The Yersinia pseudotuberculosis Cytotoxic Necrotizing Factor (CNF\textsubscript{Y}) Selectively Activates RhoA* 

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The cytotoxic necrotizing factors (CNF)1 and CNF2 from pathogenic Escherichia coli strains activate RhoA, Rac1, and Cdc42 by deamidation of Gln\textsubscript{61} (RhoA) or Gln\textsubscript{63} (Rac and Cdc42). Recently, a novel cytotoxic necrotizing factor termed CNF\textsubscript{Y} was identified in Yersinia pseudotuberculosis strains (Lockman, H. A., Gillespie, R. A., Baker, B. D., and Shakhnovich, E. (2002) Infect. Immun. 70, 2070–2071). We amplified the cnf\textsubscript{Y} gene from genomic DNA of Y. pseudotuberculosis, cloned and expressed the recombinant protein, and studied its activity. Recombinant GST-CNFY induced morphological changes in HeLa cells and caused an upward shift of RhoA in SDS-PAGE, as is known for GST-CNFA and GST-CNFB. Mass spectrometric analysis of GST-CNFA-treated RhoA confirmed deamidation at Gln\textsubscript{63}. Treatment of RhoA, Rac1, and Cdc42 with GST-CNFB decreased their GTPase activities, indicating that all of these Rho proteins could serve as substrates for GST-CNFA in vitro. In contrast, RhoA, but not Rac or Cdc42, was the substrate of GST-CNFY in culture cells. GST-CNFY caused marked stress fiber formation in HeLa cells after 2 h. In contrast to GST-CNFA, formation of filopodia or lamellipodia was not induced with GST-CNFY. Accordingly, effector pull-down experiments with lysates of toxin-treated cells revealed strong activation of RhoA but no activation of Rac1 or Cdc42 after 6 h of GST-CNFB-treatment. Moreover, in rat hippocampal neurons, GST-CNFA results in the retraction of neurites, indicating RhoA activation. In contrast, no activation of RhoA or Cdc42 was found. Altogether, our data suggest that CNFY from Y. pseudotuberculosis is a strong selective activator of RhoA, which can be used as a powerful tool for constitutive RhoA activation without concomitant activation of Rac1 or Cdc42.

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CNF1 polyclonal antibody was not able to inhibit the CNF<sub>Y</sub> cytopathic effect (7).

Here, we cloned and expressed CNF<sub>Y</sub> from <i=Y. pseudotuberculosis</i> as a recombinant GST-fusion protein (GST-CNFR<sub>Y</sub> = 146 kDa; GST = 26 kDa) and investigated its effects on the Rho GTPases RhoA, Rac1, and Cdc42, both in vitro and in intact cells. From our studies, we conclude that in intact cells, GST-CNFR<sub>Y</sub> is a selective activator of RhoA.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Lysates—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (12 mM glucose) supplemented with 10% fetal calf serum, penicillin (40 IU/mL), and streptomycin (40 μg/mL) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

For intoxication with GST-CNFR<sub>Y</sub> or GST-CNFR<sub>Y</sub>, cells were treated with 400 ng of GST-CNFR<sub>Y</sub> per mL of medium 1 day after seeding. For preparation of cell lysates, cells were washed twice with ice-cold phosphate-buffered saline, lysed in buffer A (10% glycerol, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Igepal, 2 mM MgCl<sub>2</sub>, and 0.5 mM phenylmethylsulfonyl fluoride) for 5 min at 4 °C and harvested with a rubber policeman. Lysates were cleared by centrifugation (20 min, 20,800 × g, 4 °C), and supernatants were subjected to pull-down assays.

Primary cultures of neurons were prepared from hippocampi of newborn rats, as described previously (11). The dissociated neuronal cells were seeded on poly-D-lysine/amin-coated coverslips. Incubation medium consisted of neurobasal medium supplemented with B27. Fresh medium was mixed 1:1 with medium, which had been conditioned for 3 or 4 days with hippocampal astroglial cultures. Cultures were incubated for 3 days at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Neurobasal medium and B27 were obtained from Invitrogen.

Actin-staining—Formaldehyde-fixed cells were washed three times with phosphate-buffered saline. The cells were then incubated with rhodamine-conjugated phalloidin (1 unit per coverslip) at room temperature for 1 h, washed again, and applied for fluorescence microscopy (bleaching preserving was Kaiser’s glycerol gelatin from Merck). Micrographs were taken with an Axiozoom camera (Zeiss).

