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Identification of the Region of Rho Involved in Substrate Recognition by *Escherichia coli* Cytotoxic Necrotizing Factor 1 (CNF1)*

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The *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1) and the *Bordetella* dermonecrotic toxin (DNT) activate Rho GTPases by deamidation of Gln^{63} of RhoA (Gln^{61} of Cdc42 and Rac). In addition, both toxins possess *in vitro* transglutaminase activity in the presence of primary amines. Here we characterized the region of Rho essential for substrate recognition by the toxins using Rho/Ras chimeras as protein substrates. The chimeric protein Ras55Rho was deamidated or transglutaminated by CNF1. Rat pheochromocytoma PC12 cells microinjected with Ras55Rho developed formation of neurite-like structures after treatment with the CNF1 holotoxin indicating activation of the Ha-Ras chimera and Ras-like effects in intact cells. The Ras59Rho78Ras chimera protein contained the minimal Rho sequence allowing deamidation or transglutamination by CNF1. A peptide covering mainly the switch II region and consisting of amino acid residues Asp^{59} through Asp^{78} of RhoA was substrate for CNF1. Changes of amino acid residues Arg^{68} or Leu^{72} of RhoA into the corresponding residues of Ras (R68ARhoA and L72QRhoA) inhibited deamidation and transglutamination of the mutants by CNF1. In contrast to CNF1, DNT did not modify Rho/Ras chimeras or the switch II peptide (Asp^{59} through Asp^{78}). Glucosylation of RhoA at Thr^{37} blocked deamidation by DNT but not by CNF. The data indicate that CNF1 recognizes Rho GTPases exclusively in the switch II region, whereas the substrate recognition by DNT is characterized by additional structural requirements.

Rho GTPases (*e.g.* Rho, Rac, and Cdc42) participate in the regulation of the actin cytoskeleton (1, 2). Whereas Rho subtype proteins induce formation of stress fibers and adhesion complexes, Rac is involved in formation of lamellipodia and Cdc42 induces microspikes (3–5). Beside their roles in the organization of the actin cytoskeleton, Rho proteins act as molecular switches in various signal transduction processes (6, 7).

Rho proteins are the preferred substrates for several bacterial protein toxins. Exoenzyme C3 from *Clostridium botulinum* and related C3-like transferases ADP-ribosylate RhoA, B, and C at Asn^{41} thereby inhibiting the biological activity of the GTPases (8–11). Rho proteins are monoglucosylated by members of the family of large clostridial cytotoxins (*e.g. Clostridium difficile* toxins A and B) (12–14). The toxins modify RhoA at Thr^{37} (Thr^{35} of Rac and Cdc42), a modification which blocks the interaction of the GTPases with their effectors (15, 16).

Rho family GTPases are also the targets for cytotoxic necrotizing factors (CNF)[1] 1 and 2 from *Escherichia coli* and the dermonecrotizing toxin (DNT) produced by various *Bordetella* species. CNF and DNT are ~115 and ~165 kDa proteins which share a region of homology at their C termini harboring the enzyme domain of the toxins (17). In culture cells, the toxins induce actin polymerization and inhibit cytokinesis resulting in formation of multinucleated cells (18–20). Recently it has been reported that CNF and DNT act on Rho GTPases by deamidation of glutamine 63 of RhoA, thereby inhibiting the GTPase activity of Rho. Because Gln^{63} is essential for GTP hydrolysis, deamidation causes persistent activation of the GTPase resulting in strong formation of stress fibers of CNF- or DNT-treated cells. In addition to their deamidase activity, both toxins possess *in vitro* transglutaminase activity to attach primary amines onto Rho GTPases. Substrates of CNF and DNT are Rho subfamily members including Rac and Cdc42.

In the present communication the substrate recognition of Rho GTPases by CNF1 and DNT was studied. Using GTPase chimeras of RhoA and Ha-Ras which is not a substrate of the toxins, we identified the switch II region of Rho as being sufficient for recognition by CNF1. Accordingly, a peptide consisting of amino acid residues Asp^{59} through Asp^{78} of RhoA was deamidated and/or transglutaminated by CNF1. By contrast, the structural requirements for substrate recognition by DNT are more stringent.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Proteins—For protein purification, *E. coli* strains carrying pGEX plasmids with the coding sequence for the respective GTPases, GTPase chimera, CNF1 (either as full-length or as the catalytic C-terminal part (ΔCNF1, amino acid residues 709–1014), or DNT (the catalytic C-terminal part (ΔDNT, amino acid residues 1136–1451) were grown in LB medium and induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at OD 0.5. The cells were harvested after 3–6 h and the glutathione *S*-transferase (GST) fusion proteins were purified by means of glutathione-Sepharose (Amersham Pharmacia Biotech). Unstable proteins were kept as GST fusion proteins (Rho115Ras, Ras59Rho115Ras, ΔDNT, and full-length CNF1); otherwise, the proteins were subjected to thrombin cleavage to remove GST.

