Change in Substrate Specificity of Cytotoxic Necrotizing Factor Unmasks Proteasome-independent Down-regulation of Constitutively Active RhoA*5

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Cytotoxic necrotizing factors CNF1 and CNF2 are produced by pathogenic Escherichia coli strains. They constitutively activate small GTPases of the Rho family by deamidation of a glutamine, which is crucial for GTP hydrolysis. Recently, a novel CNF (CNFγ) encompassing 65% identity to CNF1 has been identified in Yersinia pseudotuberculosis. In contrast to the E. coli toxins, which activate several isoforms of Rho family GTPases, CNFγ is a strong and selective activator of RhoA in vivo. By constructing chimeras between CNF1 and CNFγ, we show that this substrate specificity is based on differences in the catalytic domains, whereas the receptor binding and translocation domains have no influence. We further define a loop element (L8) on the surface of the catalytic domains as important for substrate recognition. A single amino acid exchange in L8 is sufficient to shift substrate specificity of CNF1. Moreover, it is shown that RhoA activation by CNF1 is transient, which may be the consequence of the broader substrate specificity of the E. coli toxin, leading to cross-talk between the activated GTPases.

The cytotoxic necrotizing factors CNF1 and CNF2 from pathogenic Escherichia coli strains and CNFγ from Yersinia pseudotuberculosis belong to a family of protein toxins that constitutively activate small GTPases of the Rho family. Rho GTPases are key regulators of a wide variety of cellular functions, including regulation of actin structures, endocytosis, secretion, integrin- and phospholipid signaling, control of transcription, cell cycle progression, and cell transformation (for review, see Refs. 1 and 2). Like all members of the Ras superfamily of small GTPases, Rho GTPases cycle between the GDP-bound inactive and GTP-bound active forms. The exchange of GDP for GTP is catalyzed by guanosine nucleotide exchange factors, whereas inactivation of Rho results from hydrolysis of the bound GTP, a process that is stimulated by GTPase-activating proteins. In the cytoplasm Rho is complexed with the guanosine nucleotide dissociation inhibitor (for review, see Ref. 3).

CNF1 and CNF2 catalyze the deamidation of RhoA at Gln63 (Gln61 of Rac1 and Cdc42), thereby inhibiting the intrinsic and GTPase-activating protein-stimulated GTPase activity and, thus, blocking the inactivation step of the GTPase cycle (4, 5). In contrast to the E. coli toxins, Y. pseudotuberculosis CNFγ (6) selectively deamidates and activates RhoA but not Rac or Cdc42 in vivo (7).

All CNFs are identical in size and are composed of an N-terminal receptor binding domain, a central translocation domain, and a C-terminal catalytic domain. The predicted amino acid sequence of CNFγ is about 65% identical to that of CNF1 from E. coli. The amino acid residues essential for catalytic activity in CNF1, Cys-866 and His-881, are conserved in CNFγ with the same spacing between them as in CNF1 and CNF2 (6, 8). The permanent activation of Rho GTPases by the toxins leads to a variety of different cellular responses including rearrangement of cellular actin microfilaments. Activation of RhoA induces the formation of stress fibers, whereas Rac activation leads to membrane ruffling. Active Cdc42 induces the formation of filopodia. Moreover, CNF1-treated cells are characterized by flattening and polynucleation (for review, see Ref. 9). Although deamidation leads to constitutive activation of the modified GTPases, there is at least one regulatory mechanism reducing the overwhelming activity of the GTPases on the cellular level. Recently, we and others showed that the amount of cellular Rac1 decreases after CNF1 treatment. The activated form of Rac1 is ubiquitinated and subsequently degraded by a proteasome-dependent mechanism (10, 11).

CNF1 has been shown to be endocytosed by receptor-mediated endocytosis and released from the late endosomes into the cytosol (12). It is not yet known whether the toxin stays associated with endosomal membranes and whether the holotoxin or only a catalytic fragment translocates into the cytosol (12).