Immunocytochemistry—Neurons were fixed with 4% paraformaldehyde at room temperature for 20 min. They were washed with phosphate-buffered saline and permeabilized with 0.1% (v/v) Triton X-100. Normal goat serum was used to block unspecific reactions. Thereafter, the neurons were incubated with the monoclonal mouse anti-β-tubulin III antibody (Sigma). The resulting immune complexes were visualized with a Cy® 3-conjugated F(ab’)<sub>2</sub> fragment of goat anti-mouse IgG (Dianova, Hamburg, Germany).

Confocal Image Analysis—Neurons were imaged by using an MRC 1024 confocal system (version 3.2, Bio-Rad) with a krypton-argon laser (Dianova, Hamburg, Germany) and was allowed to air-dry several minutes at room temperature, rehydrated, and was stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) at 4 °C, 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.

Sample Preparation for MALDI-TOF Mass Spectrometry—Four-Hydroxy-a-cyanoacrylic acid (Aldrich) was recrystallized from hot methanol and stored in the dark. Saturated matrix solution of 4-hydroxy-a-cyanoacrylic acid in a 1:1 solution of acetonitrile/aqueous 0.1% trifluoroacetic acid was prepared. 2 μl of the proteolytic peptide mixture was mixed with 2 μl of saturated matrix containing marker peptides (5 pmol of human ACTH (18–39), dip (MW 2468, Sigma), and 5 pmol of human angiotensin II (MW 1047, Sigma)) for internal calibration. Using the dried-drop method of matrix crystallization, 1 μl of the sample matrix solution was placed on the MALDI-TOF stainless-steel target and was allowed to air-dry several minutes at room temperature, resulting in a thin layer of fine granular matrix crystals.

Mass Spectrometry—MALDI-TOF mass spectrometry was performed on a Bruker Biflex mass spectrometer equipped with a nitrogen laser (λ = 337 nm) to desorb and ionize the samples. Mass spectra were recorded in the reflector positive mode in combination with delayed extraction.

Western Blot Analysis—For Western blotting, samples were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membrane. 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 Biotechnology, Santa Cruz, CA) and for Cdc42 detection, a monoclonal antibody (anti-Cdc42, Upstate Biotechnology, Lake Placid, NY) was used. Binding of the second horseradish peroxidase-coupled antibody was detected with enhanced chemiluminescence detection reagent (100 mM Tris-HCl, pH 8.0, 1 mM luminol (Fluka, St. Gallen, Switzerland), 0.2 mM p-aminocarboxylic acid, 3 mM H<sub>2</sub>O<sub>2</sub>.

RESULTS

Recently, the nucleotide sequence of CNFR<sub>Y</sub> from <i=Y. pseudotuberculosis</i> was described (7). It was shown that bacterial cell extracts of <i>Terrinia</i> induce multination in Hep-2 cells as described for CNF1 produced by pathogenic <i>E. coli</i> strains.
Selective Activation of RhoA

Cloning and Expression of CNFγ—To analyze the activity of CNFγ, the genomic DNA of Y. pseudotuberculosis was purified. We amplified the cnfy gene by PCR and cloned it into the pGEX vector. The proper construct was checked by DNA sequencing. Sequencing revealed some differences in the N-terminal part of the protein (S193N, G305Q, and T395A), as compared with the sequence published by Lockman (7). Repeated PCR gave the same results, indicating that the differing sequence is not due to mistakes of the polymerase. CNFγ was then expressed as GST-fusion protein in BL21 cells and purified by affinity chromatography (Fig. 1A, lane 1).

Activity of Recombinant GST-CN FY—The activity of the recombinant toxin was first studied by the change in electrophoretic mobility of modified RhoA. As shown in Fig. 1B, incubation of recombinant RhoA with GST-CN F1 (Fig. 1B, lane 2) or with GST-CN FY (Fig. 1B, lane 2) but not with buffer (Fig. 1B, lane 1) caused an upwards shift of the GTPase in SDS-PAGE, indicating deamidation of RhoA by GST-CN F1 and GST-CN FY. After incubation with GST-CN FY, the RhoA peptide Glu52-Arg88 results in a mass shift of the peptide of 1 Da (to 2009.86 Da).