Construction of GTPase Chimeras—The GTPase chimeras were constructed using the splicing by overlap extension method described pre-

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[1] The abbreviations used are: CNF1, *E. coli* cytotoxic necrotizing factor 1; ΔCNF1, the active fragment of CNF1 consisting of amino acid residues 709 through 1014; DNT, *Bordetella* dermonecrotic toxin; ΔDNT, the active fragment of DNT consisting of amino acid residues 1136 through 1451; GST, glutathione *S*-transferase; PC12, pheochromocytoma cells; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption-ionization time of flight mass spectrometry; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

TABLE I
Primers used for construction of chimeras

Ras55Rho:Ras	5′-CACAAAGCCAACAGGCACGTCTC-3′
Ras55Rho:Rho	5′-GCCTGTTGGCTTTGTGGGACAC-3′
Ras59Rho:Ras	5′-CCAGCTGTGTCCAGGATGTCCAAC-3′
Ras59Rho:Rho	5′-GACATCCTGGACACAGCTGGGC-3′
Rho70Ras:Rho	5′-GTACTGGTCCCGCAGGCGATC-3′
Rho70Ras:Ras	5′-GATCGCCTGCGGGACCAGTAC-3′
Rho78Ras:Rho	5′-GAGGAAGCCATCGGTATCTGGG-3′
Rho78Ras:Ras	5′-GATACCGATGGCTTCCTCTGTG-3′
Rho115Ras:Rho	5′-CTTGTTCCCCACCAGGATGATG-3′
Rho115Ras:Ras	5′-CATCATCCTGGTGGGGAACAAG-3′
pGEX 5′ of MCS[a]	5′-TAGCATGGCCTTTGCAGGG-3′
pGEX 3′ of MCS	5′-TGTGTCAGAGGTTTTCACCG-3′

[a] MCS, multiple cloning sequence.

TABLE II
Sense primers used for site-directed mutagenesis

R68A RhoA	5′-GGAAGATTATGATGCCCTGAGGCCCCTCTCC-3′
L72Q RhoA	5′-GCCTGAGGCCCCAGTCCTACCCAGATACC-3′
K98Q RhoA	5′-GAAAACATCCCAGAACAGTGGACCCCAG-3′
H105R RhoA	5′-CCAGAAGTCAAGCGTTTCTGTCCCAACG-3′
A66R Ha-Ras	5′-GAAGAGTATAGTCGCATGCGGGACCAG-3′
Q70L Ha-Ras	5′-CATGCGGGACCTGTACATGCGCACAG-3′

viously (21). In brief, two polymerase chain reactions (PCR) were performed, each amplifying the sequences 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-Ha-Ras (wild-type) as templates with the different primers listed in Table I and pGEX primers either annealing 5′ or 3′ of the multiple cloning site of pGEX (Table I). The product of the second PCR was subjected to *Bam*HI and *Eco*RI cleavage and subcloned into a pGEX2TGL vector. The sites of splicing are numbered by Rho nomenclature. The "sandwich" chimeras Ras55Rho70Ras and Ras59-Rho70Ras were constructed with splicing by overlay extension by using the primers for Ras55Rho and Ras59Rho, respectively, with Rho70Ras serving as template. Ras59Rho115Ras was constructed by using the primers for Ras59Rho with Rho115Ras as a template. All constructs were checked for proper sequences.

Mutagenesis—Mutants were constructed from pGEX2T-RhoA (wild-type) or pGEX2T-Ha-Ras (wild-type) by PCR in the presence of two primers (sense and corresponding antisense) carrying a base mismatch encoding the proper mutant. The parental DNA was eliminated using the restriction enzyme *Dpn*I, which digests methylated DNA. The PCR products were transformed into Epicurean Blue XL-1 ultracompetent cells (Stratagene). After verifying the mutation by sequencing, the plasmids were transformed into *E. coli* BL21 for protein expression. The sense primers for the various mutants are listed in Table II.

Cell Culture and Microinjection—Rat pheochromocytoma cells (PC12) were cultivated on plastic dishes in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% fetal calf serum, penicillin (4 mM), and streptomycin (4 mM) and kept at 37 °C in a humidified atmosphere with 10% CO_2. The cells were microinjected with 0.5 μg/μl GST-Ras55Rho or buffer by means of a microinjector 5242 (Eppendorf) and subsequently treated for 48 h with full-length GST-CNF1 (300 ng/ml).

Transglutamination Assay—GTPase or chimeric protein (3–6 μg) was incubated with or without CNF1 or DNT in a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 50 mM monodansylcadaverine for 15 min at 37 °C. 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 in each lane.

Nucleotide Binding Assay—RhoA, Ha-Ras, RhoA mutants, or Rho/Ras chimeras (each 0.5 μM) were incubated at 37 °C in a buffer containing 150 mM NaCl, 2.5 mM $MgCl_2$, 10 mM triethanolamine (pH 7.5). After addition of 2 μM 2′(3′)-*O*-(*N*-methylanthraniloyl)-GDP which was synthesized as described (22), the increase in light emission at 444 nm, due to the higher intensity of bound 2′(3′)-*O*-(*N*-methylanthraniloyl)-nucleotide excited at 357 nm, was monitored in a Perkin-Elmer LS 50B luminescence spectrometer.

Release of Ammonia—To determine the release of ammonia from the GTPases during incubation with CNF1, we utilized a coupled enzymatic reaction. In the presence of glutamate dehydrogenase and NADH, ammonia reacts with α-ketoglutarate to form L-glutamate, thereby oxidating the NADH. The amount of NADH oxidized in the reaction is stoichiometric to the amount of ammonia produced. RhoA, Ha-Ras, or Rho/Ras chimeras (each 10 μM) were incubated in a buffer containing 5 mM Tris-HCl (pH 7.4), 10 mM $MgCl_{2,}$, 1 mM dithiothreitol, 1 mM EDTA and the components of the coupled enzyme reaction system (Roche Molecular Biochemicals, Ammonia Test Combination for Food Analysis): 140 μM NADH, 7 mM α-ketoglutarate, and 10 units of glutamate dehydrogenase. The samples were equilibrated at 37 °C prior to addition of CNF1 as indicated in the figure legends. The decrease in NADH concentration was measured fluorimetrically (excitation at 340 nm, emission at 460 nm) in a Perkin-Elmer LS 50B luminescence spectrometer.

