Rho-Specific *Bacillus cereus* ADP-Ribosyltransferase C3cer Cloning and Characterization

Christian Wilde, Martin Vogelsgesang, and Klaus Aktories*

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Universität Freiburg, Otto-Krayer-Haus, Albertstrasse 25, D-79104 Freiburg, Germany

Received April 11, 2003; Revised Manuscript Received June 4, 2003

ABSTRACT: C3-like ADP-ribosyltransferases represent an expanding family of related exoenzymes, which are produced by *Clostridia* and various *Staphylococcus aureus* strains. Here we report on the cloning and biochemical characterization of an ADP-ribosyltransferase from *Bacillus cereus* strain 2339. The transferase encompasses 219 amino acids; it has a predicted mass of 25.2 kDa and a theoretical isoelectric point of 9.3. To indicate the relationship to the family of C3-like ADP-ribosyltransferases, we termed the enzyme C3cer. The amino acid sequence of C3cer is 30 to 40% identical to other C3-like exoenzymes. By site-directed mutagenesis, Arg^{59}, Arg^{97}, Tyr^{151}, Arg^{155}, Thr^{178}, Tyr^{180}, Gln^{183}, and Glu^{185} of recombinant C3cer were identified as pivotal residues of enzyme activity and/or protein substrate recognition. Precipitation experiments with immobilized RhoA revealed that C3cerTyr^{180}, which is located in the so-called "ADP-ribosylating toxin turn-turn" (ARTT) motif, plays a major role in the recognition of RhoA. Like other C3-like exoenzymes, C3cer ADP-ribosylates preferentially RhoA and RhoB and to a much lesser extent RhoC. Because the cellular accessibility of recombinant C3cer is low, a fusion toxin (C2IN−C3cer), consisting of the N-terminal 225 amino acid residues of the enzyme component of C2 toxin from *Clostridium botulinum* and C3cer was used to study the cytotoxic effects of the transferase. This fusion toxin caused rounding up of Vero cells comparable to the effects of Rho-inactivating toxins.

Various bacterial proteins interfere with eukaryotic cell functions by acting as ADP-ribosyl transferases on essential cellular proteins. A typical example for this type of protein is the family of C3-like ADP-ribosyltransferases (*1*−*3*). It comprises the prototype *Clostridium botulinum* exoenzyme C3bot (*4*−*6*), including the isoforms C3bot1 and C3bot2, the exoenzyme from *Clostridium limosum* (C3lim) (*7*), and various transferases from *Staphylococcus aureus* including C3stau1, formerly known as EDIN A (*8*), C3stau2 (EDIN B) (*9*), and C3stau3 (EDIN C) (*10*). The members of this family are 23−25 kDa proteins with an isoelectric point between 9 and 9.6 and are 29−77% identical to each other in their amino acid sequences. Generally, the exoenzymes are produced as single-chain proteins with a signal sequence of about 40 amino acids, which is cleaved during secretion into the medium.

C3-like ADP-ribosyltransferases specifically modify the small GTP-binding proteins RhoA, B, and C by transferring the ADP-ribose moiety of NAD onto the acceptor amino acid Asn41 (*1*, *11*, *12*). This leads to the biological inactivation of the Rho proteins and to inhibition of signal processes governed by the GTPases (*7*, *13*−*15*). Additionally, C3stau2 from *S. aureus* strain HMI6 modifies RhoE and Rnd3, which are two recently identified GTP-binding proteins (*9*). Like other ADP-ribosyltransferases, C3-like exoenzymes possess NAD glycohydrolase activity of unknown biological significance.

Although C3-like ADP-ribosyltransferases share only limited sequence similarity, key amino acid residues and short peptide stretches are conserved in all of them. Recently, the crystal structures of C3bot1 either unbound or bound to NAD were solved. Together with data derived from biochemical studies and site-directed mutagenesis (*16*, *17*), the crystal structure analysis largely improved our understanding of the mechanism of the ADP-ribosylation reaction of Rho-ADP-ribosylating transferases. Accordingly, the active site of the exoenzymes is formed by a α-helix bent over a *β*-strand, surrounded by two *β*-strands, in which key amino acids are located. The key residues include (i) a C-terminal located glutamate, termed "catalytic glutamate", (ii) a glutamine residue two positions upstream, which is thought to increase the nucleophilicity of the acceptor residue asparagine, and (iii) an aromatic residue (Phe or Tyr) three positions upstream of the glutamine, which appears to be involved in recognition of the substrate RhoA. Due to this architecture, the C3-like ADP-ribosyltransferases were grouped as toxins with an "ADP-ribosylation-toxin turn-turn (ARTT) motif" (*18*). A further structural motif was postulated as being essential for NAD-binding. It was termed the phosphate nicotinamide (PN) loop (*19*) and covers residues 137−146 in C3bot (note that the counting of the residues is without the signal sequence). This motif closes the NAD binding pocket together with the ARTT-motif thereby stabilizing the binding of the NAD molecule.

