Rho GTPases are the preferred targets of various bacterial cytotoxins, including *Clostridium difficile* toxins A and B, *Clostridium sordellii* lethal toxin, the cytotoxic necrotizing factors (CNF1) from *Escherichia coli*, and the dermonecrotizing toxin (DNT) from *Bordetella* species. The toxins inactivate or activate specific sets of Rho GTPases by mono-O-glucosylation and deamidation/transglutamination, respectively. Here we studied the structural basis of the recognition of RhoA, which is modified by toxin B, CNF1, and DNT, in comparison with RhoD, which is solely a substrate for lethal toxin. We found that a single amino acid residue in RhoA and RhoD defines the substrate specificity for toxin B and lethal toxin. Change of serine 73 to phenylalanine in RhoA turned RhoA into a substrate for lethal toxin. Accordingly, change of the equivalently positioned phenylalanine 85 in RhoD with serine allowed glucosylation by toxin B. Comparable results were achieved with the Rho-activating and transglutaminating enzymes CNF1 and DNT. Here, amino acid glutamate 64 of RhoA and the equivalent aspartate 76 of RhoD define substrate specificity for CNF1 and DNT, respectively. These data indicate that single amino acid residues located in the switch II region of Rho proteins determine enzyme specificity for diverse bacterial toxins.

The family of Rho GTPases comprises ~20 proteins that act as molecular switches in a network of cellular processes responsible for regulation of the cytoskeleton, cell polarity, secretion, gene transcription, and cell cycle progression (1, 2). Highly specific functions have been described for individual members of the Rho GTPase family, e.g. RhoA, Rac, and Cdc42 are involved in formation of stress fibers, lamellipodia, and filopodia, respectively (3). Within their functional networks, Rho GTPases may act in a concerted action, for example to promote directed cell migration by RhoA, Rac, and Cdc42 (4). Antagonistic functions of Rho GTPases have also been described. For example, RhoD, which reportedly plays a role in endocytic trafficking (5), may act in an antagonistic manner to RhoA (6). Microinjection of constitutively active RhoD leads to the disassembly of actin stress fibers, and cell migration is diminished after expression of constitutively active RhoD in 10T1/2 cells (6). So far, the structural basis for these specific actions of Rho GTPases is not well understood.

The activity of Rho proteins is regulated by GTP binding initiated by guanine nucleotide exchange factors, which turn GTPases into an active conformation (7). Only this conformation allows high affinity binding to effector proteins. In the case of RhoA, downstream effector molecules such as ROCK, mDia, or PKN are activated to elicit multiple cellular functions (8, 9). For RhoD it was recently shown that only human Dia2C exhibits a proper binding site and leads in concert with c-Src tyrosine kinase to a reduction in early endosomal motility along actin filaments (10).

Some bacterial protein toxins alter the activity of Rho/Ras GTPases in a highly specific manner. Clostridial glucosylating toxins like toxin B of *Clostridium difficile* and *Clostridium sordellii* lethal toxin mono-glucosylate low molecular mass GTPases and inhibit their function (11); other toxins like *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1) and *Bordetella* dermonecrotic toxin (DNT) activate Rho GTPases (12) (see Fig. 1). The glucosylating toxins modify a conserved threonine residue within the switch I region (position 37 in RhoA) that is essential for nucleotide binding (13–15). Toxin B modifies the Rho family members RhoA, -B, -C, Rac1, Cdc42, RhoG, and TC10 (14, 16). Lethal toxin modifies Ras family members H-/R-Ras, RaLA/-B, Rap1/-2, and the Rho subfamily members Rac1, Cdc42, RhoD (16–18). The glucosylation prevents interaction with downstream effectors and guanine nucleotide exchange factors (19, 20). The Rho-activating toxins CNF1 and DNT deamidate and transglutaminate their substrate GTPases at the conserved residue glutamine 63 in RhoA (glutamine 61 in RhoB) (21, 22). This turns the small GTPase into a constitutive active form with blocked intrinsic and GAP-stimulated GTPase activity. Like toxin B, CNF1 and DNT modify RhoA, -B, -C, Rac1, and Cdc42, but not RhoD.