The crystal structure of the catalytic domain of CNF1 has been solved recently (13). It forms a single compact domain consisting of two mixed β-sheets of 6 and 7 β-strands surrounded by 4 α-helices. Flexible loop elements at the entrance to the deep catalytic pocket restrict access of possible substrates to the catalytic amino acids. In this context it has been shown that some of these flexible loop elements are implicated in RhoA recognition and binding because their deletion results in decreased catalysis of RhoA deamidation in vitro (14). In this study we analyze the molecular basis for the difference in sub-

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2 The abbreviations used are: CNF, cytotoxic necrotizing factor; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; aa, amino acids.
strate specificity between the two toxins in the living cell by constructing toxin chimeras and by exchanging single loop elements on the surface of the catalytic domains. Moreover, we show that besides degradation a further regulatory mechanism exists in the living cell that reduces the activity of deamidated RhoA.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Lysates—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (12 mM 1-glutamine) supplemented with 10% fetal calf serum, penicillin (4 mM), and streptomycin (4 mM) in a humidified atmosphere of 5% CO₂ at 37 °C.

For intoxication with CNF1 or CNF₇, cells were treated with 400 ng of GST-CNf/BL21. Cells were harvested by centrifugation, and the resulting pellet was lysed in Rhotekin lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, 0.5% Triton X-100, 0.1 mM PMSF, 1 μg/μl aprotinin, 1 μg/μl leupeptin, 1 mM dithiothreitol). The GST-coupled Rhotekin receptor binding domain or PAK-CRB was purified by affinity chromatography with glutathione-Sepharose. Loaded beads were washed three times with Rhotekin lysis buffer and once with buffer A (10% glycerol, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 2 mM MgCl₂, and 0.5 mM PMSF). Cells were treated with toxin preparations as indicated in the figures. Where indicated, the proteasome inhibitor MG132 (Sigma, 30 μM final concentration) was added together with CNF1.

Cells were lysed in buffer A, and the lysates were cleared by centrifugation. A fraction of the cleared lysates (50 μg of total protein) was analyzed by Western blot to detect total amounts of the respective GTPases. 500–1000 μg of total protein was incubated with protein-loaded beads for 1 h at 4 °C by head-over-head rotation. After incubation, beads were washed once with buffer A. Samples were boiled in Laemmli buffer and separated by SDS-PAGE. RhoA, Rac1, and Cdc42 were analyzed by immunoblotting with their specific antibodies.

Western Blot Analysis—For Western blotting samples were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Rac was detected with a specific antibody (anti-Rac, BD Biosciences). Rho was detected with a monoclonal antibody directed against the insert region (anti-RhoA, Santa Cruz, CA), and for Cdc42 detection a monoclonal antibody (anti-Cdc42, Upstate, Lake Placid, NY) was used. Binding of the second horseradish peroxidase-coupled antibody was detected using enhanced chemiluminescent detection reagent (100 mM Tris-HCl, pH 8.0, 1 mM luminol (Fluka, St. Gallen, Switzerland), 0.2 mM p-coumaric acid, 3 mM H₂O₂). Western blots were repeated more than three times with similar results.

Quantification of CNF-modified Recombinant RhoA—Recombinant RhoA (8.6 μM) was incubated with wild type GST-CNf1 (0.1 μM) or preparations of the GST-CNf1 loop mutants (0.1 μM) in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol for 30 min at 37 °C. The reaction was stopped by the addition of 5× Laemmli buffer, and samples were boiled for 5 min at 95 °C. RhoA (2 μg/lane) were loaded onto 12.5% SDS-gel containing 1 μl urea. Gels were stained with Coomassie Brilliant Blue, destained with 10% acetic acid, and dried. CNF-modified RhoA runs at a higher apparent molecular mass than unmodified RhoA. Gels were scanned, and the amount of modified RhoA was determined as a percentage of the total RhoA in the respective lane using ImageQuant Version 5.2. Values obtained for CNF1 loop mutants were normalized to the CNF1 value in each individual experiment. The experiment was repeated three times, and the results are shown as the mean ± S.D.

Actin Staining—Formaldehyde-fixed cells were washed three times 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).
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RESULTS

Toxin Chimeras—To clarify the different substrate specificities of the Y. pseudotuberculosis toxin, CNF1, and the E. coli toxin CNF1 in the living cell, we first studied the role of the N-terminal toxin part encompassing the receptor binding domain (aa 53–190) (15) and a putative translocation domain (located between aa 190 and 720, (16). We intended to clarify whether these domains influence substrate specificity, possibly by restricting the localization of the toxin within the host cell.