* To whom correspondence should be addressed: Dr. Klaus Aktories, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Otto-Krayer-Haus, Albertstrasse 25, D-79104 Freiburg, Germany. Phone: +49-761-2035301. Fax: +49-761-2035311. E-mail: Klaus.Aktories@pharmakol.uni-freiburg.de.

10.1021/bi034583b CCC: $25.00
Published on Web 07/26/2003

Bacillus cereus is an important pathogen involved in local infections, bacteriemia, central nervous system infections, respiratory infections, and severe food poisoning (*20*). The pathogen produces several toxins including at least four different hemolytic and non-hemolytic enterotoxins, several phospholipases, proteases, and hemolysins (*20*−*24*). Recently, it was reported that *B. cereus* also produces an ADP-ribosyltransferase, which modifies Rho GTPases at Asn41 (*25*). Previous partial protein sequencing suggested a rather distant structural relationship to other C3-like transferases. Here we report on the coding sequence of the ADP-ribosyltransferase from strain 2339 showing that C3cer forms a distant subfamily of C3 transferases. We studied the substrate specificity and the structure−function analysis of the enzyme by site-directed mutagenesis, and we investigated the cytotoxicity of C3cer using the fusion toxin C2IN−C3cer.[1]

MATERIALS AND METHODS

Material and Chemicals. Oligonucleotides were obtained from MWG (Ebersberg, Germany). The pGEX-2T vector and the glutathione *S*-transferase Gene Fusion system were from Pharmacia Biotech (Uppsala, Sweden), and the Quick-Change kit was from Stratagene (Heidelberg, Germany). Restriction endonucleases were from NEB (Schwalbach, Germany) and [^{32}P]-NAD was from NEN (Belgium). All other chemicals were from commercial sources.

Cloning of the Recombinant C3cer ADP-Ribosyltransferase. Alignment of the peptides derived from protease-digested, native ADP-ribosyltransferase from *B. cereus* (*26*) with other known C3-like ADP-ribosyltransferases showed that peptide 4 is probably located in the C-terminal part of the transferase. First, we designed a primer related to the N-terminus of the ADP-ribosyltransferase and a second one related to the C-terminally located glutamate of peptide 4. Both primers were constructed in consideration of the codon-usage of several *Bacillus* strains. Using Taq-polymerase (NEB, Germany), a 567-bp fragment was amplified from genomic DNA of *B. cereus* strain 2339. The fragment was subcloned into the pCR2.1 vector (Invitrogen, The Netherlands) and sequenced with the Cycle Sequencing Ready Reaction kit (Perkin-Elmer, USA). For determination of the remaining base pairs, genomic DNA from *B. cereus* 2339 was totally digested with Nsi I and separated on an agarose gel. The fragments were extracted with the JetSorb kit (Genomed, Germany) and ligated in a Pst I digested pUC18 vector. PCR was performed with a specific pUC18 primer and a second primer, which was specific for the nucleotide sequence of the ADP-ribosyltransferase, covering base pairs 486−506 (5′-GGTAGGC TCTGGGACTCATGG-3′). PCR products were subcloned into pCR 2.1 TOPO-TA vector and sequenced. By this method, we identified one clone carrying the remaining 105 bp, followed by an in frame stop-codon. The complete coding sequence of the mature ADP-ribosyltransferase was amplified from genomic DNA and cloned into the pGEX 2TGL vector, followed by DNA sequencing.

