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The Rho-deamidating Cytotoxic Necrotizing Factor 1 from *Escherichia coli* Possesses Transglutaminase Activity

CYSTEINE 866 AND HISTIDINE 881 ARE ESSENTIAL FOR ENZYME ACTIVITY*

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Recently, it has been reported that cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli* induces formation of stress fibers by deamidation of glutamine 63 of RhoA (Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M., and Aktories, K. (1997) *Nature* 387, 725–729); Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C., and Boquet, P. (1997) *Nature* 387, 729–733). By using mass spectrometric analysis, we show now that the toxin transfers ethylenediamine, putrescine, and dansylcadaverine specifically onto glutamine 63 of RhoA. RhoA was also a substrate for guinea pig liver transglutaminase, which modified not only glutamine 63, but also glutamine residues at positions 52 and 136. Treatment of the fully active N-terminal fragment of CNF1 (amino acid residues 709–1014) with iodoacetamide inhibited both deamidation and transglutamination activities. Moreover, exchange of cysteine 866 with serine blocked the enzyme activity of the N-terminal CNF1 fragment. In addition, we identified histidine 881 to be essential for the enzyme activity of CNF1. The data indicate that CNF1 shares a catalytic dyad of cysteine and histidine residues with eukaryotic transglutaminases and cysteine proteases.

Rho proteins are involved in the organization of the actin cytoskeleton and act as molecular switches in various signaling processes (1–4). These GTPases are targets for various bacterial toxins and exoenzymes. RhoA, RhoB, and RhoC are ADP-ribosylated by *Clostridium botulinum* C3 (5, 6) and related C3-like exoenzymes (7). All Rho subfamily proteins are glucosylated by large clostridial cytotoxins (*e.g. Clostridium difficile* toxins A and B) (8, 9). In addition, Rho is a target for cytotoxic necrotizing factors 1 and 2 of *Escherichia coli* (10, 11). These toxins induce actin polymerization and inhibit cytokinesis, which results in formation of multinucleated cells (10–12). Recently, it was shown that CNF1[1] modifies RhoA by deamidation of glutamine 63, thereby forming a glutamic acid residue at this position (13, 14). Because glutamine 63 is essential for the intrinsic and GTPase-activating protein (GAP)-stimulated GTPase activity of Rho, the GTPase is constitutively activated by CNF1 (13, 15). A similar mechanism of Rho modification was recently reported for the CNF1-related dermonecrotic toxin (DNT) from *Bordetella* species (16).

It is known that deamidation of glutamine residues of proteins is also observed with various transglutaminases (*e.g.* tissue transglutaminase or coagulation factor XIII). In general, transglutaminases are cross-linking enzymes that catalyze the exchange of the γ-carboxamide group of glutamine residues for primary amines (*e.g.* peptide-bound lysine residues or polyamines) to form ε-(γ-glutamyl)lysine or (γ-glutamyl)polyamine bonds (17, 18). In the absence of an appropriate acceptor peptide, however, transglutaminases are capable of inducing the deamidation of glutamine residues. Therefore, we studied whether the Rho-deamidating CNF1 is somehow related to eukaryotic transglutaminases. Here, we report that CNF1 possesses *in vitro* transglutaminase activity and incorporates alkylamines into RhoA at glutamine 63. Moreover, we identified cysteine 866 and histidine 881 as functionally essential amino acid residues of CNF1.

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]NAD$^+$ was obtained from NEN Life Science Products. RhoA and p50^{RhoGAP} (obtained from A. Hall) were prepared from their fusion proteins as described (13). Guinea pig liver tissue transglutaminase, dansylcadaverine, ethylenediamine, putrescine, casein, and fibronectin were purchased from Sigma. Actin was prepared as described (19). Methanol and chloroform were of analytical grade, and trifluoroacetic acid and acetonitrile were of high pressure liquid chromatography grade.

Cloning and Purification of the Active CNF1 Fragment (Amino Acids 709–1014) and CNF1 Mutants—For construction of the active CNF1 fragment (ΔCNF1) consisting of amino acid residues 709–1014, the pGEX-CNF1 vector (13) was digested by *Bam*HI and *Sna*BI and purified from agarose gel (Jetsorb, Genomed). Sticky ends were filled up by Klenow enzyme in the presence of 0.25 mM each dNTP for 30 min at 30 °C. The vector was religated and transformed into BL21 cells by heat shock at 42 °C. The proper construct was checked by DNA sequencing. Expression of the glutathione *S*-transferase fusion protein in BL21 cells growing at 37 °C was induced by adding 0.2 mM isopropyl-β-D-thiogalactopyranoside (final concentration) at *A*0.5. Three h after induction, cells were collected and lysed by sonication in lysis buffer (20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM $MgCl_2$, and 1% Triton) and purified by affinity chromatography with glutathione-Sepharose (Amersham Pharmacia Biotech). Loaded beads were washed two times in washing buffer A (20 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 5 mM $MgCl_2$) and washing buffer B (150 mM NaCl and 50 mM Tris-HCl (pH 7.5)) at 4 °C. ΔCNF1 was eluted from the beads by thrombin digestion (200 μg/ml thrombin, 150 mM NaCl, 50 mM triethanolamine hydrochloride (pH 7.5), and 2.5 mM $CaCl_2$) for 45 min at room temperature. Thrombin was then removed by incubation with benzamidine-Sepharose beads.