We constructed chimeras of CNF1 and CNF1/1 that consist of the N-terminal receptor binding and translocation domains of one toxin fused to the C-terminal catalytic domain of the other toxin (Fig. 1, a and b). These chimeras were tested for their ability to activate the Rho GTPases RhoA, Rac, and Cdc42 in intact cells. To that end HeLa cells were intoxicated with CNF1, CNF1/1, or with the chimeric toxins CNF1/1 and CNF1/1, which contain the receptor binding and translocation domain of one toxin and the catalytic domain of the other toxin as depicted in Fig. 1. Subsequently, cells were lysed, and activated Rho GTPases were precipitated in an effector pulldown using the GTPase binding domain of Rhotekin and the CRIB domain of PAK, respectively. CNF1/1 activated RhoA only slightly less than wild type CNF1, whereas it was not able to activate Rac or Cdc42 (Fig. 1c). In contrast, CNF1/1 activated both Rac and Cdc42, whereas RhoA activation was even slightly stronger than with wild type CNF1. Staining of the actin cytoskeleton of toxin-treated cells reflected similar Rho activation patterns. In cells treated with CNF1 or with CNF1/1, strong stress fiber formation was visible corresponding to RhoA activation (Fig. 1d), whereas cells treated with CNF1 or CNF1/1 showed some stress fibers and also filopodia (Fig. 1d) and membrane ruffles on the top of the cells (not shown), indicating activation of RhoA, Cdc42, and Rac. Consequently, the exchange of the receptor binding and translocation domains between the two toxins did not alter substrate specificity in vivo, suggesting it is primarily defined by the catalytic domains.

Homology Modeling of Catalytic Domains—The crystal structure of the catalytic C-terminal part of CNF1 has been solved (13). In contrast, no such structural information is available for CNF1/1. To obtain information about the protein fold of the catalytic domain of CNF1/1, we submitted the sequence of CNF1/1 to Swiss Model (17, 18) using the crystal structure of CNF1 as a template. The resulting model shows a remarkably high similarity of CNF1/1 with CNF1 (Fig. 2a). Therefore, it can be assumed that the catalytic domains of CNF1 and CNF1/1 have similar folding. This applies also to the flexible loop elements that line the entrance to the catalytic pocket. Some of these loops have been recently implicated in recognition and binding of the substrate RhoA (14). In the modeled structure, these loops are displayed in the same position in CNF1/1 as in CNF1. However, a comparison of the amino acid sequences of CNF1 and CNF1/1 within the loop regions reveals differences in their amino acid composition (Fig. 2b).

CNF Loop Mutants—We decided to dissect the role of the differing residues in determining substrate specificity by creating loop mutants of CNF1 and CNF1/1. One or several amino acids within individual loops were exchanged between CNF1 and CNF1/1. Surface-exposed amino acids were selected that are potentially accessible to the substrate, as deduced by manual view on the CNF1 crystal structure. Substitutions were introduced by site-directed mutagenesis into loops 2 (L2), 8 (L8), and 9 (L9), which have already been proposed to play a role in substrate recognition (14), and additionally into loops 3 (L3), 6 (L6), and 7 (L7) (Fig. 2). Wild type and mutant CNFs were expressed as GST fusion proteins and purified by affinity chromatography. Expression and purity of wild type and mutant proteins were comparable (Fig. 3a).

Catalytic Activity of Loop Mutants—To test the catalytic activity of the CNF1 loop mutants, recombinant RhoA was treated with the toxin preparations, and the amount of deamidated RhoA was quantified. The amount of deamidated RhoA...
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The catalytic activity of CNF1 is involved in RhoA recognition and/or catalytic activity. Substrate Specificity of Loop Mutants in Vivo—In intact cells CNF1 targets all three GTPases, RhoA, Rac, and Cdc42. In contrast, CNFY is a strong activator of RhoA but does not activate Rac or Cdc42 with up to 6 h of toxin treatment (7). To test whether the loop elements of the CNF catalytic domains play a role in substrate specificity, we investigated the ability of the loop mutants of CNF1 and CNFY to activate RhoA, Rac, and Cdc42 in living cells. Cell lysates of toxin-treated HeLa cells were subjected to effector pulldown assays to monitor the respective activation level of RhoA, Rac, and Cdc42. All CNF1-derived mutants activated Rac in levels comparable with wild type CNF1, whereas none of the CNFY-based mutants activated Rac or Cdc42 (Fig. 4a).

