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 Bouquet, 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 92 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 Clostridium perfringens (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 modifies Rho 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-carboxamidated form of glutamine residues for primary amines (e.g. peptide-bound lysine residues or polyamines) to form ε-γ-glutamyl)lysine or (ε-glutamyl)polymamine 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—[32P]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 BamHI and Smal and purified by affinity chromatography with glutathione-Sepharose (Amersham Pharmacia Biotech). Loaded beads were washed twice in washing buffer A (20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM MgCl₂, and 1% Triton) and loaded beads were washed twice in washing buffer A (20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM MgCl₂ 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 CaCl₂) for 48 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 (QuickChange™, Stratagene) with the following sense primers and corresponding antisense primers (MWG): C866S, C866A, C881A, C881S, C866>C881A and C866>C881S.
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5′-GGAATTCATGATGATGACAATGGT-3′ (sense) and 5′-CAAT-TGTGCTGACTCACTGTTGATTC-3′ (antisense); H881Q 5′-GGA-TATTATGACACTAGTGCAGCTAC-3′ (antisense) and 5′-GTGTGAC- CAGTGTCATCCATTATAATATCC-3′ (antisense); H881A 5′-GGA- TATATGACACTAGTGCAGCTAC-3′ (antisense). The mutant 3′-CGACTAAAGAACCTGCTTACATCC-3′ (antisense) and 5′-GGGAT- AATGAGCCGCGTATTCTGTCG-3′ (sense), and 5′-CGACTAAAGAAA- TTCGTCCTTACATCC-3′ (antisense); D922N, 5′-GGGATATGATGAGCGTCTTTGTCG-3′ (antisense) and 5′-CCGACGATATGCCTACTCC-3′ (sense). 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 subconfluent on glass coverslips (CELLlocate, Eppendorf) and cultivated for 24 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in humidified CO2 at 37 °C. ΔCNF1 (2 mg/ml) and C866S ΔCNF1 (2 mg/ml) in injection buffer (140 mM KCl, 1 mM MgCl2, 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, washed 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/coverglass) at room temperature for 1 h, washed, and again for fluorescence microscopy (as bleaching preservative Kaiser's glycerol gelatin (Merck) was used). C866S ΔCNF1 or ΔCNF1, or Tissue Transglutaminase—RhoA, 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 MgCl2, 1 mM dithiothreitol, and 1 mM EDTA) for up to 3 h at 37 °C. 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 transglutammination of 5–10 μg of RhoA, casein, actin, and fibronectin, respectively. Labeling of proteins with the fluorescein analog dansylcadaverine was analyzed for fluorescence activity under UV light before staining and drying the gel.

GTase Assay—Recombinant Rho proteins that were modified by ΔCNF1 or transglutaminase in the presence of small amines were separated by SDS-PAGE and subsequently stained with Coomassie Blue. After staining, the gel was cut into 1 mm cubes and dissolved in 25 mM triethanolamine hydrochloride (pH 7.5), 2 mM MgCl2, and 2 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).

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 GTase was [32P]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 transglutammination of 10 μg of RhoA. As a control, RhoA was incubated without transglutaminases, but in the presence of substrates. B, transglutammination 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., 2466; Sigma) and human angiotensin II (M., 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 was used at 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.
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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 [32P]ADP-ribosylated by C3 and analyzed by SDS-PAGE. In the presence of ethylenediamine or putrescine, respectively, for up to 3 h. Thereafter, Rho was transglutaminated activity and compared the toxin with mammalian guinea pig liver transglutaminase. Therefore, we treated CNF1 with iodoacetic acid and the peptide solution was dried for subsequent MALDI-TOF-MS analysis. A, the Rho peptide Gln52–Arg68 (2008.9 Da) is shown. B, deamidation of RhoA by CNF1 at position 63 results in a mass increase of peptide Gln62–Arg68 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.

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. 1A 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 Gln52–Arg68 of 71 and 142 Da (Fig. 3A), respectively, indicating the modification of both glutamine residues (Gln52 and Gln136) 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 Gln136 of RhoA (Fig. 3B). When the Q63E mutant of Rho served as a protein substrate for liver transglutaminase, only the modification of two Gln residues (Gln52 and Gln136) 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. 4A) and transglutaminase (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. 4B). 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-
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 *Deamidation and Transglutamination by CNF1* 

**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 Gln52–Arg68 (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 Gln52–Arg68, 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 Gln136 (lower trace).

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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 (Cys866) 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. 4A). 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-
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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 danylcydaverine, 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^{136}, and Gln^{138}) 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 proteins were ADP-ribosylated by C. botulinum exoenzyme C3 and analyzed by SDS-PAGE and phosphoimaging (shown).

**DISCUSSION**

Microinjection of the C866S mutant of CNF1 into NIH cells did not cause the typical morphological effects observed with wild-type ΔCNF1 (Fig. 4B). 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.

![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 p50GAP-stimulated GTPase activity. CNF1-treated RhoA was loaded with \( ^{32} \)P-GTP. Thereafter, the GTPase activity was stimulated by adding p50GAP, 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 \( (\times; 2 \text{ mg/ml}) \), but not C866S ΔCNF1 \( (\times; 2 \text{ mg/ml}) \), 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.](image-url)

**Fig. 5. Influence of iodoacetamide on CNF1 activity.** ΔCNF1 (6 \( \mu \text{M} \)) 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 \( ^{32} \)P-GTP. After addition of p50GAP (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).
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 (Cys866) present in the active CNF1 fragment to serine completely inhibited the 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, transglaminases) 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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