Glucosylation and ADP-ribosylation of RhoA—RhoA was incubated in a glucosylation buffer (50 mM Hepes (pH 7.5), 100 mM KCl, 0.1 mg/ml bovine serum albumin, 2 mM $MgCl_2$, and 1 mM $MnCl_2$) in the presence or absence of *C. difficile* toxin B (6 ng/μl) or in an ADP-ribosylation buffer (40 mM Hepes (pH 7.5), 200 μM NAD, 0.1 mg/ml bovine serum albumin, 100 μM GDP, and 2 mM $MgCl_2$) in the presence or absence of *C. botulinum* C3 toxin (1 ng/μl) at 37 °C for 30 min.

Mass Spectrometry—RhoA switch II peptide (1 mM) was incubated for 3 h at 37 °C with or without CNF1 (1 μM) in the presence of 20 mM ethylenediamine in a buffer containing 25 mM triethanolamine (pH 7.5), 75 mM NaCl, 1 mM $CaCl_2$, 2 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM EDTA. To obtain a concentration of the RhoA switch II peptide of 10 μM, the reaction mixture was diluted with TA (acetonitrile, 0.1% trifluoroacetic acid, 1:1). A saturated matrix solution of recrystallized 4-hydroxy-α-cyanocinnamic acid (Aldrich) in TA was freshly prepared and marker peptides (ACTH 18–39 clip, human, and angiotensin II, human) were added for internal calibration. Matrix and peptide solution were mixed in equal amounts. Using the dried-drop method of matrix crystallization, 1 μl of the sample matrix solution was placed on the MALDI stainless steel target and allowed to air-dry several minutes at room temperature. MALDI/TOF-MS was performed on a Bruker Biflex mass spectrometer equipped with a nitrogen laser (λ = 337). Mass spectra were recorded in the reflector positive mode in combination with delayed extraction.

RESULTS

Rho But Not Ras Is Deamidated by CNF1—It has been shown that Rho, Rac, and Cdc42 are deamidated by CNF1 (23). To test whether Ha-Ras is a substrate for CNF1, the release of ammonia occurring concomitantly with deamidation was determined in a coupled enzyme assay (see "Experimental Procedures"). Fig. 1 shows that addition of the active fragment of CNF1 (ΔCNF1) caused release of ammonia in the presence of RhoA but not in the presence of Ras indicating that the latter GTPase is not modified by CNF1. Because Rho and Ha-Ras differ in the amino acid residues flanking the site of modification in Rho (Fig. 2), we tested a Ha-Ras mutant with an aspartic acid residue instead of glutamic acid at position 63 of Ha-Ras. Like wild-type Ha-Ras, the mutant E63D Ras was not deamidated by ΔCNF1 (not shown).

Identification of the Substrate Recognition Site of Rho—By exploiting the finding that Ha-Ras is not a substrate for ΔCNF1, chimeras of Rho and Ha-Ras were constructed to identify the minimal amino acid sequence of Rho allowing the modification by ΔCNF1. First, we constructed Rho115Ras and Ras55Rho chimeras (note that the Rho nomenclature is used), which both have the switch II region of RhoA. The chimeras were tested in a transglutaminase assay with monodansylcadaverine as a co-substrate. This primary amine contains a naphthalene group, which is excitable by UV light at 206 nm. Fig. 3 shows the proteins analyzed by SDS-PAGE and photographed under UV light. As observed for wild-type Rho, the Ras55Rho chimera was transglutaminated by ΔCNF. Also the Rho115Ras chimera was substrate for ΔCNF. This chimera was less stable than Ras55Rho. Therefore, we studied Rho115Ras as a GST fusion protein and compared it with GST fusion proteins of wild-type RhoA and Ha-Ras.

Hall and co-workers reported that amino acid residues 23 through 46 of Ras are sufficient to elicit Ras-dependent transformation in NIH3T3 cells (24). Constitutively active Ras is known to induce neurite formation in PC12 cells (25). Therefore, we tested whether CNF1 was able to induce neurite for-

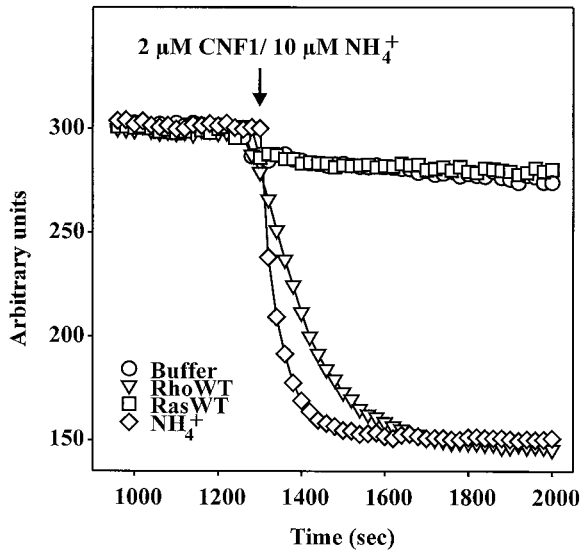

FIG. 1. **Release of ammonia from Rho induced by CNF1.** RhoA or Ha-Ras (each 10 μM) were incubated in a buffer containing the components of the coupled enzymatic reaction: NADH, α-ketoglutarate, and glutamate dehydrogenase. After the samples had equilibrated at 37 °C, ΔCNF1 (2 μM, final concentration) was added. Release of ammonia is measured by the decrease in NADH due to the coupled reaction. In one control, CNF1 was added to buffer without GTPase. In another control, 10 μM NH_4^+ was added to buffer without GTPase. The experiment was repeated 3 times with similar results.

