Identification of the C-terminal Part of Bordetella Dermonecrotic Toxin as a Transglutaminase for Rho GTPases*

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Bordetella dermonecrotic toxin (DNT) causes the deamidation of glutamine 63 of Rho. Here we identified the region of DNT harboring the enzyme activity and compared the toxin with the cytotoxic necrotizing factor 1, which also deamidates Rho. The DNT fragment (∆DNT) covering amino acid residues 1136–1451 caused deamidation of RhoA at glutamine 63 as determined by mass spectrometric analysis and by the release of ammonia. In the presence of dansylcadaverine or ethylenediamine, ∆DNT caused transglutamination of Rho. Deamidase and transglutaminase activities were blocked in the mutant proteins Cys1292→Ala, His1307→Ala, and Lys1310→Ala of ∆DNT. Deamidation and transglutamination induced by ∆DNT blocked intrinsic and Rho-GTPase-activating protein-stimulated GTPase activity of RhoA. ∆DNT deamidated and transglutamnated Rac and Cdc42 in the absence and presence of ethylenediamine, respectively. Modification of Rho proteins by ∆DNT was nucleotide-dependent and did not occur with GTP-S-loaded GTPases. In contrast to cytotoxic necrotizing factor, which caused the same kinetics of ammonia release in the absence and presence of ethylenediamine, ammonia release by ∆DNT was largely increased in the presence of ethylenediamine, indicating that ∆DNT acts primarily as a transglutaminase.

Rho GTPases including Rho, Rac, and Cdc42 isofoms are regulators of the actin cytoskeleton and act as molecular switches in a large array of signaling processes (1, 2). The GTPases are the eukaryotic substrates for various bacterial protein toxins (3, 4). C31-like exoenzymes (e.g. Clostridium botulinum exoenzyme C3) ADP ribosylate RhoA, B, and C at asparagine 41 thereby inhibiting the biological functions of the GTPases (5–7). Large clostridial toxins (e.g. Clostridium difficile toxins A and B) inhibit Rho, Rac, and Cdc42 GTPases by monoglucosylation at threonine 37 and threonine 35, respectively (8, 9). Rho family GTPases are also the targets for the Bordetella dermonecrotic toxin (DNT), which is produced by Bordetella strains (10, 11). DNT induces stress fiber formation, focal adhesion assembly, and tyrosine phosphorylation of focal adhesion kinase and paxillin (10, 12, 13). Recent studies indicate that DNT causes deamidation of glutamine 63 of RhoA (10). Glutamine 63 is essential for GTP hydrolysis by Rho. Deamidation of glutamine by DNT inhibits the GTPase activity of Rho and renders the Rho protein constitutively active.

The same mechanism of Rho activation by deamidation was reported for the cytotoxic necrotizing factor CNF1 from Escherichia coli (14, 15). Also CNF deamidates Rho at glutamine 63 and causes similar cytotoxic effects such as multinucleation of cells and stress fiber formation. CNF1 and DNT share a region of homology (amino acid residues 1250–1351 of DNT) located at the C termini of the toxins (16). Other parts of the protein sequences are not significantly similar. Recently, it was shown that a C-terminal fragment of CNF1 (∆CNF), covering the region of homology, causes the typical cytotoxic effects after microinjection and possesses full Rho-deamidating activity in vitro. In addition to deamidase activity, ∆CNF possesses transglutaminase activity. However, this activity is observed only in the presence of high concentrations of primary amines and is apparently lower than the deamidase activity (17).

Here we attempted to identify the region of DNT that harbors the enzyme activity of the toxin and characterized its biological and biochemical activities. We report that ∆DNT covering amino acid residues 1136–1451 possesses full deamidating activity. Cysteine 1292, histidine 1307, and lysine 1310 are essential for enzyme activity. As found for ∆CNF, the active fragment of DNT possesses transglutaminase activity. In contrast to CNF1, ∆DNT exhibits a higher transglutaminase than deamidase activity, indicating that DNT acts preferentially as a transglutaminase. Another difference between ∆CNF and ∆DNT is the nucleotide dependence of the deamidation/transglutamination reaction. Whereas ∆CNF modifies GDP- and GTP-loaded Rho proteins, ∆DNT exclusively accepts GDP-bound RhoA.