In contrast, several mutants showed altered levels of RhoA activation. RhoA activation was decreased after treatment of cell lysates, CNFY L8 had no effect on activity (Fig. 3b). The mutations in L6 and L7 of CNF1 had no influence on the activity of the toxins as compared with wild type CNF1 and CNFY (Fig. 3b). A single amino acid exchange in L3 and the mutations in L9 decreased the activity of the toxin toward RhoA by 50%. The amount of deamidated RhoA to 90% (Fig. 3b).

Amino acids that were targeted in the CNF loop mutants are indicated by black triangles. White characters on a black background mark identical amino acid residues, and boxed black characters indicate similar residues. The figure was generated with ESPript 2.2.

FIGURE 2. Comparison of the catalytic domains of CNF1 and CNFY. a, homology modeling of the CNF1 catalytic domain. The known crystal structure of CNF1 (aa 720–1014) was used as a template for prediction of the tertiary structure of CNFY (aa 720–1014) by Swiss Model. The resulting model shows a high homology of the catalytic domains of CNF1 (white) and CNFY (green). b, ribbon representation of the tertiary structure of CNFY (aa 720–1014) with the loop elements lining the catalytic pockets marked by colors and indicated by numbers (2, light blue; 3, orange; 6, dark blue; 7, red; 8, magenta; 9, green). The catalytic residues Cys-866 and His-881 are marked in black. The figure was created with Swiss Pdb Viewer 3.7. c, the amino acid sequences of the catalytic domains (aa 720–1014) of CNF1 and CNFY were aligned using ClustalW. The secondary structural information displayed above the sequences (α-helices as spirals, β-sheet as arrows) is derived from the CNF1 crystal structure (13). Loops are identified by the same numbers and colors as in a, shown above the sequences. Amino acids that were targeted in the CNF loop mutants are indicated by black triangles. White characters on a black background mark identical amino acid residues, and boxed black characters indicate similar residues. The figure was generated with ESPript 2.2.
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FIGURE 3. Expression and activity of CNF1 and CNFY loop mutants. a, SDS-PAGE of CNF1 and CNFY loop mutants. All mutants were expressed in BL-21 and purified by affinity chromatography as GST fusion proteins. b, deamidation of RhoA is monitored by its shift to an apparent higher molecular weight. Upper panel, SDS-PAGE of RhoA treated with CNF1 wild type (wt) and loop mutants. 1 μg of recombinant RhoA was incubated with 0.5 μg of GST-CNF1 wild type or the mutants GST-CNF1 L2, L3, L6, L7, L8, or L9 for 30 min at 37 °C. Lower panel, Western blot of RhoA from lysates treated with CNF1, wild type and loop mutants. HeLa cell lysates were incubated with 0.5 μg of GST-CNF1 wild type and loop mutants GST-CNF1 L2, L3, L6, L7, L8, or L9 for 30 min at 37 °C. Laemmli buffer was added, and the samples were boiled and separated on a 12.5% SDS gel with 1 M urea and blotted onto polyvinylidene difluoride membranes. A Western blot was performed with an anti-RhoA antibody. c, quantification of the amount of deamidated RhoA. The activity of the CNF1 loop mutants was measured by the amount of RhoA that had shifted in SDS-PAGE after 30 min using ImageQuant 5.2. The value is represented in the figure as percentage of the amount of RhoA E63 that had been formed with CNF1 wild type. Bars represent the mean of three independent experiments ± S.E. d, SDS-PAGE of recombinant RhoA treated with CNF1, CNF1 P968Q, CNF1 E969L, or CNF1 H970P as described under b.