The abbreviations used are: CNF1, cytotoxic necrotizing factor 1; lethal toxin, *C. sordellii* lethal toxin; toxin B, *C. difficile* toxin B; toxin-B546, enzyme domain covering amino acids residues 1–546 of *C. difficile* VPI10463 toxin B; lethal toxin546, enzyme domain covering amino acids residues 1–546 of *C. sordellii* lethal toxin; DNT, *Bordetella* dermonecrotic toxin; GST, glutathione S-transferase; WT, wild type; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
Glucosylation and Transglutamination of RhoA and RhoD

As known from NMR studies and co-crystal structures (23), major contact points between Rho proteins and effectors or regulators frequently include the switch I, switch II, and the α2 region. Less is known about the structural basis that defines the specificity of toxin-GTPase interaction. In this study we determined the regions within the Rho proteins RhoA and RhoD that are responsible for the recognition by glucosylating and deamidating/transglutaminating toxins. By constructing chimeras and several point mutations, we were able to narrow down the regions in RhoA and RhoD that define substrate specificity for the glucosylating and deamidating/transglutaminating toxins, respectively. We show that single amino acid residues in or near the switch II region are responsible for substrate properties of the toxins.

EXPERIMENTAL PROCEDURES

Materials—UDP-[14C]glucose (287.4 mCi/mmol) was obtained from PerkinElmer Life Sciences, and PCR primers were from Biochip Technologies (Freiburg, Germany). All other reagents were of analytical grade and purchased from commercial sources.

Construction of GTPase Chimeras—The GTPase chimeras were constructed using the splicing by overlap extension method described previously (24). In brief, two polymerase chain reactions (PCR) were performed, each amplifying the sequences that have to be fused. The products of the two first PCRs were pooled and cycled in a third PCR in which the overhanging sequences act as primers. We used pGEX2T-RhoA (wild type, WT) and pGEX2TGL-RhoD (WT) as templates with hanging sequences act as primers. We used pGEX2T-RhoA PCRs were pooled and cycled in a third PCR in which the overhanging sequences that have to be fused. The products of the two first chain reactions (PCR) were performed, each amplifying the regions in RhoA and RhoD that define substrate specificity for the toxins.

Site-directed Mutagenesis of Toxin Fragments—The QuikChange™ kit (Stratagene, La Jolla, CA) in combination with Pfu Turbo DNA polymerase was used for the replacement of one to three nucleotides in the pGEX2T-RhoA (WT) or pGEX2TGL-RhoD (WT) plasmids. Primers were constructed as follows: RhoA E64D sense/antisense (5′-GACACAGCT-GGGACAGAGTATTGATCGC-3′/5′-GGGATCATATA-TCATCTGCTGCCAGTGTGC-3′); RhoA S73F sense/antisense (5′-CCATGAGGCCCTCTTTCTACCAGATACC-3′/5′-GGTATCTGGGATAGGAGGAGGAGGCTCAGG-3′); RhoD76E sense/antisense (5′-GCAGCGAACAAAGACATAGGC-CGC-3′/5′-GGCGTCAAGTCGTTCTGCCCAGGC-3′); RhoD F85S sense/antisense (5′-CGGCCCTTTGTCTATCCGG-ATGCCC-3′/5′-GGCATGATAAGGAGCAAGGGCCG-3′); RhoA Q63E/E64D sense/antisense (5′-GACACAGCTGGGAGGACGATTATGATCCGC-3′/5′-GGGATCATATAGGAGGAGGAGGCTCAGG-3′); RhoA Q63F/E64D sense/antisense (5′-GACACAGCTGGGAGGACGATTATGATCCGC-3′/5′-GGGATCATATAGGAGGAGGAGGCTCAGG-3′); RhoD Q75E/D76E sense/antisense (5′-GCGAAGACGCGGAGGAGGAGGAGGCTCAGG-3′/5′-GGGATCATATAGGAGGAGGAGGCTCAGG-3′); RhoA S73F sense/antisense (5′-GCGAAGACGCGGAGGAGGAGGAGGCTCAGG-3′/5′-GGGATCATATAGGAGGAGGAGGCTCAGG-3′); and subcloned into a pGEX2TGL vector. The sites of splicing are numbered according to RhoA nomenclature. The chimera RhoA-59RhoD79RhoA was constructed with splicing by overlap extension by using the primers for RhoA59RhoD with pGEX2TGL RhoD79RhoA serving as template.