EXPERIMENTAL PROCEDURES

Materials—RhoA and p50RhoGAP (obtained from A. Hall, London) were prepared from their fusion proteins as described. Dansylcadaverine and ethylenediamine were purchased from Sigma. Methanol and chloroform were of analytical grade, and trifluoroacetic acid and acetonitrile were of high pressure liquid chromatography grade.

Cloning and Purification of ∆DNT and DNT Mutants—For production of ∆DNT consisting of amino acid residues 1136–1451, the DNA was amplified from the plasmid DNT 103 (16) by polymerase chain reaction with the following primers: ∆DNT sense, 5’-GGATCCGCTTCGCGCCGGGCGCCG-3’; ∆DNT antisense, 5’-GAATTCCTCAGACCGGCGCGGGAACCA-3’.

The PCR product was purified from agarose gel (Jet sorb, Genomed) and amplified in the pcRTM II vector (Invitrogen) by means of TA cloning. From this vector the DNT fragment was cut with BamHI and EcoRI, purified, and ligated into the digested pGEX vector. The proper construct was checked by DNA sequencing. The vector was transformed into BL21 cells by heat shock at 42 °C. Expression of the GST fusion protein in E. coli BL21 cells growing at 37 °C was induced by adding 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) at OD 0.5. 6 h after induction, cells were collected and lysed by sonication in

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lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) 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 washing buffer B (150 mM NaCl, 50 mM Tris-HCl, pH 7.4). ΔDNT was cleaved from the beads as a GST fusion protein with glutathione (10 mM glutathione, 50 mM Tris-HCl, pH 7.5) for 10 min at room temperature.

Mutagenesis of ΔDNT was performed by round circle polymerase chain reaction-based site-directed mutagenesis (Quick changeTM, Stratagene) with the following sense primers and corresponding antisense primers (MWG): C1292S sense, 5’-GGCTCCTTGAGCCTGCACGAGCCGCACGGAGCTTGGG-3’; H1307A sense, 5’-GGCTTACCTGGCC-TTTACGCGACTGGACAC-3’; and K1310A sense, 5’-GGCT-TCTACACACTGGCGCCGACCACTGGG-3’. Mutations were verified by DNA sequencing using a dye terminator sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems).

Activation of FXIIIa—Activation of FXIII occurs through thrombin cleavage of its propeptide at Arg 999 to Arg 1000. The propeptide factor XIII a-chains (Centeno) were incubated with 2 µg/µl thrombin for 30 min at room temperature in reaction buffer containing 150 mM NaCl, 50 mM Tris/EDTA, and 8.5 mM CaCl₂. Thrombin was then removed by incubation with benzamidine-Sepharose for 10 min at room temperature. The activity of FXIII was tested with fibronectin as a substrate.

Measurement of Ammonia—For qualitative measurement of ammonia a coupled enzymatic reaction was used that was based on the ammonia test combination for food analysis (Roche Molecular Biochemicals). NADH was diluted to give a concentration of 50 µM with triethanolamine buffer containing 2-oxoglutarate, 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, and 1 mM EDTA. Ten units of GIDH and RhoA (final concentration 10 µM) were added. After the addition of ΔCNF1 or ΔDNT (each 1 µM), the decrease in NADH fluorescence was monitored in a Perkin-Elmer LS-50B luminescence spectrometer. The emission was measured at 460 nm with excitation at 340 nm.