Mutagenesis—Mutagenesis of ΔCNF1 (amino acids 709–1014) was performed by a round circle polymerase chain reaction-based site-directed mutagenesis kit (QuikChange™, Stratagene) with the following sense primers and corresponding antisense primers (MWG): C866S,

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[1] The abbreviations used are: CNF1, cytotoxic necrotizing factor 1; GAP, GTPase-activating protein; DNT, dermonecrotic toxin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ACTH, adrenocorticotropic hormone.

5′-GGAAATCTAAGTGGTAGTACGACAATTG-3′ (sense) and 5′-CAATTGTCGTACTACCACTTAGATTTC-3′ (antisense); H881Q, 5′-GGATATATTTATAAGGTACAGACTGGTACAAC-3′ (sense) and 5′-GTTGTACCAGTCTGTACCTTATAAATATATCC-3′ (antisense); H881A, 5′-GGATATATTTATAAGGTAGCTACTGGTACAAC-3′ (sense) and 5′-GTTGTACCAGTAGCTACCTTATAAATATATCC-3′ (antisense); N921A, 5′-GGAATAATGAGCGCTGATTTCTTAGTCG-3′ (sense) and 5′-CGACTAAGAAATCAGCGCTCATTATTCC-3′ (antisense); D922N, 5′-GGGAATAATGAGCAATAATTTCTTAGTCG-3′ (sense) and 5′-CGACTAAGAAATTATTGCTCATTATTCCC-3′ (antisense); D922A, 5′-GGGAATAATGAGCAATGCTTTCTTAGTCG3′ (sense) and 5′-CGACTAAGAAAGCATTGCTCATTATTCCC-3′ (antisense); and D926A, 5′-GATTTCTTAGTCGCTTATCTGTCGG-3′ (sense) and 5′-CCGACAGATAAGCGACTAAGAAATC-3′ (antisense). Mutations were verified by DNA sequencing using a Dye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems).

Microinjection—For microinjection, HeLa cells were seeded subconfluently on glass coverslips (CELLocate, Eppendorf) and cultivated for 24 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in humidified CO_2 at 37 °C. ΔCNF1 (2 mg/ml) and C866S ΔCNF1 (2 mg/ml) in injection buffer (140 mM KCl, 1 mM $MgCl_2$, 8 mM NaCl, and 10 mM Hepes (pH 7.4)) were microinjected into HeLa cells with an Eppendorf 5242 microinjector. Six h after microinjection, the cells were fixed with 4% formaldehyde and 0.1% Tween 20 in phosphate-buffered saline for 10 min at room temperature and stained for F-actin by rhodamine-conjugated phalloidin.

Actin Staining—Formaldehyde-fixed cells were washed intensively with phosphate-buffered saline. The cells were then incubated with rhodamine-conjugated phalloidin (1 unit/coverslip) at room temperature for 1 h, washed again, and applied for fluorescence microscopy (as bleaching preservative Kaiser's glycerol gelatin (Merck) was used).

Modification of Rho, Actin, Casein, and Fibronectin by CNF1, ΔCNF1, or Tissue Transglutaminase—RhoA, actin, casein, and fibronectin were incubated with glutathione *S*-transferase-CNF1, ΔCNF1, or guinea pig liver tissue transglutaminase in the presence of monodansylcadaverine, ethylenediamine, or putrescine (50 mM), respectively, in CNF1 reaction buffer (20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EDTA) or transglutamination buffer (20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 8 mM $CaCl_2$, 1 mM dithiothreitol, and 1 mM EDTA) for up to 3 h at 37 °C. As a control, RhoA was incubated without the toxin, but in the presence of substrate. The molar ratio of CNF1 to RhoA was 1:2; 0.01 unit of tissue transglutaminase was used for transglutamination of 5–10 μg of RhoA, casein, actin, and fibronectin, respectively. Labeling of proteins with the fluorescent lysine analog dansylcadaverine was analyzed by fluorescence activity under UV light before staining and drying the gel.

GTPase Assay—Recombinant Rho proteins that were modified by ΔCNF1 or transglutaminase in the presence or absence of small amines 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 of 12 mM) and unlabeled GTP (final concentration of 2 mM) were added. For GAP stimulation, 50 nM $p50^{GAP}$ was added to 1 μM Rho and incubated at 37 °C for 4 min. GTPase activity was analyzed by filter binding assay.

Treatment of ΔCNF1 with N-Ethylmaleimide or Iodoacetamide—ΔCNF1 (amino acids 709–1014) was incubated with iodoacetamide (1 mM) in 50 mM Tris-HCl (pH 7.5) for 30 min at room temperature. Iodoacetamide was then inactivated by adding dithiothreitol in a molar ratio of 10:1 for 10 min. For modification of RhoA, the GTPase was incubated with iodoacetamide-treated or untreated toxin in CNF1 reaction buffer for 1 h at 37 °C (molar ratio of RhoA to ΔCNF1 of 16:1).