Protein Expression—Toxin fragments, GTP-binding proteins, and their mutants and chimeras were prepared from their GST fusion proteins with the E. coli BL-21 (DE3) (Invitrogen) for expression.

Sequence—All constructs were checked by sequencing with ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit and ABI 310 Cycle sequencer (PerkinElmer).

Transglutamination Assay—Transglutamination reaction was performed as described before (26). The basis of the assay of transglutamination is the use of the autofluorescent co-substrate monodansylcadaverine that is attached to the Rho GTPase. CNF and DNT, amino acid residues 1136–1451) in a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, and 50 mM monodansylcadaverine for the indicated times and temperatures. As controls, GST-RhoA, GST-RhoD, and their corresponding mutants were incubated without the toxin but in the presence of co-substrate. The proteins were separated by SDS-PAGE, and the transglutaminated proteins were detected under UV light (206 nm). Subsequently, the gel was Coomassie stained to check the amount of proteins. Quantiﬁcation was performed with ImageQuant (GE Healthcare).
**Glucosylation Reaction**—Recombinant GTP-binding proteins (50 μg/ml) were incubated with recombinant toxin fragments (concentrations as indicated) and 10 μM UDP-[14C]glucose in a buffer containing 50 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl₂, 1 mM MnCl₂, and 100 μg/ml of bovine serum albumin for the indicated times at 30 °C. Total volume was 20 μl. Labeled proteins were analyzed by SDS-PAGE followed by phosphorimaging (GE Healthcare).

**Mass Spectrometry**—A glycosyltransfer reaction was performed with 127 pmol RhoD F85S as substrate and 1.6 pmol of the enzymatic fragments of toxin B (toxin-B546) and the toxin B mutant (toxin-B546 I383S/Q385A) in the presence of the indicated UDP sugars. The reaction was stopped and the proteins purified from salts and contaminants by precipitation in 80% acetone. After washing and drying by vacuum centrifugation, the pellet was resolved in 5 μl of chymotrypsin cleavage buffer containing 50 mM ammonium bicarbonate and 5 mM CaCl₂ at pH 7.8. Chymotrypsin (0.2 μg) (Roche Diagnostics) was added to a final volume of 10 μl, and the mixture was incubated at 37 °C for 1 h. Afterward the obtained peptide solution was mixed with a saturated matrix solution of 4-hydroxy-cyanocinnamic acid according to the dried droplet method for matrix crystallization, resulting in a fine granular matrix layer. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Bruker Biflex mass spectrometer equipped with a nitrogen laser (λ, 337 nm). Mass spectra were recorded in the reflector-positive mode in combination with delayed extraction. For calibration an external two point calibration with 5 pmol of a fragment of human adrenocorticotropic hormone (amino acids 18–39, (M+H)⁺ 2,465.20 Da; Sigma) and 5 pmol of human angiotensin II ((M+H)⁺ 1,046.54 Da; Sigma) were used. Glucosylated peptides were identified by an increase in mass by 162 Da. To prove that the increase in mass was caused by glycosylation, we performed the glycosylation assay also with the mutant toxin B (toxin-B546 I383S/Q385A), which was recently shown to catalyze the N-acetylglycosaminylation of Rho proteins in the presence of UDP-GlcNAc (25). In this case the same peptides increased by 203 Da in mass (not shown).