For quantitative analysis of the ammonia release, Rho proteins (200 µM) were incubated with ΔCNF1 (1 µM) or ΔDNT (1 µM) in a reaction buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 8 mM CaCl₂, 1 mM dithiothreitol, and 1 mM EDTA at 37 °C. The reaction was stopped at different time points by incubation for 1 min at 95 °C. Denatured proteins were removed by centrifugation, and ammonia produced was measured in the supernatant. Decrease in absorbance was measured following the instructions given for the ammonia test combination for food analysis (Roche Molecular Biochemicals).

Microinjection and Actin Staining—For microinjection, NIH3T3 cells were seeded subconfluent on glass coverslips (CELLocate, Eppendorf) and grown to 70% confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in 5% CO₂ at 37 °C. After serum starvation GST-ΔDNT (2 mg/ml) or buffer was microinjected into NIH3T3 cells with a Microjector 5242 (Eppendorf). 6 h after microinjection, cells were fixed with 4% formaldehyde and 0.1% Tween 20 in phosphate-buffered saline at room temperature for 10 min. For actin staining, formaldehyde-fixed cells were intensively washed with phosphate-buffered saline and then incubated with rhodamine-conjugated phalloidine (1 unit/coverslip) at room temperature for 1 h, followed by washing two times in washing buffer A (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl₂) and washing buffer B (150 mM NaCl, 50 mM Tris-HCl, pH 7.4). ΔDNT was eluted from the beads as a GST fusion protein with glutathione (10 mM glutathione, 50 mM Tris-HCl, pH 7.5) for 10 min at room temperature.

Aspartate transglutaminase activity was tested by incubation for 1 min at 95 °C. Denatured RhoA revealed the RhoA peptide Gln 52-Arg 68 exhibiting a mass shift of one dalton in comparison to untreated RhoA.

RESULTS

The C-terminal Fragment DNT 1136–1451 (ΔDNT) Is Sufficient for Deamidase and Transglutaminase Activity—Recently, it was shown that the CNF1 of *E. coli* activates Rho proteins by deamidating glutamine at position 63 of RhoA or 61 of Rac and Cdc42. Moreover, it has been reported that CNF1 possesses transglutaminase activity. CNF1 and DNT share a region of homology (amino acid residues 1250–1351 of DNT) located at their C termini (16). Therefore, we studied whether the C-terminal fragment ΔDNT (amino acid residues 1136–1451 of the holotoxin) is sufficient for the enzyme activity.

To analyze the activity of GST-ΔDNT, we constructed the vector pGEX-ΔDNT, expressed the toxin fragment as a GST fusion protein, and purified it by affinity chromatography. Because the fusion toxin exhibited full activity and was not cleavable without degradation of ΔDNT, we used the fusion toxin (which is termed ΔDNT in the text) throughout the entire study. After incubation of RhoA with ΔDNT for 15 min, the GTase shifted to an apparent higher molecular mass in SDS-PAGE indicating deamidation of RhoA (15) (Fig. 1). In the presence of the transglutaminase cosubstrate ethylenediamine, however, RhoA shifted slightly to an apparent lower molecular mass. Recently, we reported that a downward shift of RhoA in SDS-PAGE corresponding to transglutamination was obtained when the GTase was incubated with ΔCNF and ethylenediaine (17). Therefore, we analyzed tryptic peptides of ΔDNT-treated RhoA by mass spectrometry. As shown in Fig. 2B, the mass analysis of the tryptic digest of the upper band of ΔDNT-treated RhoA revealed the RhoA peptide Glu^52^-Arg^68^ exhibiting a mass shift of one dalton in comparison to untreated RhoA.
(Fig. 2A). Tryptic digest of the downward shifted band of RhoA resulted in identification of the same peptide (Gln62-Arg68) but with a mass shift of 43 Da as compared with the control protein. This increase in mass indicated the transglutamination of Gln63 by ethylenediamine (Fig. 2C). Also, dansylcadaverine, a fluorescent primary amine (18), served as a cosubstrate for the transglutamination of RhoA by ΔCNF1 (note that also ΔCNF1 was used as the GST fusion protein) and ΔDNT. To compare the transglutamination activity of the toxin fragments, RhoA was incubated with the enzymes in the presence of dansylcadaverine, and the amount of GTPase modified was analyzed in SDS-PAGE under UV light. As shown in Fig. 3, RhoA was danylsylated by ΔDNT to a larger extent than by ΔCNF. To compare the transglutaminase activities of both toxins in more detail, kinetic studies were performed.