FIGURE 4. In vivo substrate specificity of CNF1 and CNFY loop mutants. Effector pulldowns were performed with the Rho binding domain of Rhotekin or the CRIB domain of PAK to measure the amount of activated RhoA, Rac, and Cdc42. a, HeLa cells were intoxicated for 2 h with CNF1, CNFY, or with the respective loop mutants (400 ng/ml each). Cells were lysed, and the activated GTPases were precipitated with immobilized Rhotekin or PAK. The precipitated GTPases were detected by immunoblotting with their specific antibodies. WT, wild type. b, HeLa cells were intoxicated with 500 ng/ml of CNF1, CNF1 P968Q, or CNF1 E969L for 2 h. Note that blot exposure was short due to the strong activation of RhoA by CNF1. Longer exposure shows also activation of RhoA by CNF1. The results shown in a and b are representative of at least three independent experiments.

dered decreased (Fig. 4, compare Fig. 3c). Because these seemingly contradictory effects were most pronounced with CNF1 L8, we additionally tested the in vivo activity of the mutant CNF1 P968Q. This mutation within L8 was responsible for the drop in enzyme activity of CNF1 (Fig. 4). However, in intact cells this mutant induced a stronger RhoA activation than wild type CNF1. Intriguingly, the same mutant was not able to efficiently activate Cdc42 in vivo. In contrast, all other CNF1 mutants still activated Cdc42 with comparable activity to wild type CNF1. Taken together our results show that a single amino acid substitution within L8 of CNF1, CNF1 P968Q, is sufficient to shift the Rho GTPase activation pattern of the toxin in vivo.

Transient Activation of CNF1-modified RhoA—When HeLa cells are treated with CNF1 or CNF1 L8 for various times, deamidation proceeds somewhat slower with CNF1 L8, as monitored by the shift of RhoA from lysed cells (Fig. 5a, upper panel). This finding is in line with the lower in vitro catalytic activity of the mutant toxin (Fig. 3c). Nevertheless, the amount of active, effector-bound RhoA is significantly higher with the L8 mutant. The same phenomenon was observed with CNFY (not shown). We reasoned that RhoA from CNF1-treated cells, although already deamidated (shift, Fig. 5a) might not remain GTP-bound and/or is impaired in effector-coupling. Indeed, when HeLa cells were treated with CNF1 in a time course, we observed that RhoA activation by CNF1 wild type toxin was only transient. The highest level of active, effector-bound RhoA was reached after 60 min (Fig. 5a). RhoA activity decreased to background level at 90 min, although the total amount of cellular RhoA was deamidated, as seen by a complete shift in SDS-PAGE (Fig. 5a). In contrast, RhoA activation was strong and long-lasting after treatment of cells with CNF1 L8.

Previously, we and others showed that Rac activation is transient in CNF1-intoxicated cells due to proteasomal degradation of Rac Q63E (10, 11). To rule out the possibility that CNF1-activated RhoA is proteolytically degraded in our HeLa cell system, cells were either left untreated or were treated with MG132, a potent inhibitor of the proteasome machinery. Although Rac1 was degraded in untreated cells, MG132 inhibited the degradation of Rac after CNF1 activation (Fig. 5b, lower panel), indicating that the proteasomal system is blocked in the
presence of MG132. The time-course of CNF1 intoxication shows that RhoA activation is transient both in untreated and MG132-treated cells (Fig. 5b). Moreover, we did not observe a decrease in the protein level of RhoA in total lysate of CNF1-treated cells (Fig. 5b).

We conclude that CNF1-deamidated RhoA is only transiently activated and that the decrease in active RhoA is not due to proteasomal degradation. An alternative regulatory mechanism might be involved. As reported here, CNF1 L8 has almost completely lost its ability to activate Cdc42. At the same time, RhoA activation is increased. This led us to speculate that activated Cdc42 might negatively regulate the level of active RhoA.

The time course of cell intoxication with CNF1 or CNF1 L8 shows that activation of Cdc42 indeed negatively correlates with the level of active RhoA (Fig. 5a). In CNF1-treated cells, full Cdc42 activation was reached already after 60 min and persisted at least up to 90 min. In cells treated with CNF1 L8, a minor activation of Cdc42 occurred only after 90 min, whereas RhoA was strongly activated after 60 min (Fig. 5a).

**DISCUSSION**

We have shown previously that CNF\(_V\) targets RhoA but not Rac or Cdc42 in intact cells. However, the toxin is able to deamidate recombinant RhoA as well as recombinant Rac1 and Cdc42 in *vitro* (7). In contrast, the *E. coli* toxin CNF1 modifies all three Rho GTPases in *vitro* and in the living cell (7).