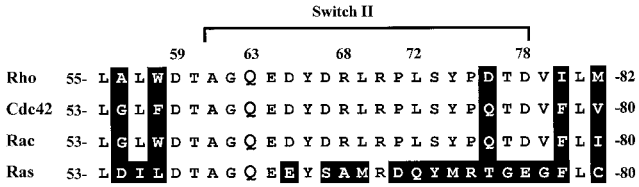

FIG. 2. **Alignment of amino acids of RhoA, Cdc42, Rac1, and Ha-Ras that cover the switch II region of the GTPases.** Gln^{63} (*bold letter*, Rho nomenclature) is the target for deamidation or transglutamination by CNF and DNT. Mutant RhoA proteins were constructed with amino acids Arg^{68} or Leu^{72} changed to the respective residues of Ha-Ras. The peptide Asp^{59} through Asp^{78} was studied as a substrate for CNF1.

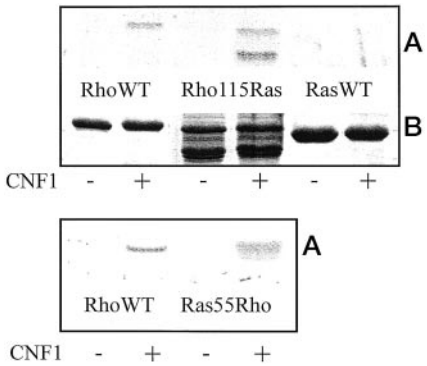

FIG. 3. **Transglutamination of Rho/Ras chimeras.** GST-RhoA, GST-Rho115Ras, or GST-Ha-Ras (each 3 μg, *upper panel*) and RhoA or Ras55Rho (each 3 μg, *lower panel*) were incubated with or without ΔCNF1 (1 μg) at 37 °C for 15 min in a buffer containing monodansylcadaverine. The samples were subjected to SDS-PAGE and the gels were photographed under UV light (*A*). Subsequently, the gels were stained with Coomassie Blue (*B*). The *lower band* of Rho115Ras, which is also modified by ΔCNF1, may be a degradation product of the protein. The experiment was repeated more than 3 times with similar results.

mation in PC12 cells microinjected with Ras55Rho prior to toxin treatment. PC12 cells were grown on plastic dishes, microinjected with 0.5 μg/μl GST-Ras55Rho, and treated with

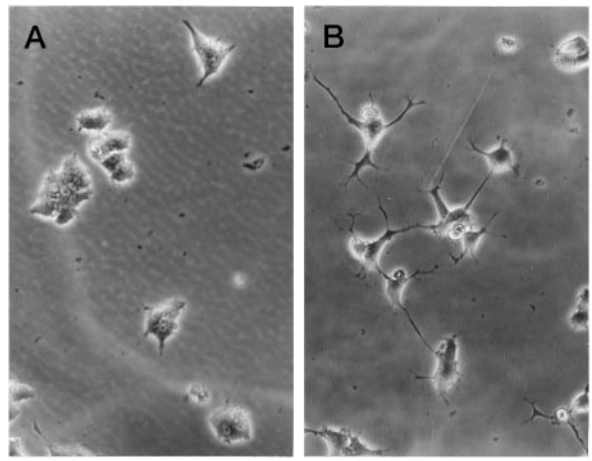

FIG. 4. **Neurite outgrowth of PC12 cells after microinjection of the GST-Ras55Rho chimera and activation by CNF1.** Subconfluent PC12 cells growing on plastic dishes were microinjected with 0.5 μg/μl GST-Ras55Rho (*A* and *B*) and subsequently incubated for 48 h without (*A*) or with (*B*) 300 ng/ml full-length GST-CNF1. The experiment was repeated 3 times with similar results.

GST fusion protein of the CNF1 holotoxin (300 ng/ml) for 48 h. Whereas Ras55Rho-injected cells without CNF1 showed only small processes (Fig. 4*A*), injected cells developed long neurite extensions after CNF1 treatment (Fig. 4*B*) indicating an activation of the Ras chimera by CNF1.

Because experiments with the Ras55Rho and Rho115Ras chimeras suggested that neither the N terminus nor the C terminus of Rho are essential for substrate recognition by ΔCNF1, we studied whether sandwich (Ras/Rho/Ras) chimeras are modified by the toxin. The sandwich chimera, Ras59Rho115Ras, was substrate for deamidation by ΔCNF1 (not shown). Further reduction of the length of the RhoA insert in Ha-Ras resulted in the chimera Ras59Rho78Ras. We tested this chimera in the transglutamination assay at increasing concentrations of ΔCNF1. The chimera was a substrate for transglutamination, however, whereas wild-type Rho was significantly transglutaminated at 10 nM ΔCNF1, higher concentrations of CNF1 (about 10-fold) were required for transglutamination of Ras59Rho78Ras (Fig. 5). Chimeras containing very short Rho sequences of only 8 or 5 amino acid residues (Ras55Rho70Ras and Ras59Rho70Ras) did not serve as substrates of CNF1 (data not shown). To exclude that these results were due to incorrect protein folding, we tested the ability of the chimeras to bind nucleotide. Both chimeras (Ras55Rho70Ras and Ras59Rho70Ras) were able to bind 2′(3′)-*O*-(*N*-methylanthraniloyl)-GDP indicating a proper folding of the proteins (not shown). The results obtained with the different chimeras are summarized in Fig. 6.