**ΔDNT Is Preferentially a Transglutaminase**—To compare kinetics of the deamidation and transglutamination reaction of ΔCNF1 and ΔDNT, the time course of ammonia release induced by the toxins was studied. The ammonia release assays were performed with a substrate concentration of 200 μM RhoA and an enzyme (GST-ΔDNT or GST-ΔCNF1) concentration of 1 μM. As shown in Fig. 4A, no difference in the production of ammonia was observed with or without ethylenediamine when RhoA was modified by ΔCNF1. On the contrary, ΔDNT released a higher amount of ammonia in the presence of ethylenediamine than in the absence of the primary amine (Fig. 4B). A similar result was obtained in the presence of increasing concentrations of ethylenediamine. As shown in Fig. 5A, with ΔDNT the production of ammonia increased in an ethylenediamine concentration-dependent manner. In contrast, the addition of ethylenediamine at increasing concentration had no effect on ammonia production by ΔCNF1. Thus, all these data indicate that ΔDNT is preferentially a transglutaminase. Similarly, blood clotting factor FXIII, which is a mammalian transglutaminase (19), released a higher amount of ammonia in the presence of the primary amine than in its absence (not shown).

It is known that the activity of mammalian transglutaminases including FXIII is dependent on calcium ions (19). To test whether Ca2+ ions affect the activity of ΔDNT, we measured the ammonia release induced by the toxin fragment in the absence and presence of EGTA. Ammonia release caused by FXIII was dependent on the presence of Ca2+ ions, whereas the presence of EGTA had no (5 mM) or a very small (10 mM) effect on the activity of ΔDNT (not shown). In the presence of EGTA, both ΔDNT and ΔCNF1 modified RhoA by dansylation (not shown).

**ΔDNT Modifies Gln63 of RhoA and Gln61 of Cdc42 and Rac**—Blood clotting factor FXIII, which modifies various protein substrates such as fibrinogen, actin, and casein, transglutaminates three of the five glutamine residues of RhoA, whereas CNF1 is specific for Gln63 of RhoA and Gln61 of Cdc42 and Rac1 (17). To investigate the specificity of ΔDNT, we incubated RhoA, Rac1, Cdc42, the respective Q63E/Q61E mutants, actin, and casein in the presence of dansylecadaverine with ΔDNT for 30 min at 37 °C. Thereafter, transglutaminated proteins were analyzed by SDS-PAGE and UV light exposure. As shown in Fig. 6, ΔDNT modified the wild-type GTPases RhoA, Cdc42, and Rac1 but not the Q63E/Q61E mutants, actin, or casein. In contrast, FXIII modified wild-type and mutant GTPases, actin, and casein (not shown). In line with the above observations, no ammonia was released during incubation of the Q63E mutant with the toxins (not shown).

**Deamidation Kinetics**—To compare kinetics of the deamidation/transglutamination reactions of RhoA, Rac1, and Cdc42 induced by the toxin fragments, we measured ammonia release in a time course. The reactions were performed with a protein substrate concentration of 200 μM, an enzyme concentration of 1 μM and 20 mM ethylenediamine. In Fig. 7, the time courses of ΔDNT-induced ammonia release of RhoA, Cdc42, and Rac1 are shown as the mean of three independent experiments. All Rho proteins exhibited similar kinetics of ammonia release. Similarly, ΔCNF1 did not show major differences in the kinetics of ammonia release between the three GTPases (not shown).