ADP-ribosylation—For ADP-ribosylation, modified RhoA proteins (30 μg/ml) were incubated with *C. botulinum* exoenzyme C3 (hereafter referred to as C3; 0.5 μg/ml) and 1 μM [^{32}P]NAD^+ (0.1 μCi/tube) in 50 μl of ribosylation buffer (25 mM triethanolamine hydrochloride (pH 7.5), 2 mM $MgCl_2$, and 1 mM dithiothreitol) for 15 min at 37 °C. The reaction was terminated by adding 10 μl of stop reagent (10% SDS and 10 mM dithiothreitol) and incubation for 5 min at 95 °C. After cooling down, *N*-ethylmaleimide was added to a final concentration of 4 mM; the mixture was incubated at room temperature for 15 min; and proteins were analyzed by SDS-PAGE and subsequent phosphoimaging (Molecular Dynamics, Inc.).

Stability of Wild-type ΔCNF1, C866S ΔCNF1, and H881Q ΔCNF1 to Trypsin—To compare the stability of wild-type ΔCNF1 (amino acids 709–1014) and the mutants C866S ΔCNF1 and H881Q ΔCNF1, we digested 4 μg of purified protein with 40 ng of trypsin (Boehringer Mannheim) in 50 mM Tris-HCl (pH 7.5) at 37 °C for 10–60 min. Thereafter, the digest was analyzed by SDS-PAGE.

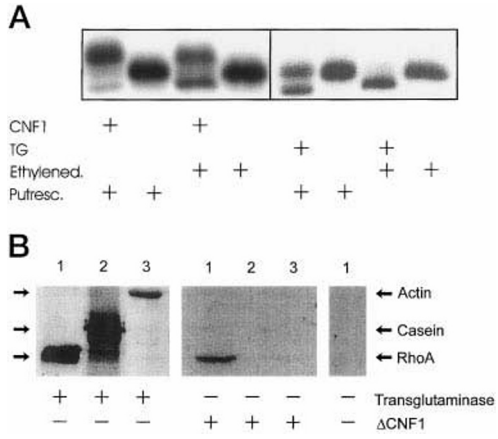

FIG. 1. **Transglutamination of RhoA by CNF1 and tissue transglutaminase.** *A*, SDS-PAGE analysis of modified RhoA. After treatment of RhoA with ΔCNF1 or guinea pig liver transglutaminase (*TG*) in the presence of putrescine (*Putresc.*) or ethylenediamine (*Ethylened.*) (50 mM) for 3 h at 37 °C, the GTPase was [^{32}P]ADP-ribosylated by *C. botulinum* exoenzyme C3 and, thereafter, analyzed by SDS-PAGE and subsequent phosphoimaging (shown). Upward shifts indicate deamidation of RhoA; downward shifts indicate transglutamination. The molar ratio of CNF1 to RhoA was 1:2, and 0.01 unit of tissue transglutaminase was used for transglutamination of 10 μg of RhoA. As a control, RhoA was incubated without transglutaminases, but in the presence of substrates. *B*, transglutamination by ΔCNF1 and tissue transglutaminase with dansylcadaverine as a substrate. RhoA (*lanes 1*), casein (*lanes 2*), and actin (*lanes 3*) (5 μg each) were treated with ΔCNF1 (2 μg) or tissue transglutaminase (0.01 unit) for 2 h at 37 °C with the fluorescent lysine analog dansylcadaverine; the labeled proteins were analyzed by SDS-PAGE; and before staining and drying the gel, the UV-activated fluorescence was detected. Fluorescence intensities of ΔCNF1- and transglutaminase-treated samples were amplified 50- and 20-fold, respectively.

Proteolytic Digestion for Mass Spectrometric Analysis—The proteins, previously precipitated by the chloroform/methanol method, were dissolved in 20 μl of 50 mM Tris-HCl (pH 7.8). Thereafter, 2.5 μl of acetonitrile and 0.5 μg of modified trypsin (sequencing grade, Boehringer Mannheim) dissolved in 2.5 μl of 1 mM HCl were added, and digestion was carried out for 12 h at 37 °C.

Proteolytic Digestion in the Gel Matrix—The excised gel plugs of RhoA were destained for 1 h in 40% acetonitrile and 60% hydrogen carbonate (50 mM; pH 7.8) to remove Coomassie Blue, gel buffer, SDS, and salts. The plug was subsequently dried in a vacuum centrifuge for 15 min. Thereafter, 30 μl of digestion buffer with trypsin was added, and digestion was carried out for 12 h at 37 °C. The proteolytic peptide mixture was extracted into 100 μl of 60% acetonitrile overnight at room temperature. Finally, the gel plug was removed, and the peptide solution was dried for subsequent MALDI-TOF-MS analysis.

Sample Preparation for MALDI-TOF-MS—4-Hydroxy-α-cyanocinnamic acid (Aldrich) was recrystallized from hot methanol and stored in the dark. A saturated matrix solution of 4-hydroxy-α-cyanocinnamic acid in a 1:1 solution of acetonitrile and aqueous 0.1% trifluoroacetic acid was prepared fresh each day. Four μl of the proteolytic peptide mixture was mixed with 1 μl of 10% trifluoroacetic acid, 2 μl of acetonitrile, 8 μl of saturated matrix, and 2 μl of marker peptides (5 pmol of human ACTH-(18–39) (CLIP; M_r 2466; Sigma) and human angiotensin II (M_r 1047; Sigma), respectively) for internal calibration. 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 for several minutes at room temperature, resulting in a thin layer of fine granular matrix crystals.