Expression of the Recombinant ADP-Ribosyltransferases. For expression of recombinant C3cer wild type (rWT C3cer) as glutathione *S*-transferase fusion protein, the pGEX 2TGL-C3cer vector was transformed into *Escherichia coli* TG1 cells by standard methods. 1 L of LB medium containing 100 μg/mL ampicillin was inoculated with 30 mL of an overnight culture, and cells were grown at 37 °C until the optical density at 600 nm reached 0.8. IPTG was added to a final concentration of 0.2 mM, and cells were grown for another 6 h at 37 °C. Cells were collected by centrifugation, resuspended in lysis buffer (50 mM HEPES [pH 7.5] and 1 mM PMSF), and broken with a French press (SLM Aminco, Spectronic Instruments). The lysate was centrifuged, and glutathione-Sepharose beads were added to the supernatant and incubated for 45 min at 4 °C. Beads were washed with 100-fold beads volume wash buffer (50 mM HEPES [pH 7.5] and 150 mM NaCl). GST-C3 was eluted with a buffer containing 10 mM reduced glutathione, 50 mM HEPES [pH 7.5], and 100 mM NaCl. The GST-carrier was cleaved with thrombin followed by its removal with benzamidine beads. C3bot1, C3lim, C3stau1 were purified as described elsewhere (*4*, *7*, *9*). All purification steps were checked by SDS−PAGE.

SDS−PAGE. SDS−polyacrylamide gel electrophoresis was performed according to the method of Laemmli (*27*). Gels were stained with Coomassie Brilliant Blue R250, dried, and further analyzed.

Construction and Expression of Mutant Recombinant C3cer. C3cer mutants were constructed by site-directed mutagenesis with the pGEX-2T-C3cer plasmid as a template and the respective oligonucleotides using the Quick-Change kit according to the manufacturer's instructions. From two complementary primers needed, only one is indicated: C19A, 5′-CAA ATA TAA GCT GGC TAC AAA CAA AG-3′; R59K, 5′-GAA TTT TTA AAA ATG CAT GCG G-3′; R97K 5′-TAT AAA AGT GTA TAA AGG GGA TGA TGC-3′; Y151V, 5′-ATT GAT GCC GGA GTT GCC AAA ACA AGA CC-3′, R155E, 5′-TAT GCC AAA ACA GAA CCA GTT ATG ACA GAA-3′; T178A, 5′-CAG ATG ACT TGG CTG CGT ACC CAG-3′; Y180T, 5′-GAC TTG ACT GCG ACA CCA GGG CAA TAC G-3; Q183E, 5′-CGT ACC CAG GGG AAT ACG AAT TAT TAT TG-3′; E185Q, 5′-CCA GGG CAA TAC CAA TTA TTA TTG C-3′. Mutated plasmids were transformed in *E. coli* TG1 cells, and all mutations were confirmed by DNA sequencing. Proteins were expressed as thrombin-cleaved proteins as described above.

ADP-Ribosylation Reaction. 2 μM recombinant RhoA, RhoB, RhoC, or RhoC I43V, Rac1, Cdc42, RalA, RhoE, Rnd3, or Ras were incubated with rWT C3cer or mutant protein at various concentrations in a buffer containing 50 mM HEPES (pH 7.3), 2 mM $MgCl_2$, and various concentrations of [adenylate-^{32}P] NAD at 37 °C as indicated. Radiolabeled proteins were analyzed by phosphorimaging, and the amount of incorporated [^{32}P] ADP-ribose was calculated from Phosphorimager data.

Glucosylation Reaction. 2 μM recombinant RhoA, B, and C were incubated with the enzymatically active fragment CDB 1−546 (5 nM final concentration) from *Clostridium difficile* toxin B as described previously (*28*). Radiolabeled proteins were analyzed by phosphorimaging.

NAD Glycohydrolase Reaction. For detection of glycohydrolase activity, 200 μM [adenylate-^{32}P] NAD were

[1] Abbreviations: rWT C3cer, recombinant wild-type C3cer expressed and purified from *E. coli*; C2IIa, activated C2II toxin from *Clostridium botulinum*; C2IN−C3cer, fusion toxin consisting of the N-terminal half of C2I toxin from *C. botulinum* and the complete rWT C3cer.

incubated with various concentrations of either rWT C3cer or mutant C3cer in a buffer containing 50 mM HEPES (pH 7.3) and 2 mM $MgCl_2$ at 37 °C for up to 3 h. Three microliter aliquots of the reaction mix were separated by TLC on Silica Gel 60_{F254} (Merck, Germany) with 66% 2-propanol and 0.33% ammonium sulfate, followed by phosphor imaging. The amount of cleaved [adenylate-^{32}P] ADP-ribose was calculated from Phosphorimager data.