**Effects of Transglutamination of RhoA—Gln63 of RhoA is known to be important for the intrinsic and GAP-stimulated GTPase mechanism of the protein** (20). To analyze whether transglutaminated protein is still able to hydrolyze GTP, we measured its p50RhoGAP-stimulated GTPase activity. Fig. 5B illustrates the effects of ΔCNF1 and ΔDNT on the GTPase activity of RhoA in the presence of increasing concentrations of ethylenediamine. Similar as observed for the ammonia release (Fig. 5A), inhibition of the GTPase with ΔCNF1 was independent of the ethylenediamine concentration, whereas the blockade of the GTP hydrolysis with ΔDNT increased with increasing concentration of the primary amine. Thus, inhibition of GTPase activity and ammonia release induced by ΔDNT correlated very well, indicating that the transglutamination inhibits GTPase activity of RhoA.

**In Vivo Effects of ΔDNT (Microinjection Experiments)**—It has been shown by Horiguchi et al. (10) that treatment of cells with DNT leads to actin polymerization and stress fiber formation because of activation of RhoA. To investigate whether ΔDNT possesses the same cytotoxic effect in intact cells, we microinjected the toxin fragment as a GST fusion protein into quiescent NIH3T3 cells. The toxin fragment caused formation of stress fibers after 6 h of incubation. However this effect was not as strong as observed with ΔCNF1 (not shown). This may be because of instability of the GST-ΔDNT fusion protein, which significantly decreased in activity after a few days of storage at 4 °C or after incubation for 30 min at 37 °C.

**Structure-Function Analysis of ΔDNT**—Recently, cysteine was identified to be a functionally essential residue in ΔCNF1, which is most likely located in the active site of the enzyme. Like ΔCNF, ΔDNT contains a single cysteine residue in a protein region highly similar to ΔCNF (Fig. 9). According to the findings with CNF, treatment of the toxin fragment with iodoacetamide or N-ethylmaleimide blocked the enzyme activity of ΔDNT (not shown). Exchange of cysteine 1292 with serine or alanine largely decreased or completely inhibited the enzyme activity of ΔDNT, respectively. Moreover the exchange of histidine 1307 with alanine blocked the enzyme activity of ΔDNT in analogy to CNF1 (not shown). A nucleotide binding motif has been described for DNT (not present in CNF) covering residues 1304–1311 (AFYHTGKS) with the consensus (A/G)XXGK(S/T) (16). To study the relevance of this motif for ΔDNT activity, we changed lysine 1310 to alanine. This mutation blocked ΔDNT activity of the toxin fragment as already.
Matrix-assisted laser desorption ionization/time of flight-mass spectrometry spectra of in gel digestion of modified RhoA. Gel plugs of unmodified RhoA (A) and RhoA modified by GST-ΔDNT in the absence (B) or presence of 20 mM ethylenediamine (C) were excised and destained for 1 h in 40% acetonitrile, 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. A, the RhoA peptide Gln63-Arg68 (2009 Da) is shown. B, deamidation of Gln52 of RhoA by GST-ΔDNT results in a mass shift of the peptide of 1 Da. C, transglutamination of Gln52 of RhoA by GST-ΔDNT in the presence of ethylenediamine results in a mass shift of the peptide of 43 Da. aa, amino acid.

DISCUSSION

Recently Horiguchi et al. (10) showed that DNT from Bordetella modifies Rho GTPases by deamidation of Gln63. A similar deamidation of Rho at Gln63 was reported for CNF1 from E. coli (14, 15). DNT and CNF share a significant sequence homology in a rather small part of the proteins, suggesting that the deamidase activity is located in this region of the toxins. Therefore, we constructed ΔDNT, which covered this homologous region (Fig. 9). This fragment consisting of amino acid residues 1136–1451 possessed full deamidase activity and typically caused an upward shift of RhoA in SDS-PAGE. This change in migration in SDS-PAGE was not observed with the Q63E mutant of RhoA, confirming that exclusively Gln63 was deamidated. Thus, the active fragment ΔDNT exhibited the same biochemical properties as reported for the holotoxin DNT. Moreover, similarly as observed for the holotoxin but to a smaller extent microinjection of GST-DNT caused formation of stress fibers in fibroblasts.