Mass Spectrometry—MALDI-TOF-MS was performed on a Bruker Biflex mass spectrometer equipped with a nitrogen laser (length = 337 nm) to desorb and ionize the samples. Mass spectra were recorded in the reflector positive mode in combination with DE. External calibration was routinely used, and internal calibration with two points that bracketed the mass range of interest was additionally performed to consolidate peptide masses further. The computer program MS-digest (Peter Baker and Karl Clauser, University of California San Francisco Mass Spectrometry Facility) was used for computer-assisted comparison of the tryptic peptide mapping data with the expected set of peptides.

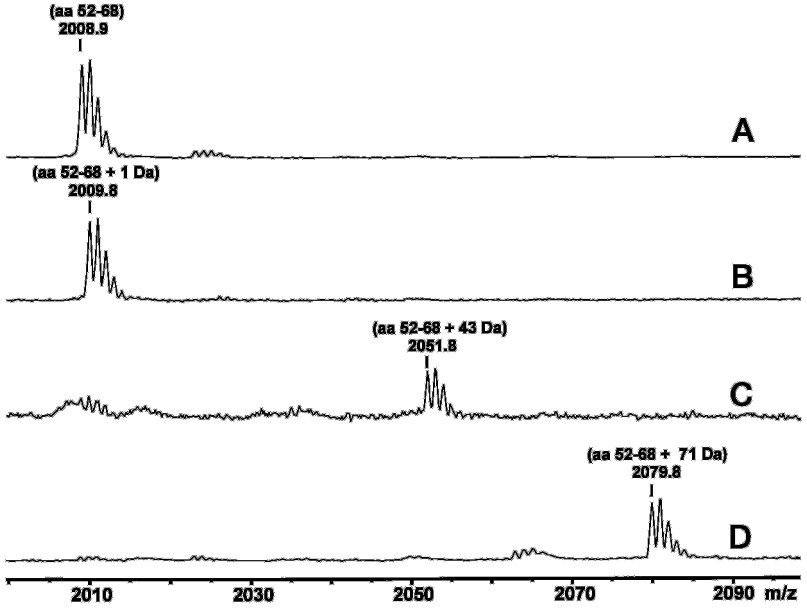

FIG. 2. **MALDI-TOF-MS spectra of in gel digestion of modified RhoA.** Gel plugs of unmodified RhoA (*A*) and RhoA modified in the absence (*B*) or presence of ethylenediamine (*C*) or putrescine (*D*) were excised and destained for 1 h in 40% acetonitrile and 60% hydrogen carbonate (50 mM, pH 7.8). The plugs were subsequently dried in a vacuum centrifuge for 15 min. Thereafter, trypsin digestion was carried out for 12 h at 37 °C. The proteolytic peptide mixture was extracted into 100 μl of 60% acetonitrile overnight at room temperature. Finally, the gel plugs were removed, and the peptide solution was dried for subsequent MALDI-TOF-MS analysis. *A*, the RhoA peptide Gln^{52}–Arg^{68} (2008.9 Da) is shown. *B*, deamidation of RhoA by CNF1 at position 63 results in a mass increase of peptide Gln^{62}-Arg^{68} of 1 Da. *C*, transglutamination of RhoA by CNF1 in the presence of putrescine increases the mass of the peptide by 43 Da. *D*, transglutamination by CNF1 in the presence of ethylenediamine increases the mass of the peptide by 71 Da. *aa*, amino acids.

RESULTS

Transglutamination of RhoA by CNF1—We tested CNF1 for transglutaminase activity and compared the toxin with mammalian guinea pig liver transglutaminase. To this end, Rho was incubated in the presence of CNF1 with ethylenediamine and putrescine, respectively, for up to 3 h. Thereafter, Rho was [^{32}P]ADP-ribosylated by C3 and analyzed by SDS-PAGE. In the presence of ethylenediamine or putrescine, CNF1 treatment resulted in an upward shift of the Rho protein band on the SDS-polyacrylamide gel that was also observed with CNF1 in the absence of the alkylamines, indicating the deamidation of Rho. In addition, treatment of RhoA with CNF1 and alkylamines partially resulted in a downward shift of RhoA, suggesting a different type of modification of the GTPase (Fig. 1*A*). To identify this type of modification induced by CNF1, we applied mass spectrometry to gel plugs of modified RhoA (Fig. 2). As reported recently (13), mass spectrometric analysis of the proteolytic Rho peptide Gln^{52}–Arg^{68} (QVELALWDTAGQEDYDR) identified the 1-Da shift caused by CNF1-induced deamidation of Gln^{63} (Fig. 2*B*). In addition, mass shifts of this peptide of 43 Da (Fig. 2*C*) and 71 Da (Fig. 2*D*) were detected after treatment of RhoA with CNF1 in the presence of putrescine and ethylenediamine, respectively, indicating a transglutamination reaction. No change in mass of RhoA or RhoA peptides was detected in the absence of CNF1. Also, dansylcadaverine served as a cosubstrate for transglutamination of RhoA catalyzed by CNF1. The incorporation of this fluorescence label into RhoA was detected by UV analysis of the SDS-polyacrylamide gel (Fig. 1*B*) and also by mass spectrometric analysis showing an increase in mass of 318 Da (data not shown). By contrast, no labeling by CNF1 was observed with typical substrates of transglutaminases like casein and actin (Fig. 1*B*) or with fibronectin (data not shown). To further test the specificity of RhoA transglutamination, we treated the Q63E RhoA mutant with CNF1 in the presence of putrescine. This RhoA mutant was not modified by CNF1 (data not shown). Changes in migration of deamidated and transglutaminated Rho on the SDS-polyacrylamide gel allowed us to compare the kinetics of the deamidation and transglutamination reactions. The rate constant of the deamidation reaction was 0.32 ± 0.07/min. The specific activity of CNF1 for the transglutamination with ethylenediamine as a cosubstrate was estimated to be decreased by ~70% as compared with the deamidation reaction.