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Fig. 1. A, expression of recombinant GST-CN FY, SDS-PAGE (12.5%) of recombinant GST-CN FY (lane 1) and the catalytic inactive mutants GST-CN FY(C866S) (lane 2) and GST-CN FY(H881A) (lane 3). The proteins were expressed from pGEX-2TGL in E. coli BL21 and purified by affinity chromatography as GST-fusion proteins. 2 μg of protein were loaded per lane. B, activity of recombinant GST-CN F1, GST-CN FY, and mutants. The activity of the recombinant toxin was studied by the change in electrophoretic mobility of modified RhoA. Therefore, RhoA was incubated with buffer (lane 1), recombinant GST-CN F1 (lane 2), recombinant GST-CN FY (lane 3), mutant GST-CN FY(C866S) (lane 4), and mutant GST-CN FY(H881A) (lane 5). Incubation of recombinant RhoA with both wild-type toxins, but not with the mutants, changes the electrophoretic mobility of the GTPase.

To study the activity of the toxin in the living cell, HeLa cells were incubated with GST-CN F1, GST-CN FY, and the inactive mutant GST-CN FY(C866S) respectively, and morphological changes induced by the toxins were monitored by phase contrast microscopy. Moreover, the actin cytoskeleton of fixed cells was stained with rhodamine-phalloidin and analyzed by fluorescent microscopy. As expected, GST-CN FY induced the rearrangement of the actin cytoskeleton of HeLa cells and led to polynucleation. Notably, the morphology of cells and toxin-induced actin rearrangements observed with GST-CN F1 and GST-CN FY, respectively, were different. As shown in Fig. 4, incubation of HeLa cells with GST-CN F1 for 2 h led to cell spreading (Fig. 4C). Also, the formation of membrane ruffles and, to a lesser extent, the formation of actin stress fibers and filopodia was evident already after 2 h of incubation with GST-CN FY, indicating strong activation of Rac and activation of RhoA and Cdc42. In contrast, incubation with GST-CN FY for the same time period led to the formation of actin stress fibers, but no membrane ruffling, filopodia formation, or cell spreading were detected (Fig. 4D), indicating activation of RhoA but not of Rac and Cdc42 by CNFγ. However, after 24 h of incubation with GST-CN FY, cells were flattened and showed mem-
brane ruffling (Fig. 4E). As expected, the catalytically inactive mutant GST-CNFC(S866S) had no effect on the morphology of HeLa cells (Fig. 4A). From our in vitro data presented above, the slower flattening could be explained by the minor catalytic activity of GST-CNFC as compared with GST-CNFC1. However, an even stronger stress fiber formation was observed after 2 h of GST-CNFC treatment than was observed after GST-CNFC1 treatment. This finding indicates that GST-CNFC has even higher activity toward RhoA than GST-CNFC1 in the living cell.

**GST-CNFC Selectively Activates RhoA in HeLa Cells**—To clarify the in vivo substrate specificity of GST-CNFC, we performed pull-down experiments with bead-coupled GTPase-binding domains of rhotekin, PAK, or POSH. These effector domains exclusively bind to the GTP-bound form of the GTPases. HeLa cells were treated with the two toxins in a time course and then lysed. The pull-down experiments were performed with increasing incubation times to follow activation of the respective GTPase. In addition, toxin-treated HeLa cells were fixed and stained with rhodamine-phalloidin after the same time periods to allow comparison of GTPase activation and morphology. As shown in Fig. 5, incubation of the cells for 2 h with GST-CNFC1 led to strong activation of Rac and activation of RhoA and Cdc42 with the corresponding formation of membrane ruffles, stress fibers, and filopodia in HeLa cells (Fig. 5A, lane 1). With GST-CNFC2, a strong activation of RhoA was evident already after 1 h of incubation and was still present after 24 h of toxin treatment. In contrast, no activation of Rac and Cdc42 could be detected during the first 6 h of treatment with GST-CNFC2. In line with this, GST-CNFC2-treated HeLa cells showed a strong formation of stress fibers but no membrane ruffling or filopodia formation (Fig. 5, A and B, lanes 3–6). After 8–24 h of treatment with GST-CNFC2, a slight activation of Rac occurred, which was accompanied by the formation of membrane ruffles (Fig. 5, A and B, lanes 7 and 8). Thus, activation of the GTPases detected in the pull-down experiments clearly depicts the morphological changes induced by GST-CNFC2 in HeLa cells. All together, the data indicate that GST-CNFC1 and GST-CNFC2 have different substrate specificity in vivo, with GST-CNFC2 specifically modifying RhoA.