Recently, we reported that CNF1 possesses transglutaminase activity and modifies Rho GTPases in the presence of primary amines (17). In the presence of ethylenediamine, transglutamination of RhoA by CNF caused a downward shift of the GTPase in SDS-PAGE. However, this activity of CNF was only observed at high concentrations of the primary amine and occurred slower than deamidation. Similarly as with CNF, we detected a downward shift of RhoA in SDS-PAGE after treatment with ΔDNT in the presence of ethylenediamine. The transglutamination of Rho by ΔDNT was verified by mass spectrometric analysis. To further characterize the enzyme activity of ΔDNT in more detail and to compare it with ΔCNF, we applied an ammonia release assay. Interestingly, we observed that the release of ammonia by ΔDNT was largely dependent on the presence of ethylenediamine. Almost no ammonia was released in the absence of the primary amine. Increasing concentration of ethylenediamine also increased ammonia production. In contrast, CNF-induced ammonia release was hardly changed in the presence and absence of the primary amine. These data suggest that (at least under the conditions used) DNT acts preferentially as a transglutaminase, whereas CNF behaves preferentially as a deamidase. In fact, differences in the activities of CNF and DNT are obvious from studies in intact cells. Treatment of intact cells with CNF causes an upward shift of RhoA in SDS-PAGE indicating a deamidase reaction (15, 21). By contrast, Horiguchi et al. (10) reported that DNT caused a downward shift of RhoA after treatment of cells for 1–3 h. Longer incubation of cells with DNT (e.g., for up to 6 h) resulted in an occurrence of an additional upward shift. These data can be interpreted to indicate that DNT causes preferentially a transglutaminase reaction also in intact cells.
cells. Because we did not succeed in the expression of a recombinant full-length DNT preparation, which was biologically active, we are at present not able to verify this hypothesis.

Because deamidation- or transglutamination-induced changes in migration of GTPases in SDS-PAGE are less pronounced with Rac and Cdc42, we used the ammonia release assay to study the substrate specificity of DDT. These data indicated that all Rho GTPases including Rac and Cdc42 are modified by DDT. We did not detect major differences in the ability of the various Rho proteins to serve as substrate for DDT. A similar substrate specificity was recently reported for CNF1 (17). However, we observed differences between DDT and CNF1 in respect to the nucleotide dependence of the deamidation/transglutamination reactions. Whereas DDT catalyzed the deamidation reaction with a similar velocity in the presence of GDP or GTPγS-loaded RhoA, DDT accepted GDP-loaded RhoA or GDP-loaded V14RhoA but did not modify GTPγS-bound RhoA or V14RhoA that is not able to hydrolyze GTP. The slight modification of GTPγS-loaded RhoA with DDT and the low modification of V14RhoA GDF may be because of an incomplete exchange of the nucleotides. As binding of nucleotides largely changes the conformation of the switch II region of the GTPases, these findings suggest that the structural requirements for modification by DNT are more restricted. Another possibility would be that free nucleotides interact with the enzyme to alter its activity. In fact, a nucleotide binding motif has been described for DNT but not for CNF covering residues 1304–1311 (AFYHTGKS) with the consensus (A/G)XXXXHK(S/T) (11, 22). To study the relevance of this motif for DNT activity, we changed lysine 1310 to alanine. This mutation blocked DDT activity as reported earlier for the holotoxin (11). The role of lysine 1310 is not clear because this residue is not present with similar spacing in CNF1. It is conceivable that the loss in activity of the K1310A mutant is caused by structural changes of the toxin not directly involving catalysis, because K1310 is located in the vicinity of the catalytically important residue His1307. Although a putative nucleotide binding motif is present in DNT, we did not obtain evidence for a control of DNT activity by direct interaction of the enzyme with nucleotides.

