRho1 and HsRhoA. Thus, it is likely that the glucosylation of EhRho1 also affects the interaction of the GTPase with downstream effector proteins. An interesting aspect of our work is the finding that in contrast to toxin B, toxin A was not able to glucosylate EhRho1. So far, it was suggested that toxin A and toxin B target the same proteins (17), while our study indicates a difference in substrate recognition by these two toxins. Another important finding was the labeling of additional proteins by lethal toxin in *Entamoeba histolytica* cell lysate, whereas EhRho1 was not modified. Deduced from the protein substrate specificity of the toxins in mammalian cells, we can expect a spectrum of Rho/Ras-like GTPases, which are specific targets for these toxins.

Treatment of *Entamoeba histolytica* with the toxins did not affect the behavior of the amoeba. We were also not able to find evidence for a modification of Rho-like GTPases in the intact protozoa. Therefore, we assume that the toxins are not able to enter amoebae from outside the cells. So far, we do not know whether the toxin receptors are missing or whether the endocytic uptake machinery of *Entamoeba histolytica* does not allow entry of the toxin. Recently, it was reported that C3 exoenzyme from *Clostridium botulinum*, which ADP ribosylates mammalian Rho protein at Asn41 (3), is not able to modify EhRho1 (13). In contrast, expression of C3 in intact amoebae caused functional consequences, e.g., cell killing by *Entamoeba histolytica* was inhibited (14). Therefore, it is important to study the functional consequences of the expression of clostridial glucosylating cytotoxins in *Entamoeba histolytica*.

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