Modification of RhoA by Tissue Transglutaminase—Because our studies indicated that CNF1 possesses transglutaminase activity, we tested whether guinea pig liver transglutaminase is also capable of modifying RhoA. Fig. 1*A* shows that in the presence of ethylenediamine or putrescine, the transglutaminase induced a downward shift of the RhoA band. The mass spectra of the trypsin digest of RhoA previously treated with guinea pig liver transglutaminase in the presence of putrescine are given in Fig. 3. The transglutaminase induced mass shifts of peptide Gln^{52}–Arg^{68} of 71 and 142 Da (Fig. 3*A*), respectively, indicating the modification of both glutamine residues (Gln^{52} and Gln^{63}) of the peptide by transglutamination. Moreover, we identified a shift of 71 Da in peptides 134–145 and 136–145, indicating the modification also of Gln^{136} of RhoA (Fig. 3*B*). When the Q63E mutant of Rho served as a protein substrate for liver transglutaminase, only the modification of two Gln residues (Gln^{52} and Gln^{136}) was observed (data not shown). Thus, the data suggest that the transglutaminase modifies three of the five glutamine residues present in RhoA.

Structure-Function Analysis of CNF1—To get more insights into structure-function analysis of CNF1, we deleted the N terminus of the enzyme and constructed a CNF1 fragment (ΔCNF1) of amino acids 709–1014 of the holotoxin. This C-terminal fragment of CNF1 possessed full deamidation (Fig. 4*A*) and transglutamination (data not shown) activities. Moreover, microinjection of ΔCNF1 into HeLa cells induced the typical cytotoxic effects observed after treatment of cells with the holotoxin (Fig. 4*B*). It has been proposed that a functionally essential cysteine residue is located in the active site of various transglutaminases. Therefore, we treated ΔCNF1 with iodoac-

