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 sordelli* did not glucosylate EhRho1 but labeled several other substrate proteins in lysates from *Entamoeba histolytica* in the presence of UDP-[14C]glucose.
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).

**Expression and purification of EhRho1.** Expression and purification of EhRho1 were performed from *Entamoeba* genomic DNA (23), and the full-length coding sequence (636 bp) was subcloned into pGEX-2TK. The recombinant plasmid was transformed into *Escherichia coli* XL1-Blue and the sequence confirmed by automated sequencing. The mutated recombinant protein was purified as described above for EhRho1.

**In vitro glucosylation assay.** In vitro glucosylation was performed with the active toxin B and *Clostridium novyi* alpha-toxin in vitro, although the holotoxins do not affect the *Entamoeba histolytica* trophozoites in culture.

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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 coincided 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-32P]GTP binding comparable to that of HSrhoA. The GTPase activities of the two proteins, which were measured by hydrolysis of [γ-32P]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 Thr35 residue. 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-[14C]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).

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 Thr35 or Thr37 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-
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 [14C]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 14C-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 14C-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
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 comparatively 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 naive 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-
EhRho1 has biochemical properties similar to those of *amoeba histolytica* are not well established (22, 23). EhRho1 shares high sequence work. 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 cytoxins. 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 protzoa. 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 cytoxins in *Entamoeba histolytica*.

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**REFERENCES**