Differences in toxin uptake and, consequently, in subcellular localization might contribute to differences in substrate specificity of CNF1 and CNF\(_V\). Therefore, we analyzed the influence of the N-terminal receptor binding and translocation domains of the toxins on substrate specificity by exchanging them between CNF1 and CNF\(_V\). The exchange of the receptor binding/translocation domains of CNF1 and CNF\(_V\) had no major influence on the set of substrates that are targeted. We conclude that it is not the uptake of the toxins, that may influence its cellular localization but, rather, the catalytic domain itself that defines substrate specificity.

Recently, some of the loop elements lining the entrance to the catalytic pocket of CNF1 were implicated in substrate recognition and binding, because their deletion resulted in decreased catalysis of RhoA in *vitro* (14). Here, we exchanged selected amino acids in loops 2, 3, 6, 7, 8, and 9 of CNF1 for the corresponding residues of CNF\(_V\) to study their role in defining substrate specificity. Equivalent mutations were introduced into CNF\(_V\).

The enzymatic activity of the CNF1 L8 mutant dropped most dramatically, whereas the activity of CNF1 L3 and L9 was reduced to about half that of wild type CNF1. Remarkably, single amino acid substitutions in CNF1 were sufficient to cause the reported decrease in activity (L814Q, which is identical to L3 and P968Q in L8, respectively). We cannot exclude that the mutation alters the folding of the loop. Nevertheless, it is unlikely that a point mutation in a surface loop changes the overall folding of the toxin. This assumption is supported by a comparable pattern of protein bands in a tryptic digest of the wild type and the mutant protein.

Intriguingly, L814Q and P968Q together with L9 are positioned in the CNF1 structure on one side of the entrance to the catalytic pocket. The initial contact between the toxin and its substrate may occur via these loops of the CNFs, and certain amino acids within RhoA that have not been identified so far. It was shown earlier that a peptide corresponding to the switch II region of RhoA (aa 58–70 (19)) and an even shorter peptide (aa 59–69 (20)) was sufficient for recognition and deamidation by CNF1. However, catalysis was about 100-fold reduced with the switch II peptide as compared with the full protein (19). Therefore, additional regions of RhoA located outside the switch II region must be required for proper substrate binding.

Importantly, higher selectivity of CNF\(_V\) as compared with CNF1 was found *in vivo*, whereas both toxins modified recombinant RhoA, Rac, and Cdc42 (7). We, therefore, tested the influence of the various loop mutants on substrate specificity in living cells. As CNF1 wild type, all CNF1 mutants activated Rac very robustly, whereas none of the CNF\(_V\)-derived mutants could activate Rac. The specificity of CNF1 for Rac might be defined by different or additional contact sites. Equally to CNF\(_V\) wild type, none of the mutants activated Cdc42 either, whereas most CNF1 mutants still activated Cdc42. However, CNF1 L8 was not able to activate Cdc42 to the same extend as all other mutants. Importantly, CNF1 L8, and more specifically the point mutant P968Q, showed much stronger RhoA activation in comparison to wild type CNF1. These results are seemingly in contrast to our *in vitro* data in which CNF1 L8 and CNF1P968Q had the lowest catalytic activities toward recombinant RhoA (Fig. 3). Interestingly, also in the living cell the stronger activation of RhoA induced by CNF\(_V\), or CNF1 L8 is clearly not due to faster modification of the GTPase, as monitored by the shift of deamidated RhoA to higher molecular weight. On the contrary, modification of RhoA occurs slower than by CNF1. Thus, deamidation and activation (GTP loading and signaling) of RhoA are dissociated. The Rho shift indicates deamidation, but it is not indicative for the activation status of RhoA. RhoA activation by CNF1 in HeLa cells was only transient, although the total amount of cellular RhoA was deamidated over time, as indicated by a complete shift in SDS-PAGE (Fig. 5a). In contrast to the transient activation with CNF1, RhoA activation was strong and long-lasting after treatment of cells with CNF1 L8 or CNF1 L9. Interestingly, these toxins do not activate Cdc42. Our data suggest that, rather, it is the modification and activation of Cdc42 that influences RhoA activity than the kinetics of RhoA deamidation. In line with our data, it was shown that expression of active Cdc42 reduces LAP-induced RhoA activation in endothelial cells (21). Moreover, activated Rac or Cdc42 leads to down-regulation of RhoA in NIH3T3 fibroblasts, Swiss 3T3 fibroblasts, and neuronal cells (22–25). In all these studies the physiological activation of RhoA may be inhibited by reducing exchange factor activity or by enhancing the activity of GTPase-activating proteins. In CNF1-treated cells the deamidated form of RhoA cannot hydrolyze GTP even in the presence of GTPase-activating proteins and is expected to act constitutively active (4). However, in HeLa cells treated with CNF1 RhoAQ63E activity is only transient. Deamidated RhoA might not remain GTP-bound and/or could be impaired in effector coupling due to a Cdc42-dependent pathway. Consistent with this hypothesis, expression of dominant active Cdc42 but not expression of dominant active Rac inhibits activation of cellular
RhoA by CNFY. In contrast, deamidation of RhoA is not influenced by Cdc42 expression.³ Taken together, cross-talk between activated Cdc42 (and probably additional GTPases) and constitutively active RhoA seems to be a regulatory mechanism, which besides proteasomal degradation could orchestrate deamidated GTPases.