Precipitation Assay. Wild-type RhoA was expressed as recombinant glutathione *S*-transferase fusion protein in *E. coli* and coupled to glutathione beads. About 1.5 μg of RhoA was incubated with 300 ng of toxin (rWT C3cer or mutant) in a buffer containing 50 mM HEPES (pH 7.3), 2 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM PMSF and Nonidet P40 (final concentration 0.5%) for 45 min at 4 °C. Beads were washed 3 times with buffer and subjected to SDS−PAGE. After transferring proteins onto nitrocellulose, bound toxin was detected with a specific polyclonal antibody against rWT C3cer (1:10 000 in PBST).

Construction of C2IN−C3cer Fusion Toxin. The C2IN−C3cer fusion toxin was constructed as described for C2IN−C3lim fusion toxin (*29*) using the Bgl II/BamH I site flanking the ADP-ribosyltransferase gene. The recombinant fusion toxin was expressed as glutathione *S*-transferase fusion protein and purified as thrombin-cleaved recombinant protein. Purification was checked by SDS−PAGE.

Cytotoxicity Assay. Vero cells were grown to subconfluency at 37 °C, 5% CO_2 in Dulbecco's minimum essential medium (DMEM) containing 10% FCS, 2 mM L- glutamate, 100 U of penicillin/mL, and 100 μg/mL streptomycin. For cytotoxic assays, medium was changed to serum-free DMEM and subconfluent cells were treated with either activated C2II or C2IN−C3cer alone or with activated C2II in combination with increasing concentrations of C2IN−C3cer or C2IN−C3lim as control. Pictures were taken after 3.5 h. For differential ADP-ribosylation, Vero cells were treated as described above. Cells were washed three times with PBS, scraped off the plates, and lysed by sonication. About 30 μg of lysate (Bradford) was used for [^{32}P]-ADP-ribosylation by C2IN−C3cer as described above. For the quantification of the total amount of RhoA, about 80 μg of lysate (Bradford) was probed with a specific RhoA antibody (Clone 26C4, 1:500, Santa Cruz Biotechnology).

RESULTS

Nucleotide Sequence of C3cer. The native ADP-ribosyltransferase from *B. cereus* was purified recently, and amino acid sequences of peptide fragments were determined by peptide sequencing (*26*). Here we cloned the transferase from the genomic DNA of *B. cereus* strain 2339 with primers deduced from the N-terminus of the purified, native transferase and the 3′ end of the complete gene. Sequencing analysis revealed an open reading frame covering 657 bp, followed by an in-frame stop-codon with a GC-content of 33%. Recombinant wild-type (rWT) C3cer covered 219 amino acids; it has a mass of 25.2 kDa and a theoretical isoelectric point of 9.30. Furthermore, the deduced amino acid sequence matches four peptides, which were previously generated by proteolytic digestion of the native transferase and determined by peptide sequencing (*26*).

Alignment of C3cer with Other C3-Like ADP-Ribosyltransferases. Comparison of the amino acid sequence of

A

	aa	MG [kDa]	IP
C3bot 1	211	23.56	9.60
C3bot 2	204	23.20	9.46
C3lim	205	23.01	9.57
C3stau1	212	23.79	9.02
C3stau2	212	23.64	9.42
C3stau3	221	25.07	8.99
C3cer	219	25.25	9.30

B

	C3bot 1	C3bot 2	C3lim	C3stau1	C3stau2	C3stau3	C3cer
C3bot 1	100	65	63	34	34	33	38
C3bot 2	65	100	67	34	35	33	36
C3lim	63	67	100	35	34	31	36
C3stau1	34	34	35	100	77	65	29
C3stau2	34	35	34	77	100	69	29
C3stau3	33	33	31	65	69	100	31
C3cer	38	36	36	29	29	31	100

FIGURE 1: (A) Properties of C3-like ADP-ribosyltransferases. aa: amino acids; IP: theoretical isoelectric point. Accession numbers are P15879 [C3bot1], Q00901 [C3bot2], X87215 [C3lim], P24121 [C3stau1], AJ277173 [C3stau2], AP003088 [C3stau3], AJ429241 [C3cer]. (B) Amino acid identity between C3-like ADP-ribosyltransferases. Sequences (without signal sequence) were aligned using the program "pair wise blast" at NCBI and identity is given in %.