**RESULTS**

**Glucosylation of RhoA and RhoD by C. difficile Toxin B and C. sordellii Lethal Toxin**—As shown in Fig. 2A, toxin B glucosylated RhoA, but not RhoD. Vice versa, when lethal toxin was used at a concentration of 50 nM, RhoD was a substrate, but no glucosylation was detectable with RhoA (Fig. 2G). To investigate the structural basis for substrate recognition of RhoA and RhoD by toxin B and lethal toxin, we constructed several RhoA-RhoD chimeras and single amino acid mutant proteins as shown in Fig. 3. The toxins were used as recombinant truncated N-terminal parts (amino acids 1–546) that harbor the fully active enzymatic domain (27, 28). In the case of toxin B, this domain is released into the cytosol by proteolytic cleavage (29, 30) and exhibits in *in vitro* experiments the same substrate specificity as described for the corresponding holotoxins (27).

After replacement of the N-terminal part of RhoA (amino acids 1–103) by the corresponding RhoD domain (amino acids 1–115, see Fig. 3), this chimera displayed the typical RhoD enzyme specificity, e.g. no modification occurred with toxin B and minor glucosylation was obtained with lethal toxin (Fig. 2, B and H). The same results were obtained with the construct RhoD79RhoA (Fig. 2, C and I; note, RhoA numbering). This chimera was comprised of the N-terminal part of RhoD (amino acids 1–91), which was fused to the C-terminal part of RhoA. These results localized the substrate recognition sites of toxin B and lethal toxin in the N-terminal parts of the small GTPases RhoA and RhoD.

When the small RhoD part, comprising amino acids 71–91 (RhoA numbering amino acids 59–79), was introduced into RhoA, the sandwich chimera was not modified by toxin B (Fig. 2B and H). The same results were obtained with the construct RhoD79RhoA (Fig. 2, C and I; note, RhoA numbering). This chimera was comprised of the N-terminal part of RhoD (amino acids 1–91), which was fused to the C-terminal part of RhoA. These results localized the substrate recognition sites of toxin B and lethal toxin in the N-terminal parts of the small GTPases RhoA and RhoD.

**FIGURE 2. Modification of RhoA, RhoD, and their mutants and chimeras by C. difficile toxin B and C. sordellii lethal toxin. In vitro glucosylation of small GTPases and their chimeras (1 μg) by the recombinant enzyme domain of toxin B (toxin-B546, 10 mM) (A–F) and lethal toxin (lethal toxin 546, 50 mM) (G–L) for 10 min at 30 °C. The proteins were separated by SDS-PAGE and Coomassie stained, and the radiolabeled proteins were detected by PhosphorImager analysis.
RhoA include the switch II region, the α2 helix, and a part of the following loop. A comparison of this region among Rho GTPase family members showed that only 5 amino acid residues differed between RhoD and the other Rho proteins (Fig. 4A). Considering surface-exposed amino acids within the group of variant amino acid residues, we focused our next studies on analysis of the role of glutamine 64 and serine 73 of RhoA and the corresponding residues aspartic acid 76 and phenylalanine 85 of RhoD (Fig. 4B).

When serine 73 of RhoA was exchanged to phenylalanine (RhoA S73F), the GTPase was not modified by toxin B (Fig. 2E). However, the mutant GTPase was now glucosylated by lethal toxin (Fig. 2K). Thus, these results demonstrated that the switch in the substrate recognition was based on a single amino acid exchange. Accordingly, RhoD F85S became a substrate for toxin B (Fig. 2F). A weak modification by lethal toxin remained (Fig. 2L).