Identification of Amino Acid Residues in RhoA Which Are Essential for CNF1 Substrate Recognition—Deduced from recent crystal structure analysis of Rho (26) and by means of the "Rasmol" program we identified four amino acids (Arg^{68}, Leu^{72}, Lys^{98}, and His^{105}) between residues 59 through 115 of Rho which are surface exposed and are identical in Rho, Rac, and Cdc42. These amino acids were changed to the corresponding Ras residues. All mutant proteins were capable of binding 2′,3′-*O*-*N*-methylanthraniloyl-GDP in the nucleotide binding assay (not shown) indicating a correct protein folding. We tested the deamidation of the mutant RhoA proteins by ΔCNF1 in the ammonia release assay. As shown in Fig. 7, *A* and *B*, whereas the K98Q and the H105R mutants were deamidated by ΔCNF1, L72Q and R68A mutants did not serve as substrates or were only marginally modified by the toxin. The rate of the modification of the mutant K98Q varied between differ-

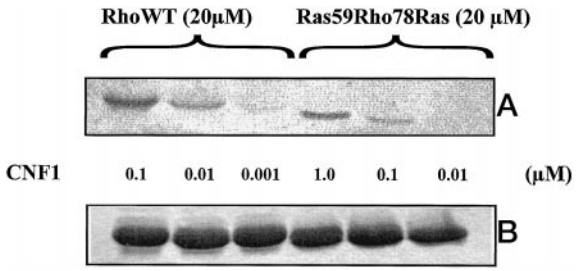

FIG. 5. **Transglutamination of the GTPase chimera Ras59Rho78Ras.** Wild-type RhoA (RhoWT) or the chimera Ras59Rho78Ras (each 20 μM) were incubated for 15 min in the presence of monodansylcadaverine at the indicated concentrations of ΔCNF1. The samples were separated on SDS-PAGE and the gel was photographed under UV light (*A*), thereafter, the gels were stained with Coomassie Blue (*B*). Repetition of the experiment gave similar results.

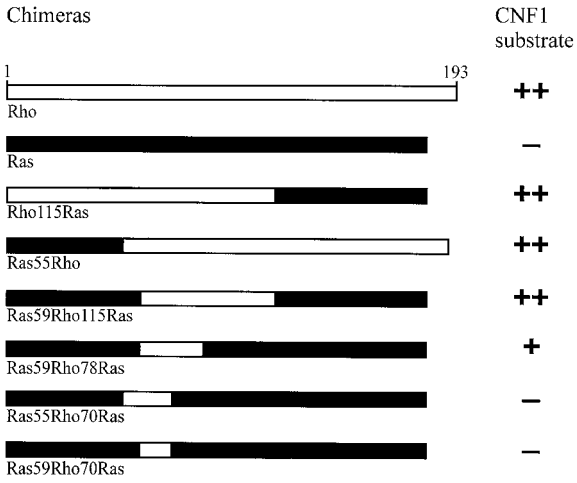

FIG. 6. **Properties of Rho/Ras chimeras to serve as substrates for ΔCNF1.** Substrate properties were tested at least 3 times by the ammonia release and/or the transglutamination assay. ++, substrate properties similar as wild-type RhoA; +, lower rate of transglutamination than with wild-type RhoA; –, no substrate for ΔCNF1.

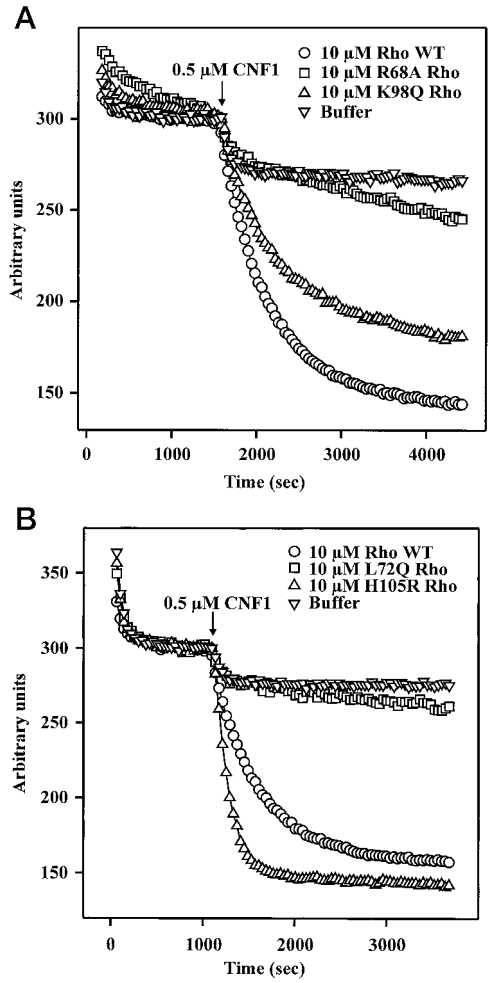

FIG. 7. **Release of ammonia from RhoA mutants induced by ΔCNF1.** Wild-type RhoA (RhoWT, *A* and *B*), R68A RhoA (*A*), K98Q RhoA (*A*), L72Q RhoA (*B*), or H105R RhoA (*B*) were incubated in a buffer containing the components of the coupled enzymatic reaction: NADH, α-ketoglutarate, and glutamate dehydrogenase. After equilibration of the samples at 37 °C, ΔCNF1 (0.5 μM) was added. Ammonia release was determined by the decrease in NADH fluorescence (arbitrary units) caused by the coupled reaction. In control experiments (*A* and *B*), ΔCNF1 was added to buffer without Rho protein. The experiment was repeated with similar results.