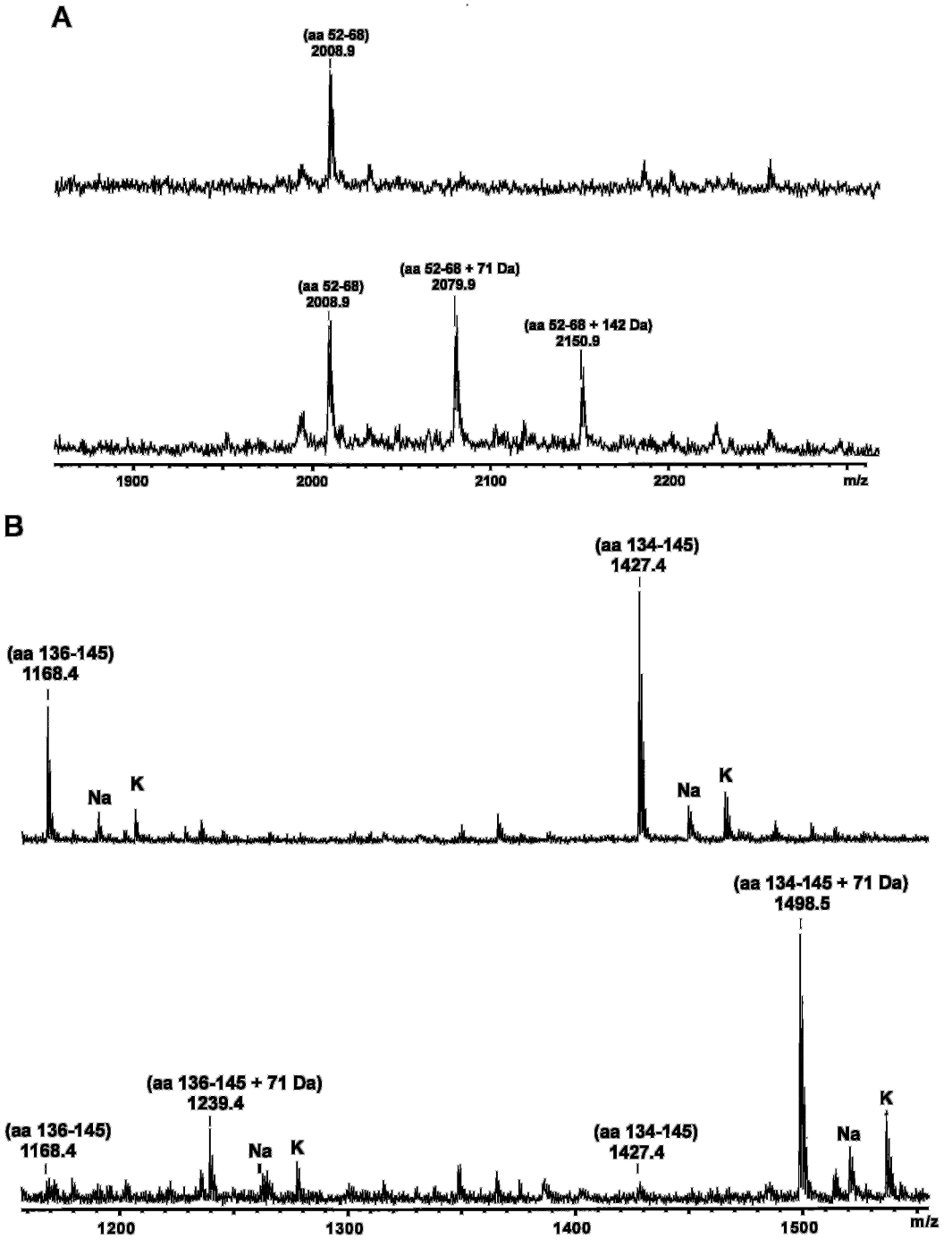

FIG. 3. **MALDI-TOF-MS of RhoA treated with tissue transglutaminase.** RhoA was treated in the presence of putrescine with (*lower trace*) or without (*upper trace*) guinea pig liver transglutaminase. Treated RhoA was precipitated and trypsin-digested for 12 h at 37 °C. Thereafter, the proteolytic peptide mixture was analyzed by MALDI-TOF-MS as described under "Experimental Procedures." The mass spectrogram shows the mass range of 1900–2200 (*A*) and 1150–1550 (*B*), respectively. *A*, the mass of 2008.9 Da gives the RhoA peptide Gln^{52}–Arg^{68} (amino acids (*aa*) 52–68; *upper trace*). After treatment of RhoA with guinea pig liver transglutaminase in the presence of putrescine, the increases in mass by ~71 and 142 Da indicate transglutamination of one and two Gln residues of peptide Gln^{52}–Arg^{68}, respectively (*lower trace*). *B*, the masses of 1168.4 and 1427.4 Da indicate the RhoA peptides consisting of residues 136–145 and 134–145, respectively (*upper trace*). Sodium (*Na*) and potassium (*K*) adducts are indicated. After treatment of RhoA with guinea pig liver transglutaminase in the presence of putrescine, the increase in mass by 71 Da indicates transglutamination of Gln^{136} (*lower trace*).

etamide (1 mM) for 30 min. Thereafter, the unreacted SH reagent was inactivated by adding dithiothreitol. As shown in Fig. 5, iodoacetamide treatment blocked the ΔCNF1-induced changes in RhoA migration on the SDS-polyacrylamide gel and prevented the inhibition of GTPase activity. To corroborate the essential role of cysteine in enzyme activity, we exchanged the single cysteine residue (Cys^{866}) present in the active CNF1 fragment with serine. Whereas wild-type ΔCNF1 induced the typical upward shift of RhoA on the SDS-polyacrylamide gel, no change in migration of RhoA was observed with the C866S mutant of the CNF1 fragment (data not shown). Accordingly, treatment of Rho with the ΔCNF1 mutant did not result in any inhibitory effects on the intrinsic and GAP-stimulated GTPase activity of RhoA (Fig. 4*A*). Moreover, the C866S mutant of ΔCNF1 was not able to induce the transglutamination of RhoA in the presence of alkylamines (data not shown). Finally, mi-