For each of the mutant proteins the time course of glucosylation by toxin B and lethal toxin, respectively, was studied (Fig. 5). These studies showed that the kinetics of glucosylation were linear and that different substrate properties of the mutants were not caused by changes in the stability of the proteins.

To obtain further evidence for attachment of glucose to RhoD F85S, we performed MALDI-TOF mass spectrometric analysis. After glucosylation of RhoD F85S by toxin B in the presence and absence of UDP-glucose, we purified the proteins by acetone precipitation and digested the proteins with chymotrypsin. The obtained peptide fragments were analyzed by MALDI-TOF mass spectrometry. Two peptides (equivalent to amino acids 38–51 and amino acids 38–54) with masses of m/z...
FIGURE 4. Structural determinants for modification of Rho GTPases by glucosylating and transglutaminating toxins. A, alignment of the region defining the substrate specificity of RhoD, including RhoA (Swissprot accession code P61586), RhoB (P62745), RhoC (P08134), RhoD (P97348), Rac1 (P63000), and Cdc42 (P60953). Sequences were aligned using ClustalW. Additionally, the sequence of H-Ras (AAH99130) is presented for comparison. The region defined by β3 to α2 to β4 shows *: :********: ********: : : : : : : : :

RhoA 49 DGKQVELALWDTAGQEDYDRLRPLSYSPTDVILMCF 84
RhoB 49 DGKQVELALWDTAGQEDYDRLRPLSYSPTDVILMCF 84
RhoC 49 DGKQVELALWDTAGQEDYDRLRPLSYSPTDVILMCF 84
RhoD 61 KGKVHLOQIDTAGQDDYDRLRPLFYPDANVLLLCF 96
Rac1 47 DGKPVNLGLWDTAGQEDYDRLRPLSYSPTDVFLICF 82
Cdc42 47 GGEYTLGFLDGTAQEDYDRLRPLSYSPTDVFLVCVF 82
(H-Ras 47 DGETCQLDILDTAGQEEYSAMRDQYMRTGECFLCVF 82)

RhoA E64/ RhoD D76
RhoA S73/ RhoD F85

B

RhoA S73/ RhoD F85

Gln63

RhoA E64/ RhoD D76

Lys27

Thr37

sw1

N

C

swl

swll

β3

α2

β4

Threonine 37 acceptor amino acid for glucosylation by toxin B (14); glutamine 63 acceptor amino acid for transamidation by CNF1 and DNT; lysine 27 responsible for substrate specificity between Rac1 and RhoA (34); glutamic acid 64 responsible for substrate specificity of CNF1 and DNT between RhoA and RhoD; serine 73 responsible for the discrimination between RhoA and RhoD by toxin B. Images are shown as ribbon presentation and were prepared using Pymol (DeLano Scientific LLC).
Glucosylation and Transglutamination of RhoA and RhoD

1500.72 and 1948.97, respectively, obtained from modified RhoD F85S were shifted to a higher mass of plus 162 Da, indicating a modification of the peptides by a hexose (Fig. 6).

Identification of Amino Acids in RhoD and RhoA That Define the Substrate Specificity of E. coli CNF1 and Bordetella DNT—Comparable with toxin B, CNF1 and DNT modify RhoA but not RhoD (Fig. 7). However, CNF1 and DNT possess deamidation and transglutamination activity, and both toxins are unrelated to the glucosylating toxins with respect to enzyme activity and amino acid sequences (21, 22). Here, we studied the transglutamination reaction for both CNF1 and DNT. The acceptor amino acid for transglutamination and deamidation of RhoA is glutamine 63 (21, 22). This amino acid residue is also located within the switch II region. Lerm et al. (31) showed that a peptide covering amino acids 59–78 of RhoA is sufficient to be accepted as a substrate for CNF1. Apparently, DNT needs additional structural requirements to recognize its protein substrates (31). The alignment in Fig. 4 shows that within the CNF recognition peptide of amino acids 59–78 of RhoA, the amino acids glutamate 64, serine 73, threonine 77, and aspartate 78 differ in RhoD. Based on the report that even a peptide including amino acids 59–69 of RhoA can be modified by CNF1 (32), we examined whether change of glutamate 64 of RhoA by aspartate (RhoA E64D) affected transamidation of RhoA by...
we studied why RhoA, but not RhoD, is a substrate for toxin B and lethal toxin (RhoD F85/RhoA S73, see residues recognized to be essential for modification of the GTPases by toxin B and lethal toxin. Further- more, the amino acids that were identified to be crucial for the modification by the transglutaminating toxins.