ent experiments and was not consistently slower than the wild-type. To investigate whether arginine 68 and leucine 72 are sufficient for CNF1 substrate recognition, a Ha-Ras double mutant carrying an arginine residue at position 66 and a leucine residue at position 70 was constructed and tested for activity in the transglutaminase and ammonia release assay. However, this mutant was not modified by ΔCNF1 (not shown).

A Peptide Corresponding to the RhoA Switch II Region Is Deamidated by CNF1—A peptide of the sequence ^{59}DTAGQEDYDRLRPLSYPDTD^{78} which covers the switch II region of RhoA was tested for its ability to serve as substrate for CNF1 in the ammonia release assay. The switch II peptide was compared with RhoA (10 μM) at two different concentrations (10 and 100 μM) in the presence of 1 μM ΔCNF1. As shown in Fig. 8, after addition of ΔCNF1, ammonia was released from the peptide, however, at a slower rate than with RhoA. The difference in the modification rates was calculated as a 110-fold decrease for the peptide in comparison with the recombinant protein (Sigma Plot). A shorter peptide with the sequence ^{59}DTAGQEDYDRLR^{70} did not release ammonia after addition of ΔCNF1 (not shown). To study whether the switch II peptide (Asp^{59}-Asp^{78}) was modified by transglutamination, the peptide was treated with CNF1 in the presence of ethylenediamine. Thereafter, the sample was analyzed by MALDI-TOF mass spectrometry. As shown in Fig. 9, after ΔCNF1 treatment a new peptide characterized by an increase in mass by 43 Da was detected indicating the attachment of ethylenediamine onto the switch II peptide.

Comparison of CNF1 with the Related Toxin DNT—Next we compared ΔCNF with the related *Bordetella* deamidase DNT in respect to substrate specificity and substrate recognition. The active C-terminal part of *Bordetella* DNT (amino acids 1136–1451, ΔDNT) was applied as a GST fusion protein because GST-ΔDNT proved to be more stable than ΔDNT. For comparison of the two enzymes, ΔCNF1 was also used as a GST fusion protein. As observed for GST-ΔCNF1, GST-ΔDNT deamidated RhoA, Rac 1, and Cdc42 (not shown).

To investigate whether DNT requires the same amino acid residues of Rho for substrate recognition as CNF1, the transglutaminase activities of both toxins were studied in the presence of monodansylcadaverine with the RhoA mutants (R68A, L72Q, K98Q, and H105R) mentioned above (Fig. 10). Like ΔCNF1, ΔDNT did not modify the mutants R68A RhoA and L72Q RhoA with alterations in the switch II region. K98Q RhoA and H105R RhoA were transglutaminated by both toxins.

We tested the Ras55Rho and Rho115Ras chimeras with GST-ΔCNF1 and GST-ΔDNT in the transglutamination assay. In contrast to GST-ΔCNF1, both chimeras were not modified by GST-ΔDNT (not shown). It has been reported that unlike CNF1, DNT modifies RhoA only in the GDP-bound form (27). To exclude the possibility that GTP binding caused inhibition

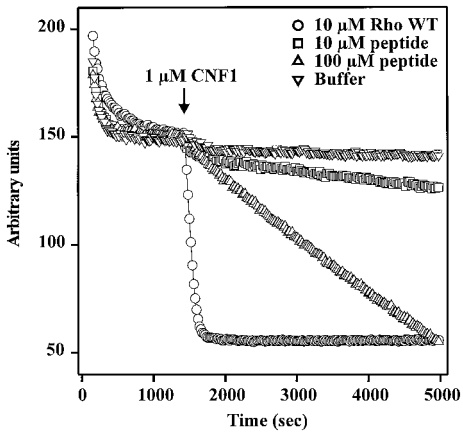

FIG. 8. **Release of ammonia from the RhoA peptide Asp^{59} through Asp^{78}.** RhoA (10 μM) or the RhoA peptide (10 and 100 μM) were equilibrated at 37 °C in a buffer containing the components of the coupled enzymatic reaction, then ΔCNF1 (1 μM) was added. Ammonia release was determined by the decrease in NADH fluorescence (arbitrary units) caused by the coupled reaction. In controls, ΔCNF1 was added to buffer without Rho protein or peptide. Repetition of the experiment gave similar results.