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REFERENCES

1. Aspenstrom, P. (1999) Curr. Opin. Cell Biol. 11, 95–102
2. Ridley, A. J. (1996) Curr. Biol. 6, 1256–1264
3. Mackay, D. J. G., and Hall, A. (1998) J. Biol. Chem. 273, 20685–20688
4. Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M., and Aktories, K. (1997) Nature 387, 725–729
5. Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C., and Boquet, P. (1997) Nature 387, 729–733
6. Lockman, H. A., Gillespie, R. A., Baker, B. D., and Shakhnovich, E. (2002) Infect. Immun. 70, 2708–2714
7. Hoffmann, C., Pop, M., Leemhuis, J., Schirmer, J., Aktories, K., and Schmidt, G. (2004) J. Biol. Chem. 279, 16026–16032
8. Schmidt, G., Selzer, J., Lerm, M., and Aktories, K. (1998) J. Biol. Chem. 273, 13669–13674
9. Hoffmann, C., and Schmidt, G. (2004) Rev. Physiol. Biochem. Pharmacol. 152, 49–63
10. Lerm, M., Pop, M., Fritz, G., Aktories, K., and Schmidt, G. (2002) Infect. Immun. 70, 4053–4058
11. Doye, A., Mettouchi, A., Bossis, G., Clément, R., Buisson-Touati, C., Flatau, G., Gagnoux, L., Piechaczyk, M., Boquet, P., and Lemichez, E. (2002) Cell 111, 553–564
12. Contamin, S., Galmiche, A., Doye, A., Flatau, G., Benmerah, A., and Boquet, P. (2000) Mol. Biol. Cell 11, 1775–1787
13. Buetow, L., Flatau, G., Chiu, K., Boquet, P., and Ghosh, P. (2001) Nat. Struct. Biol. 8, 584–588
14. Buetow, L., and Ghosh, P. (2003) Biochemistry 42, 12784–12791
15. Fabbri, A., Gauthier, M., and Boquet, P. (1999) Mol. Microbiol. 33, 108–118
16. Pei, S., Doye, A., and Boquet, P. (2001) Mol. Microbiol. 41, 1237–1247
17. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) Nucleic Acids Res. 31, 3381–3385
18. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
19. Lerm, M., Schmidt, G., Goehring, U.-M., Schirmer, J., and Aktories, K. (1999) J. Biol. Chem. 274, 28999–29004
20. Flatau, G., Landraud, L., Boquet, P., Bruzzone, M., and Munro, P. (2000) Biochem. Biophys. Res. Commun. 267, 588–592
21. Moreau, V., Tatín, F., Varon, C., and Genot, E. (2003) Mol. Cell. Biol. 23, 6809–6822
22. Sander, E. E., ten Klooster, J. P., van, D. S., Van der Kammen, R. A., and Collard, J. G. (1999) J. Cell Biol. 147, 1009–1022
23. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995) Mol. Cell. Biol. 15, 1942–1952
24. Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997) Mol. Cell. Biol. 17, 1201–1211
25. Leeuwen, F. N., Kain, H. E., Kammen, R. A., Michiels, F., Kranenburg, O. W., and Collard, J. G. (1997) J. Cell Biol. 139, 797–807