C3cer with related C3-like ADP-ribosylating enzymes revealed some characteristics of this family of enzymes: (i) all transferases have approximately the same size of 204−221 amino acids, (ii) their molecular masses range from 23 to 25 kDa, and (iii) all of them are basic proteins, having theoretical isoelectric points between 8.99 and 9.60 (Figure 1A). In contrast, their amino acid identity is rather weak, ranging from 29 to 77% (Figure 1B), although several residues are conserved between all C3-like ADP-ribosyltransferases (Figure 2). From those, C3cer Thr^{178}, Tyr^{180}, Gln^{183}, and Glu^{185} are localized at the C-terminus and are highly conserved between all C3-like exoenzymes. The glutamate residue is pivotal for enzymatic activity of all known ADP-ribosyltransferases, while the glutamine residue is present in all transferases, which modify their target proteins at an asparagine residue. The aromatic and the hydrophilic residue are part of the so-called "ARTT"-motif, which is involved in the recognition of RhoA (*30*). A third region, described as the "STS" motif, which is critical for enzymatic activity of several ADP-ribosylating toxins, covers residues C3cer Ser^{142}, Thr^{143}, and Ser^{144}. Furthermore, three arginine residues, C3cer Arg^{59}, Arg^{97}, and Arg^{155} are conserved among the family of C3-like transferases.

Substrate Specificity of the Recombinant ADP-Ribosyltransferase. Using high concentrations of the transferases, we found that recombinant C3cer ADP-ribosylated only RhoA, B, and C. Rac1 was only modified in traces, while Cdc42, RhoE, Rnd3, RalA, and Ras were not modified (Figure 3A,B or data not shown). These findings are in agreement with previously reported data. In more detail, we found that the specific ADP-ribosyltransferase activity of rWT C3cer for RhoA was 3−5 times lower if compared to the other C3-like ADP-ribosyltransferases (Figure 3E). Moreover, we were surprised to find that the various Rho subtypes Rho A, B, and C differed considerably in their substrate properties. Therefore, the K_m values for NAD and the V_{max} of the recombinant exoenzyme-catalyzed ADP-