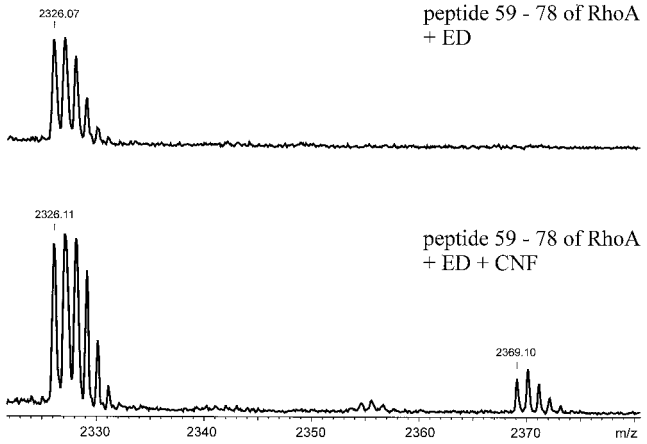

FIG. 9. **MALDI-TOF-MS spectra of the transglutaminated RhoA peptide Asp^{59}-Asp^{78}.** The RhoA peptide Asp^{59}-Asp^{78} was treated in the presence of ethylenediamine (20 mM) without (*upper line*) or with ΔCNF1 (1 μM, *lower line*) for 3 h. Thereafter, the peptides were analyzed by MALDI-TOF-MS. Shown is the Rho peptide Asp^{59}-Asp^{78} (2326 Da) and a new peptide with a mass of 2369.1 Da caused by ΔCNF1-catalyzed attachment of ethylenediamine (43 Da) onto the Rho peptide. Note the increase in mass of 1 Da by deamidation was not detected, because the reaction was not complete.

of modification by DNT, chimera Ras55R40 was loaded with GDP prior to toxin treatment. However, the Rho chimeras were not modified by DNT even after loading with GDP (not shown). The above findings suggested that the substrate recognition by DNT is more stringent than for CNF1. Because nucleotide binding causes major conformational changes in the switch I region of Rho, we studied the effects of glucosylation of RhoA at Thr^{37} by *C. difficile* toxin B on modification by DNT. As shown in Fig. 11, prior glucosylation of Rho by toxin B inhibited the transglutamination by DNT but not by CNF1. By contrast, ADP-ribosylation of Rho at Asn^{41} by exoenzyme C3, a modification that occurs downstream of the switch I region did not affect transglutamination by CNF1 or DNT.

DISCUSSION

In contrast to various bacterial protein toxins inactivating small GTPases including large clostridial cytotoxins and exoenzyme C3 (28), the *E. coli* cytotoxic necrotizing factors CNF1 and CNF2 activate Rho GTPases. The latter toxins deamidate Rho

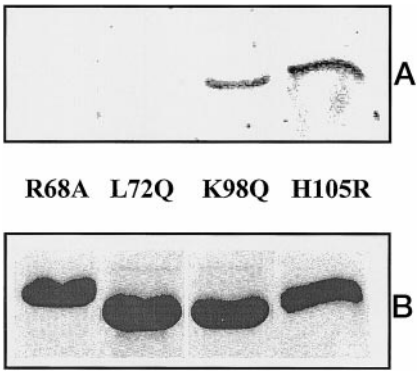

FIG. 10. **Transglutamination of Rho mutants by ΔDNT.** The Rho mutants R68A, L72Q, K98Q, and H105R (each 6 μg) were incubated for 15 min in the presence of monodansylcadaverine and GST-ΔDNT (2 μg). The samples were separated on SDS-PAGE and the gel photographed under UV light (*A*) before staining the gels with Coomassie Blue (*B*). The experiment was repeated more than 3 times.

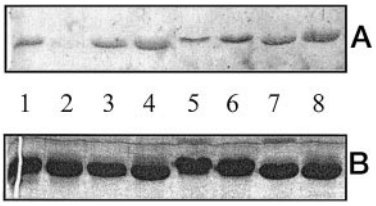

FIG. 11. **Transglutamination of glucosylated and ADP-ribosylated RhoA by CNF1 and DNT.** RhoA was incubated in a glucosylation buffer in the presence (*1* and *2*) or absence (*3* and *4*) of *C. difficile* toxin B (6 ng/μl) and subsequently transglutaminated by GST-ΔCNF1 (1 μM, *1* and *3*) or GST-ΔDNT (2 μM, *2* and *4*). Similarly, Rho A was incubated in a ADP-ribosylation buffer in the presence (*5* and *6*) or absence (*7* and *8*) of C3 toxin (1 ng/μl) prior to CNF1 (*5* and *7*) or DNT (*6* and *8*) transglutamination. The samples were subjected to SDS-PAGE and the gel was photographed under UV light (*A*). The Coomassie staining is shown in *B*. Repetition of the experiment gave similar results.

GTPases at Gln^{63} of Rho (Gln^{61} of Rac and Cdc42) to inhibit the endogenous and GAP-stimulated GTP hydrolysis thereby blocking the switch off reaction of the GTPase (29, 30). Recently, we reported that CNF1 possesses *in vitro* transglutaminase activity in addition to deamidase activity (31, 32). Here we attempted to identify the recognition site of Rho GTPases for CNF1.

Rozengurt and co-workers (33) reported that CNF1 does not activate the mitogen-activated protein kinases p42^{mapk} or p44^{mapk} in Swiss 3T3 cells, suggesting that Ras is not activated by the toxin. In line with this notion, we confirmed that recombinant Ras is not modified by CNF1. To get more information about the structural requirements for substrate recognition by CNF1, we constructed several chimeras consisting of Rho and Ras. The enzymatically active toxin fragment ΔCNF1 (amino acid residues 709–1014 of CNF1) deamidated and transglutaminated all chimeras consisting of Ras harboring the switch II region (Ala^{61} through Asp^{78}) of Rho.