**GST-CNFC Induces RhoA Morphology in Rat Hippocampal Neurons**—To further substantiate our observation that CNFy selectively activates RhoA, we studied the morphology of a different type of cell and tested the effect of GST-CNFC on rat hippocampal neurons. In these cells, it is possible to distinguish morphological changes induced by active RhoA from changes induced by active Rac and Cdc42 activation. It has been shown that RhoA activation in hippocampal neurons leads to the retraction of dendrites, whereas constitutive activation of Rac or Cdc42 induces the formation of small finger-like extensions along the dendrites (12). We treated rat hippocampal neurons with GST-CNFC1 or GST-CNFC2 and studied the morphology induced. As shown in Fig. 6, incubation of the neurons for 4 h with GST-CNFC1 or with GST-CNFC2 led to retraction of dendrites from the total neurite length of ~500 μm to <100 μm in toxin-treated cells, indicating RhoA activation.

Moreover, the induction of small finger-like extensions, indicating activation of Rac and/or Cdc42 from control levels with 3 spikes per neuron, increased to 12 spikes in mean only when the cells were treated with GST-CNFC1. In contrast, incubation of the neurons with GST-CNFC2 for 4 h led to a clear retraction of dendrites but not to the formation of small extensions, suggesting that RhoA was activated exclusively. Activation of RhoA in this cell system was verified by pull-down experiments with rhotekin. Moreover, no activation of Rac or Cdc42 by GST-CNFC2 was found in PAK pull-down experiments (data not shown).

All together, our data strongly suggest that CNFy can be used as a new tool for permanent and selective activation of RhoA in mammalian cells without concomitant activation of Rac and Cdc42.

**DISCUSSION**

We and others analyzed the molecular mechanism and the structure-function relationship of the cytotoxic necrotizing factor (CNF1) of *E. coli* (for review, see Ref. 4). Recently, the nucleotide sequence of the CNFy from *Y. pseudotuberculosis* was described (7). CNFy shows 61% identity in its amino acid sequence with CNF1 all over the protein. The activity of the toxin was analyzed using bacterial cell extracts. In these studies, GST-CNFCy induced multinucleation in Hep-2 cells in the same manner as described for GST-CNFC1.

In the present study, we cloned and expressed the CNFy of *Y. pseudotuberculosis* for the first time and investigated the activity of the recombinant toxin in vitro and in vivo. We used different methods established for the analysis of CNF1 to analyze the activity of CNFy. First, we analyzed the GST-CNFCy-catalyzed modification of recombinant RhoA by determination of the different migration behavior of unmodified and deamidated RhoA in SDS-PAGE. Incubation of the GTPase with the
recombinant toxin led to the same shift as induced by GST-CN FY, indicating the same modification. The same activity was found for the thrombin-cleaved toxins (not shown). Deamidation at Glu\textsuperscript{63} of RhoA by GST-CN FY was further verified by the increase in mass of one Dalton in a peptide corresponding to amino acids 52–68 of the GTPase detected by MALDI-TOF mass spectrometry. This increase in mass corresponds to deamidation of Glu\textsuperscript{63}. Deamidation of RhoA leads to the block of intrinsic and GAP-stimulated GTPase activity, which can be analyzed in a filter-binding assay. We show that GST-CN FY deamidates RhoA, Rac, and Cdc42 in vitro and leads to the block of the GTP-hydrolyzing activity of the respective GTPase. In CNF1, a catalytic dyad of Cys\textsuperscript{866} and His\textsuperscript{881} was identified (9). These residues are identical in CNF Y. Mutation of these amino acids in GST-CN FY completely blocks the catalytic activity of the Yersinia toxin. As expected from the overall identity of 61\%, including the catalytic amino acids, the data indicate that CNF1 and CNF Y catalyze the same modification, namely the deamidation of Glu\textsuperscript{63} or Glu\textsuperscript{61} of RhoA, Rac, and Cdc42, respectively.

**Fig. 4.** Toxin-induced morphological changes and reorganization of the actin cytoskeleton of HeLa cells. HeLa cells growing on glass coverslips were untreated (B), treated with 400 ng/ml GST-CN FY(C866S) for 8 h (A), 400 ng/ml GST-CN F1 for 2 h (C), or 400 ng/ml GST-CN FY for 2 h (D) or for 24 h (E). After phase contrast photography (magnification, 32×), the cells were fixed and stained with rhodamine-phalloidin for fluorescence microscopy (100×).