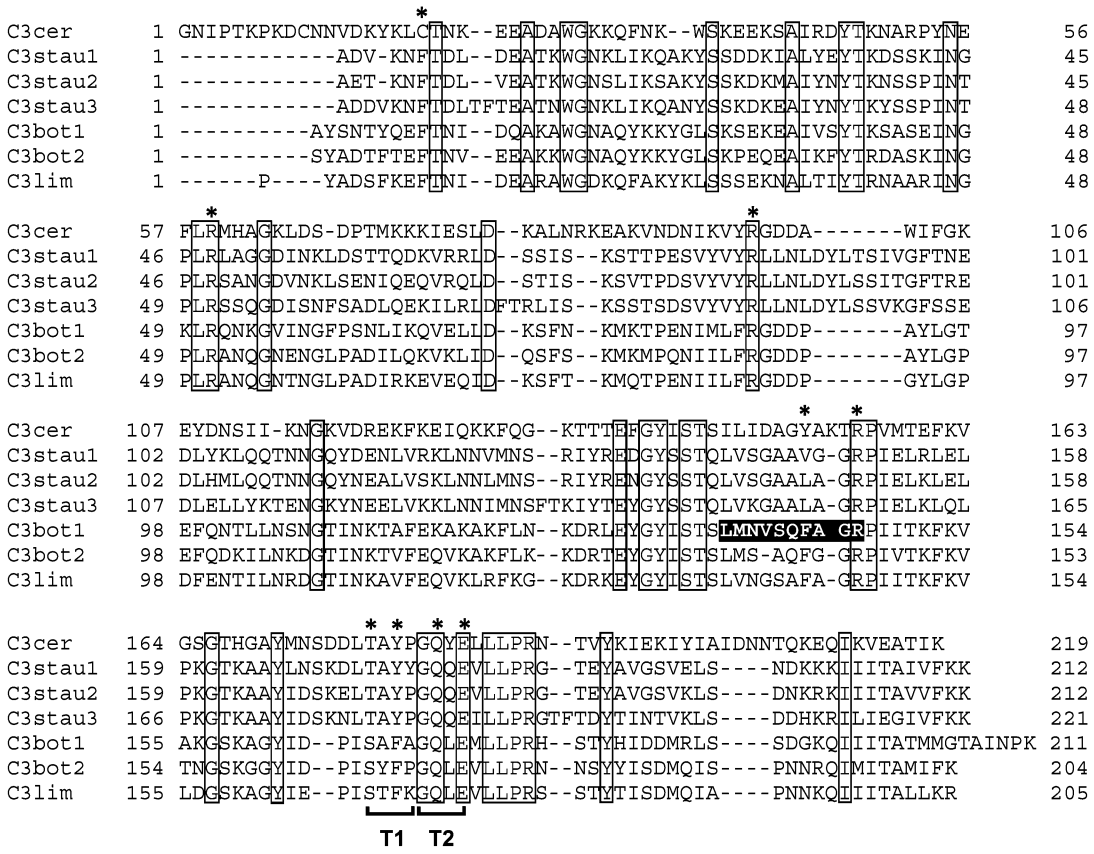

FIGURE 2: Alignment of C3cer amino acid sequence with other C3-like ADP-ribosyltransferases. Numbers indicate the position of the residues in the whole protein. Conserved residues are boxed, and an asterisk marks mutated residues in the C3cer sequence. The ADP-ribosylating toxin turn-turn ("ARTT")-motif is indicated under the C3lim sequence. T1: Turn1, T2: Turn2. Amino acid residues covering the phosphate nicotinamide loop (PN loop) are boxed in C3bot.

ribosylation of RhoA, B, and C were determined. Whereas the K_m values for NAD were in the same range for the Rho isoforms (RhoA 20.8 μM; RhoB 56.4 μM and RhoC 17.1 μM), the specific ADP-ribosyltransferase activity of rWT C3cer for RhoA was determined to be 22.25 mol (mol of toxin)$^{-1}$ min^{-1}, while it was 4.19 mol (mol of toxin)$^{-1}$ min^{-1} for Rho B and only 0.99 mol (mol of toxin)$^{-1}$ min^{-1} for RhoC (Figure 3D). To study whether this rank order for modification of Rho proteins was typical for C3cer, we compared the ADP-ribosylation of Rho isoforms by other C3-like exoenzymes. All recombinant exoenzymes tested modified Rho GTPases with the rank order RhoA > RhoB > RhoC in respect to V_{max} (Figure 3E and data not shown). Exchange of only one residue in RhoC (Ile43Val) resulted in an increase in the V_{max} value of ADP-ribosylation catalyzed by the various C3 ADP-ribosyltransferases, indicating that this structural feature might be responsible for the low rate of modification of RhoC as compared to RhoA (Figure 3B−D and data not shown). As a control for protein integrity, RhoA, B, and C were tested in the glucosylation reaction catalyzed by the active fragment CDB 1−546 from *C. difficile* toxin B. In contrast to the ADP-ribosylation, RhoA, B, and C were glucosylated with the same kinetic (Figure 3F). Interestingly, we found that C3stau1 (EDIN A) also modified RhoE/Rnd 3, as it has been described for C3stau2 (EDIN B).

Structure−Function Analysis of Recombinant C3cer. Using photoaffinity labeling and protein sequencing, first indications for the localization of the active site of C3cer were obtained recently (*26*). Here we extended the structure−function analysis of the ADP-ribosyltransferase. Therefore, we generated several C3cer mutants, and expressed and purified them as thrombin-cleaved proteins from *E. coli*. The specific ADP-ribosyltransferase activity of rWT C3cer was 22.25 mol (mol of toxin)$^{-1}$ min^{-1} as determined with recombinant RhoA as a protein substrate (Table 1). Whereas rWT C3cer modified recombinant RhoA in a time-dependent manner, C3cer R59K, R97K, R59K/R97K, T178A, T178A/Q183E, Q183E, and E185Q possessed strongly decreased or no detectable ADP-ribosyltransferase activity (Figure 4A and/or Table 1), confirming the essential role of these residues in enzymatic activity. Recently, Han et al. described an "ADP-ribosylating toxin turn-turn" (ARTT) motif in the transferase C3bot, which is part of the loop that links β-strands 5 and 6. This motif is suggested to be important for the ADP-ribosylation of RhoA (*18*). Within this motif, an aromatic residue (Tyr^{180} in C3cer) is proposed to play an important role in the recognition of RhoA. A C3cer mutant in whom this tyrosine is changed to threonine showed decreased binding to RhoA and no detectable ADP-ribosyltransferase activity (Figure 4A,B and/or Table 1). Further mutations were done in the "PN loop" (phosphate nicotinamide loop), which links β-strands 3 and 4 and is suggested to stabilize one phosphate and the nicotinamide moiety of NAD. Exchange of Tyr^{151} or Arg^{155} in C3cer, which are both located in this region, abolished the ADP-ribosyltransferase activity of these recombinant mutants (Table 1).