FIGURE 7. Transglutamination of wild-type RhoA, wild-type RhoD, and RhoA/RhoD mutants by CNF1 (A) or DNT (B). RhoA, RhoD, and their mutants (3 μg each) were incubated with and without CNF1 (1 μg) or DNT (1 μg) at 37 or 30 °C, respectively, for 30 min in a buffer containing monodansylcadaverine. The experiments were repeated more than three times with identical results. C, time course of transglutamination of wild-type GTPases RhoA (●) and RhoD (▲) and their mutants RhoA E64D (○) and RhoD D76E (▼) (all GST fusion proteins). The GTPases were incubated with GST-CNФ1 in the presence of the autofluorescent co-substrate monodansylcadaverine at 37 °C for the indicated times. The samples were subjected to SDS-PAGE, and the gels were photographed under UV light. The intensity of fluorescence was measured by ImageQuant software. Mean values of three experiments are shown.

CNF1. As shown in Fig. 7, A and C, RhoA E64D was no longer modified by CNF1. The same was true for DNT (Fig. 7B). As shown in the alignment of Fig. 4, an aspartate residue (aspartate 76) is located at the equivalent position in RhoD. When aspartate 76 of RhoD was changed to glutamate, the mutant RhoD D76E was modified by CNF1 (Fig. 7, A and C) as well as by DNT (Fig. 7B). These results demonstrated that a single amino acid exchange was sufficient to allow recognition of RhoD as a protein substrate for CNF1 and DNT or, in the case of RhoA, prevented the modification by the transglutaminating toxins. However, the amino acids that were identified to be crucial for modification by CNF and DNT were not identical with those residues recognized to be essential for modification of the GTPases by toxin B and lethal toxin (RhoD F85/RhoA S73, see Fig. 7, A and B).

DISCUSSION

In this study we investigated the structural basis for the different substrate properties of RhoA and RhoD for glucosylation by C. difficile toxin B and C. sordellii lethal toxin. Furthermore, we studied why RhoA, but not RhoD, is a substrate for E. coli cytotoxic necrotizing factor CNF1 and Bordetella dermonecrotic toxin DNT. Using chimeras of RhoA and RhoD, we narrowed down the region that determined recognition by toxin B and lethal toxin to amino acids 59–79 in RhoA and amino acids 71–91 in RhoD, respectively. Single amino acid exchanges in RhoA and RhoD within this region showed that serine 73 in RhoA and phenylalanine 85 in RhoD were responsible for the different properties of these GTPases to serve as substrates for toxin B and lethal toxin. Change of serine 73 in RhoA to the aromatic phenylalanine allowed recognition and modification by lethal toxin, whereas modification by toxin B was blocked. Vice versa, when phenylalanine was introduced at the equivalent position of RhoD, the GTPase was modified by toxin B. Mass analysis identified attachment of a hexose in RhoD catalyzed by toxin B, and kinetic data indicated that in fact the rates of glucosylation were changed by the amino acid changes made.