**Fig. 5.** Activation of GTPases by GST-CN FY mirrors toxin-induced morphological changes in HeLa. A, HeLa cells were treated with GST-CN F1 (lane 1, 2 h) or GST-CN FY (lanes 3-8) in a time course (lane 3, 2 h; lane 4, 2 h; lane 5, 4 h; lane 6, 6 h; lane 7, 8 h; and lane 8, 24 h). Toxin-treated HeLa cells were fixed and stained with rhodamine-phalloidin. B, HeLa cells were treated with GST-CN F1 (lane 1, 2 h) or GST-CN FY (lanes 3-8) for the same time periods as in A and lysed. For analysis of the total amount of the respective GTPase in the lysates, 1/10 of the volume was taken for Western blot analysis. 9/10 of the volume was incubated with bead-coupled GTPase-binding domains of rhotekin, PAK, or POSH. The amounts of activated RhoA, Rac1, and Cdc42 were analyzed by immunoblotting with specific antibodies. The total amount of the GTPases was analyzed by Western-blotting of the lysates.
Notably, we observed marked differences in cell morphology induced by GST-CNF1 compared with GST-CNFY. HeLa cells treated with GST-CNFY showed strong formation of stress fibers, but no flattening, membrane ruffling, or formation of filopodia within the first 6 h of toxin treatment, indicating activation of RhoA, but suggesting that Rac and Cdc42 are not activated. After 24 h of treatment with GST-CNFY, however, the cells were spread out, and membrane ruffling was detectable. In all in vitro studies performed, we detected stronger activity of GST-CNF1 as compared with the recombinant Yersinia toxin.

In contrast, we found a stronger activation in intact cells of RhoA by GST-CNFY than after incubation with the same amount of GST-CNF1. For greater insight into the morphological differences observed, we performed pull-down experiments with bead-coupled GTPase-binding domains of rhotekin, PAK, or POSH, respectively, to exclusively precipitate activated Rho, Rac, or Cdc42 from lysates of toxin-treated cells. To allow comparison of GTPase activation and morphology, HeLa cells were treated with the toxins for the same time periods, fixed, and stained for F-actin. As known from previous studies, incubation of the cells for 2 h with GST-CNF1 leads to activation of RhoA, Rac, and Cdc42, with preference for Rac and with the corresponding formation of stress fibers, membrane ruffles, and filopodia in HeLa cells (13). In contrast, with GST-CNFY, strong activation of RhoA was evident after 1 h of incubation. RhoA activation was still present after 24 h of toxin treatment, but no activation of Rac and Cdc42 was detected by pull-down experiments during the first 6 h of treatment with GST-CNFY and, subsequently, no membrane ruffling or formation of filopodia was observed. Activation of the GTPases detected in the pull-down experiments clearly depicts the morphological changes induced in HeLa cells.

The finding of a selective activation of RhoA in intact cells is supported by the toxin-induced morphology of rat hippocampal neurons. In these cells, GST-CNF1 and GST-CNFY induced retraction of dendrites, indicating activation of RhoA. In contrast, only GST-CNF1 but not GST-CNFY induced the formation of small finger-like extensions in these cells, indicating that RhoA is activated exclusively by the Yersinia toxin.

Our data show that Rac and Cdc42 is not activated by GST-CNFY in intact cells within the first 6 h of toxin treatment, whereas RhoA is activated strongly already after 1 h of incubation with GST-CNFY. In line with this, we detected no degradation of Rac in GST-CNFY-treated cells, whereas GST-CNF1-activated Rac is degraded by a proteasome-dependent pathway (Refs. 5 and 6 and data not shown). This finding again suggests that Rac is not activated by GST-CNFY in intact cells. In contrast, we found a decrease of total RhoA in cells treated with the Yersinia toxin for 24 h (compare Fig. 5B, lane 8). Similar results were obtained with thrombin-cleaved CNFY (not shown).

There are many examples of toxins with a broader substrate specificity in vitro as compared with their actions in the living cell (14–17). Moreover, substrate specificity has been shown to be even cell-type dependent. For example, the Pseudomonas aeruginosa exotoxins ADP-ribosyltransferase modifies a different set of small GTPases in different cell lines (18).
The reason for the tighter specificity of CNF_Y in living cells might be due to a different uptake mechanism or different localization within the cell and remains to be analyzed.

All together, our data strongly indicate that CNF1 and CNF_Y have different substrate specificity in intact cells. CNF_Y is a specific and direct activator of RhoA in HeLa cells and primary hippocampal neurons and can be used as an important new tool for the permanent activation of RhoA without concomitant activation of other Rho family GTPases like Rac and Cdc42.

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