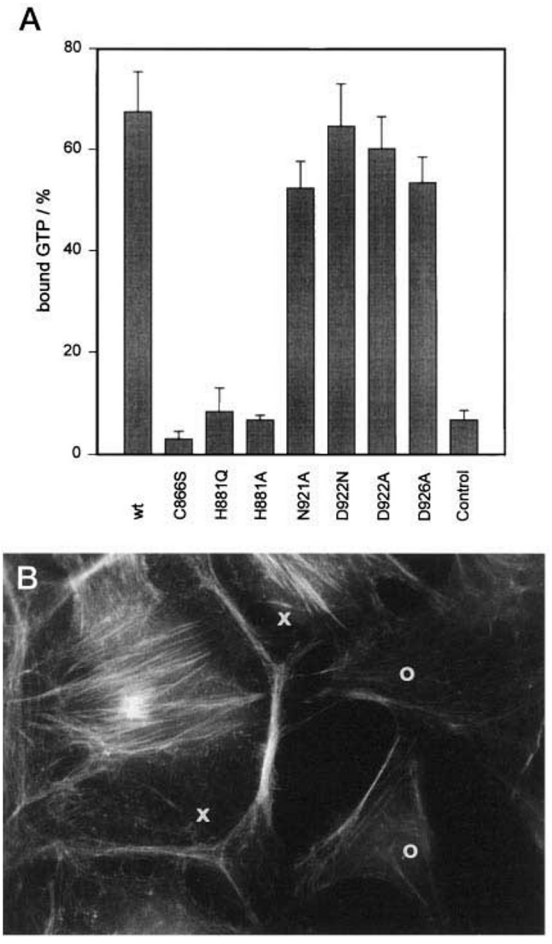

FIG. 4. **Effects of ΔCNF1 and ΔCNF1 mutants on RhoA.** RhoA was treated with wild-type ΔCNF1 (*wt*) or ΔCNF1 mutants (C866S, H881Q, H881A, N921A, D922N, D922A, and D926A) at a molar ratio 2:1 for 3 h. As a control, untreated RhoA (*control*) was used. *A*, influence of ΔCNF1 and ΔCNF1 mutants on $p50^{GAP}$-stimulated GTPase activity. CNF1-treated RhoA was loaded with [γ-^{32}P]GTP. Thereafter, the GTPase activity was stimulated by adding $p50^{GAP}$, and the hydrolysis of GTP was determined after 4 min by the filter binding assay. Shown is the remaining bound radioactivity as a percent of radioactivity bound at the start of the reaction. *B*, induction of stress fibers by microinjection of ΔCNF1 and C866S ΔCNF1 into HeLa cells. ΔCNF1 (×; 2 mg/ml), but not C866S ΔCNF1 (◎; 2 mg/ml), induced formation of stress fibers after microinjection into HeLa cells. Six h after microinjection, cells were fixed and stained for F-actin by rhodamine-conjugated phalloidin. The fluorescence micrograph is shown.

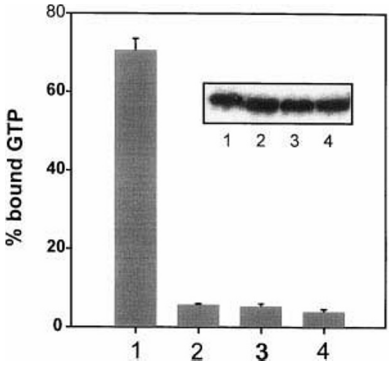

FIG. 5. **Influence of iodoacetamide on CNF1 activity.** ΔCNF1 (6 μM) was incubated with 1 mM iodoacetamide or without inhibitor for 30 min at room temperature. Unreacted iodoacetamide was inactivated by adding dithiothreitol in a molar ratio of 1:10. Thereafter, RhoA was treated with control ΔCNF1 (*bar 1*) or iodoacetamide-modified ΔCNF1 (*bar 2*) in a molar ratio of 16:1 or without CNF1 but in the presence (*bar 3*) or absence (*bar 4*) of the iodoacetamide/dithiothreitol mixture for 1 h at 37 °C. To determine GTPase activity, RhoA treated as described above was loaded with [γ-^{32}P]GTP. After addition of $p50^{GAP}$ (40 nM), GTP hydrolysis by RhoA (800 nM) was determined by the filter binding assay. Radioactivity remaining on the GTPase is given as a percent of the maximum determined at the start of the reaction. *Inset*, treated RhoA proteins were ADP-ribosylated by *C. botulinum* exoenzyme C3 and analyzed by SDS-PAGE and phosphoimaging (shown).

croinjection of the C866S mutant of ΔCNF1 into NIH cells did not cause the typical morphological effects observed with wild-type ΔCNF1 (Fig. 4*B*). Because exchange of cysteine did not change the susceptibility of ΔCNF1 to proteases (data not shown), we assume that loss of activity is not caused by gross changes in the overall structure of the CNF1 mutant and suggest that this cysteine residue is essential for deamidation and transglutamination activities and also for the cytotoxic effects of CNF1. Because the active site of transglutaminases is suggested to be formed by a catalytic triad of cysteine, histidine, and aspartic acid, we changed histidine 881, aspartic acid 922, and aspartic acid 926 to alanine, asparagine, or glutamic acid and studied the activity of the mutant proteins. Whereas the D922A, D922N. and D926A mutants of ΔCNF1 were fully active, the deamidation and transglutaminase activities of the H881A and H881Q mutants were blocked (Fig. 4, transglutamination not shown). Moreover, the H881A mutant did not cause any cytotoxic effects after microinjection (data not shown). In cysteine proteases, an asparagine (but not an aspartic acid) residue participates in catalysis. Therefore, we changed asparagine 921, which is also conserved in DNT, to alanine. However, this mutant was fully active in enzyme activity and induced cytotoxic effects after microinjection.

DISCUSSION

CNF1 was shown to induce the deamidation of Gln^{63} of RhoA (13, 14), thereby blocking its intrinsic and GAP-stimulated GTPase activity and rendering the GTPase persistently active. Here, we report that CNF1 also possesses transglutaminase activity. In the presence of ethylenediamine, putrescine, or dansylcadaverine, CNF1 induced the incorporation of the alkylamines into RhoA specifically at position 63 that is also deamidated by the toxin. The transglutamination by CNF1 is specific for Rho because the typical substrates of eukaryotic transglutaminases like actin, casein, or fibronectin were not modified. So far, the physiological role of transglutamination is not clear, and rather high concentrations of these alkylamines were necessary. With the substrates used (putrescine and ethylenediamine), the deamidation activity was clearly higher than the transglutaminase activity. However, it is feasible that an unknown physiological alkylamine is a much better cosubstrate for modification than the compounds tested in this study.