These results indicate that the switch II region of Rho is sufficient for substrate recognition by CNF1 and neither the switch I region nor the insert region (Asp^{124} through Gln^{136}) are required for interaction with CNF1. This notion was corroborated by the findings that the peptide Asp^{59} through Asp^{78} covering the switch II region of RhoA was capable to serve as a substrate for the active fragment of CNF1. Similar as found for the chimera Ras59Rho78Ras, the peptide was a poorer substrate for CNF1 than wild-type RhoA, suggesting that additional residues, although not essential for modification by the toxin, increase the substrate properties of RhoA. With one exception (Asp^{76} of Rho, which is glutamine in Rac and Cdc42), all Rho proteins including Rac and Cdc42 possess highly conserved amino acid sequences in the switch II region. Thus, the

substrate requirements observed in this study are in agreement with recent findings that Rac and Cdc42 are modified by CNF1 (23) and it seems likely that all Rho GTPases, including those not studied so far, are also substrates of the toxin.

To identify amino acids which are possibly involved in interaction with CNF1, we selected surface-exposed amino acids (Arg^{68}, Leu^{72}, Lys^{98}, and His^{105}) for site-directed mutagenesis by using the crystal structure data of RhoA (26). Replacement of Arg^{68} or Leu^{72} of RhoA with the corresponding amino acid in Ha-Ras (R68A and L72Q) prevented modification of the mutant proteins. By contrast, replacement of His^{105} and Lys^{98}, which are located outside the switch II region, with the corresponding residues of Ha-Ras did not alter the modification of RhoA, and the rate of ammonia release from H105R RhoA was greater than that from wild-type Rho. Although both Arg^{68} and Leu^{72} of RhoA are essential for recognition by CNF1, they are not sufficient for modification. This is concluded from the finding that replacement of the corresponding amino acids in Ras by arginine and leucine did not make it a substrate for CNF1.

The observation that the switch I region of Rho is not important for substrate recognition by CNF1 *in vitro* was confirmed in intact cells. Microinjection of the Ras55Rho chimera into PC12 cells and subsequent treatment with the CNF1 holotoxin, which in contrast to ΔCNF1 (amino acids 709 through 1014) is able to enter cells, caused a typical Ras-like response, *e.g.* neurite outgrowth. Addition of CNF1 to buffer-injected cells did not lead to this phenotype (not shown). These results are explained by interaction of the Ras55Rho chimera with Ras effectors mediated by the intact switch I region of Ras and the deamidation of the chimera at Gln^{63} (Rho nomenclature) thereby preventing the inactivation of the chimeric GTPase. These findings are in agreement with the recent report by Hall and co-workers (24) that a constitutively active Ras/Rho chimera, containing the switch I region of Ras caused the typical Ras effects such as cell transformation (24). Moreover the observed neurite outgrowth is clearly a Ras phenotype, because in contrast to microinjection of dominant active Ras (G12V) the injection of dominant active RhoA (G14V) does not induce neurite outgrowth in PC12 cells (not shown).

The *Bordetella* dermonecrotizing toxin DNT is another toxin which activates Rho GTPases. This toxin shares significant similarity at its C terminus (amino acids 1136–1451) with the active region of CNF1 (17, 34). Like CNF1, DNT catalyzes deamidation of Gln^{63} of Rho (35). Moreover, DNT possesses high transglutaminase activity (36). We compared the structural requirements for substrate recognition by ΔDNT and ΔCNF1. As with ΔCNF1, changes of Arg^{68} and Leu^{72} to the equivalent amino acids of Ras inhibited modification by ΔDNT. However, in contrast to ΔCNF1 (comparison between GST-ΔDNT and GST-ΔCNF1), ΔDNT did not accept any of the Rho/Ras chimeras as substrates for deamidation or transglutamination. Accordingly, the peptide representing the switch II region of RhoA (Asp59 through Asp78) was not modified by ΔDNT. Because neither the Ras55Rho nor the Rho115Ras chimera were modified by ΔDNT, it can be concluded that structural determinants located both N- and C-terminal to the switch II region are essential for substrate recognition by DNT. Thus, it is obvious that the structural requirements for substrate recognition by DNT are more stringent than for CNF1. In line with this notion are recent reports that modification of Rho by DNT, but not by CNF1, depends on the nucleotide bound to Rho (27). Using the active fragment ΔDNT, we confirmed that GDP, but not the GTPγS bound form of Rho is a substrate for toxin-catalyzed deamidation and transglutamination. These findings suggest that in addition to the switch II region, the switch I region, which undergoes major changes upon nucleotide binding, is involved in substrate recognition by DNT. A role of the switch I region in substrate recognition by DNT is also supported by the finding that prior glucosylation of Rho by *C. difficile* toxin B impaired DNT-induced deamidation or transglutamination. Toxin B glucosylates Rho at Thr^{37} which is located in the switch I region (13). ADP-ribosylation of Rho by C3 at Asn^{41}, which is located outside the switch I region, did not alter DNT effects on Rho. By contrast, as substrate recognition by CNF1 depends almost exclusively on the sequence of the switch II region, it seems logical that nucleotide binding of Rho and glucosylation or ADP-ribosylation by toxins do not alter modification of the GTPase by CNF1.

In summary, the switch II region of Rho was identified as the substrate recognition site for CNF1. Minimal structural requirements for substrate recognition by CNF1 are provided by a peptide covering amino acid residues Asp^{59} through Asp^{78} of Rho. By contrast, the substrate recognition by DNT is much more complex requiring, in addition to the switch II region, further sites including the switch I region of Rho.

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