All eukaryotic transglutaminases are characterized by a catalytic cysteine and histidine residue. Recently, we identified cysteine 866 in CNF1 as essential for deamidase and transglutaminase activity (17). Suggesting that a similar catalytic mechanism is functional in DNT, we changed cysteine 1292 of...
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Fig. 6. Dansylation of GTPases, actin, and casein with GST-ΔDNT. RhoA, Rac, Cdc42, Q63E/Q61E mutants, actin, and casein (20 μM each) were incubated with GST-ΔDNT (1 μM) in the presence of dansylcadaverine for 15 min at 37 °C and supplied to SDS-PAGE (5 μg of protein/lane). Dansylation of the proteins was analyzed under UV light.

Fig. 7. Kinetics of the modification of Rho, Rac, and Cdc by GST-ΔDNT. RhoA (200 μM, ▲), Rac1 (200 μM, ■), and Cdc42 (200 μM, ●) were incubated with GST-ΔDNT (1 μM) in transglutaminase buffer in the presence of 20 mM ethylenediamine at 37 °C. The reaction was stopped at different time intervals as indicated by heating for 1 min at 95 °C. Denatured proteins were removed by centrifugation, and the ammonia produced was measured in the supernatant. As a control, RhoA (200 μM) was incubated without the toxin fragments (▼). Cdc42 and Rac showed similar results. Shown is the ammonia produced at each time point as mean ± S.D. of three independent experiments.

DNT to serine or alanine. These mutations caused inhibition of the enzyme activity indicating an essential role in catalysis. Thus, as assumed from the amino acid sequence alignment of DNT and CNF1, cysteine 1292 of DNT is functionally equivalent to cysteine 866 of CNF1. In contrast to transglutaminases, like the blood clotting factor FXIII, the activity of ΔDNT or ΔCNF was not dependent on calcium ions. Another Ca2+-independent transglutaminase was recently cloned from *Streptomyces verticillum* (23).

The preferential transglutamination of Rho allowed studies on the GTPase activity of the cross-linked Rho protein. Gln63 of RhoA is essential for the intrinsic and GAP-stimulated GTPase mechanism of the protein. Recent crystal structure analysis of Rho and Rho-GAP in a complex with a transition state analogue GDP-ATPγS explains the function of Gln63 in stabilizing the transition state of GTP hydrolysis. To this end, the nitrogen of the carbonyl group of Gln63 is bonded to the main chain carbonyl of Arg85 of Rho-GAP and to one of the fluorides of AlF4− (20). If Gln63 is deamidated (e.g. by CNF), this interaction with GAP is not possible resulting in the blockade of GAP-stimulated GTP hydrolysis (14, 15). After transglutamination of Gln63, however, the pivotal nitrogen residue is still present. Therefore, we were surprised that after transglutamination both intrinsic and GAP-stimulated GTPase activity of Rho were blocked. The reason for this inhibition is not entirely clear but may be based on structural changes that are the prerequisite for catalysis of GTP hydrolysis. For example, binding of Rho-GAP and subsequent activation of Rho GTPase activity are accompanied by conformational changes to allow the introduction of the catalytic Arg85 of RhoGAP into Rho (20). It is feasible that this interaction is hindered by transglutamination of Gln63. Further studies are underway to analyze the influence of smaller transglutaminase cosubstrates like methylamine on the GAP-stimulated and intrinsic GTPase activity of RhoA after modification with ΔDNT.

In summary, we localized the enzyme domain of DNT to a C-terminal fragment covering amino acid residues 1136–1451 with cysteine 1292, histidine 1307, and lysine 1310 as essential residues. This active fragment acts as a deamidase and/or transglutaminase to modify Gln63 of Rho or Gln61 of Rac and Cdc42, respectively, and to activate the GTPases. Kinetic analysis indicates that ΔDNT acts preferentially as a transglutaminase. In contrast to ΔCNF, which effectively modifies Rho proteins in the GDP- and GTP-bound form, GDP-bound Rho proteins are the preferred substrates of ΔDNT.

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