The amino acids serine 73 of RhoA and phenylalanine 85 of RhoD are located on the edge of the α2 helix within the switch II region of the GTPases. As shown in the crystal structure of RhoA-GDP, ser-
Rac and Cdc42, respectively. They found that changes of several amino acids were needed to induce a switch in typical functions of the GTPases. However, exchange of a single amino acid was not sufficient to cause a change of function of any GTPase studied. Moreover, they reported that many of the relevant residues that define gain or loss of functions of GTPases were located outside the classical switch I and switch II regions or distinct from other known interaction sites, suggesting major impact of allosteric effects. Our results on the structural requirements for the substrate properties of RhoA and RhoD to be glucosylated by toxins are different from those obtained in studies with switch-of-function mutants. Only 1 amino acid residue, which was located in the switch II region, defined loss and gain of the property to serve as a substrate for glucosylation by toxin B and lethal toxin, respectively. However, we were not able to extend our findings with RhoA/RhoD on Ras subfamily proteins.

In all Ras proteins (H-Ras, R-Ras, K-Ras, Rap1, -B, Rap1, -D) the aromatic residue tyrosine 71 is strictly conserved (see also alignment in Fig. 4A). This residue is located at the same site as phenylalanine 85 of Rhod that was important for modification by lethal toxin. Because lethal toxin predominantly modifies Ras protein family members, we also tested whether this tyrosine residue in Ras is crucial for toxin recognition. However, change of the tyrosine residue of H-Ras into serine did not alter the substrate properties toward the toxins (data not shown), e.g. toxin B was still not able to modify Ras proteins. Thus, for Ras recognition by lethal toxin additional conserved amino acids are necessary.

Constructing RhoA-Rac1 chimeras, Müller and coworkers (34) found that amino acids 22–27 in Rac1 were of major impact for recognition by lethal toxin of C. sordellii strain 1522 and toxin B from C. difficile strains 1470 and 8864. Especially, lysine 27 located on helix α1 of RhoA hindered the modification by lethal toxin (see Fig. 4B). In RhoA K27T, the substrate properties were switched. This mutant protein adapted Rac1 properties. Prior to the construction of the above described chimeras, we exchanged several amino acids in helix α1 of RhoA to those of Rhod (RhoA S26D, S26A, S26E, S26H, S26K, K27D, D28G, Q29A, S26A/D28G, S26A/Q29A and RhoA D28G/Q29A) to elucidate the substrate specificity in Rhod, but none of them allowed modification of RhoA by lethal toxin or reduced the modification by toxin B (data not shown). This suggests that helix α1 of Rhod does not contain any structural feature that is decisive for modification by lethal toxin and toxin B. Nevertheless, we cannot exclude that the isoforms that were used in the reported studies behave differently.

For CNF1, it was reported that a peptide covering amino acids 59–69 of the switch II region of RhoA was sufficient to be modified by the toxin. We focused on this region to identify crucial amino acid residues that prevent modification of Rhod by CNF and DNT. By site-directed mutagenesis, we were able to show that glutamate 64 of RhoA and aspartate 76 of Rhod define the specificity for CNF1. For DNT it was shown that beyond the switch II region of Rhod other contact regions are necessary (31). Therefore, it was even more surprising that the property to serve as substrate for DNT was defined by the same amino acid residue in Rhod as for CNF1. The change of aspartate 76 to glutamate in Rhod causes only a minor change in the side chain length of one methylene group, but this was crucial for the substrate property of Rhod and allowed modification by DNT.

Taken together, a striking finding of this study is the fact that the substitution of a single amino acid can change the substrate properties of the GTPases with simultaneous and selective loss and gain of functions, respectively. Thus, single amino acids play a crucial and determining role for the interaction of specific Rho protein family members with diverse bacterial toxins. From crystal structures of Rho proteins in complexes with effectors and regulators and from biochemical studies, it was shown that glutamate 64 (35–39) and serine 73 (35, 40–45) participate in protein-protein interactions. It remains to be clarified whether these amino acid residues play a similar decisive role in the interaction of RhoA/Rhod with effectors or regulatory proteins as is shown here in the case of the interaction of the GTPases with toxins.

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