The finding that Rho is transglutaminated by CNF1 prompted us to study whether the GTPase is also a substrate for eukaryotic transglutaminases. In fact, we observed that guinea pig transglutaminase is capable of modifying RhoA by transglutamination. However, this enzyme is much less specific than CNF1 and modifies three (Gln^{52}, Gln^{63}, and Gln^{136}) of the five glutamine residues of RhoA. As deduced from the recent crystal analyses of RhoA (20) and Ras (21), the glutamine residues that are transglutaminated are localized at the surface of the protein, whereas the glutamine residues that are not modified are located inside the GTPase and are most likely not accessible for modification.

Recently, it has been reported that like CNF1, also DNT, which is produced by various *Bordetella* species (22, 23), deamidates RhoA at glutamine 63 to activate the GTPase (16). Moreover, it was reported that after DNT treatment, RhoA shifted not only to an apparently higher molecular mass on SDS-PAGE, but also downward to an apparently lower mass.

With respect to our results that the incorporation of amine residues into RhoA causes a downward shift of the GTPase, we speculate that also DNT possesses transglutaminase activity to modify Rho. Thus, it remains to be clarified whether CNF1 and DNT belong to a novel family of toxins possessing deamidation and transglutamination activities.

To obtain more insight into the structure-function relationship of CNF1, we studied the enzyme activity of a deletion construct that consisted of amino acids 709–1014 of the holotoxin. A similar CNF1 fragment (amino acids 720–1007) was recently described by Lemichez *et al.* (24). The CNF1 fragment (ΔCNF1, amino acids 709–1014) possessed deamidation and transglutamination activities and was capable of inducing the typical morphological effects after microinjection into fibroblasts. In contrast, addition of ΔCNF1 to cell culture medium did not induce any cytotoxic effects, consistent with the view that the N terminus of CNF1 is involved in receptor binding and/or translocation of the toxin into the cytosol (24).

The transamidation reaction by transglutaminases is thought to be similar to the reverse hydrolysis reaction of proteinases (18, 25). This notion is supported by site-directed mutagenesis and crystallographic data indicating that transglutaminases possess a protease-like catalytic triad consisting of cysteine, histidine, and aspartic acid (18, 26). The inhibition of the active CNF1 fragment by iodoacetamide suggests an essential role of cysteine in the enzyme activity also in this toxin. Consistently, the change of the single cysteine residue (Cys^{866}) present in the active CNF1 fragment to serine blocked deamidation and transglutamination activities. In addition, we identified histidine 881 of CNF1 as essential for catalysis of deamidation and transglutamination reactions. Exchange of this residue with alanine completely inhibited the enzyme activities. More important, mutation of each of these amino acid residues (Cys^{866} or His^{881}) blocked the cytotoxic effects of CNF1 after microinjection. Both amino acid residues (Cys^{866} and His^{881}) are also conserved in DNT (22, 23). Recently, Lemichez *et al.* (24) described four C-terminal regions that are highly homologous in CNF1 and DNT. Most conserved is region III of ~34 amino acid residues positioned between residues 855 and 888 (CNF1), covering the pivotal cysteine 866 and histidine 881 residues. These authors suggested that this area (amino acids 855–888) forms one large β-strand. This hypothesis does not support a direct catalytic interaction of cysteine 866 and histidine 881. However, using the secondary structure prediction described by Fischer and Eisenberg (program UCLA-DOE fold recognition) (27), we found that the structure of this area cannot be described as a continuous β-strand. Thus, the data available do not exclude the direct involvement of cysteine 866 and histidine 881 in catalysis.

Besides cysteine and histidine, aspartic acid is the third amino acid residue of the catalytic triad of transglutaminases (with asparagine in the related cysteine proteases (28)). Because asparagine 921 and aspartic acid 922 are also conserved in DNT, we exchanged these amino acid residues with alanine. However, the N921A and D922A mutants of CNF1 were fully active, indicating no essential role of asparagine 921 or aspartic acid 922 in catalysis. In this context, it is notable that the third amino acid residue of the catalytic triad appears to be only indirectly involved in catalysis of transglutaminase reactions (*e.g.* exchange of the "catalytic" aspartic acid residue in the transglutaminase coagulation factor XIIIa causes only partial inhibition of enzyme activity (29)) and is largely variable at least in various cysteine/histidine proteases (30). Taken together, our studies show that CNF1 possesses, in addition to deamidation activity, transglutaminase activity and indicate that cysteine 866 and histidine 881 of CNF1 play pivotal roles in these activities. Thus, the toxin appears to belong to the superfamily of hydrolases (*e.g.* cysteine proteases, transglutaminases) whose active site is composed of a cysteine and a histidine residue as part of a catalytic dyad. Future structural analysis will show whether CNF1 (probably together with DNT) forms a unique family or is directly related to any of these "cysteine/histidine" hydrolases.

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