NAD Glycohydrolase Activity. ADP-ribosyltransferases catalyze the hydrolysis of NAD in the absence of a specific protein substrate. The specific NAD glycohydrolase activity

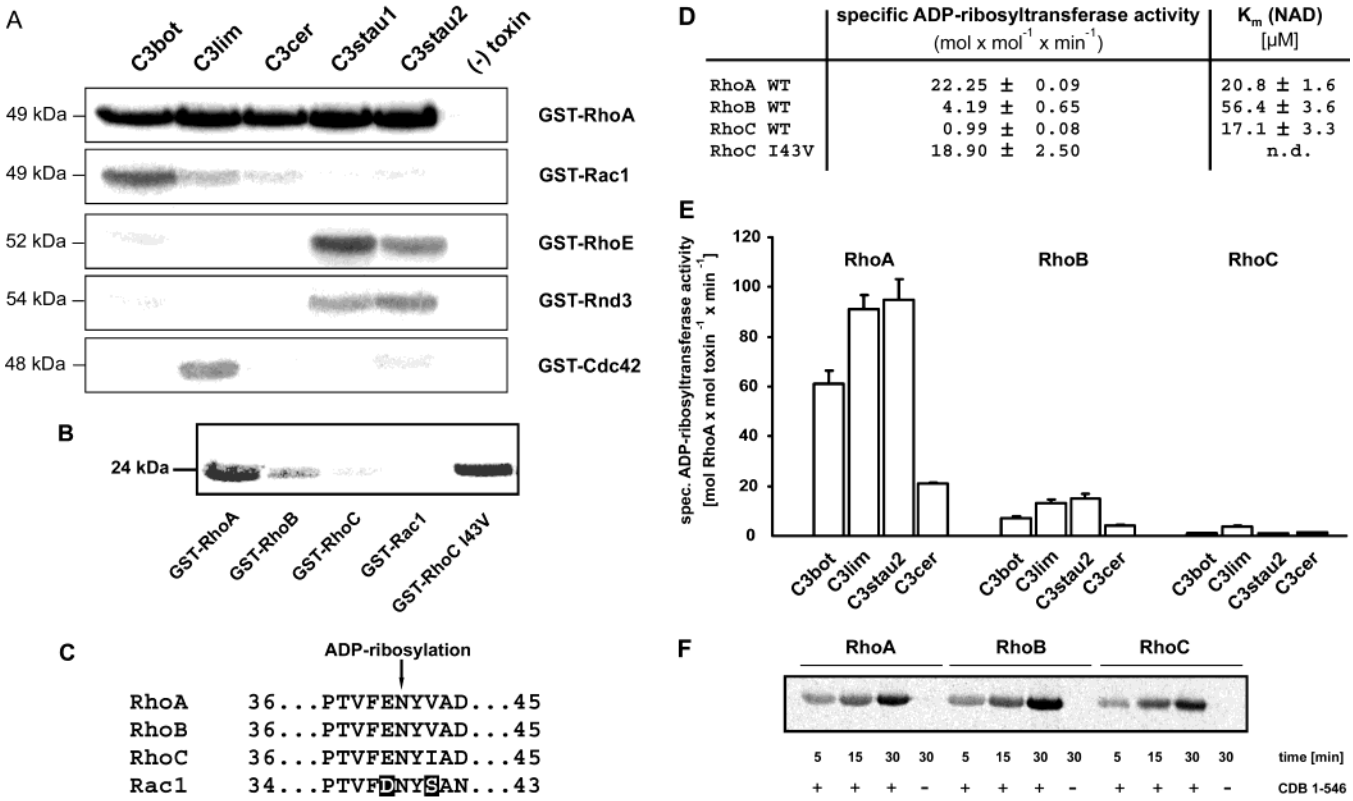

FIGURE 3: Substrate specificity of recombinant C3cer. (A) 1 μg of the indicated GTPases ($\sim$2 μM) were ADP-ribosylated by recombinant C3-like ADP-ribosyltransferases (20 nM final for 10 min at 37 °C for GST-RhoA or 200 nM final for 60 min at 37°C for other GST-GTPases) in the presence of [^{32}P]NAD. (Note the enzyme concentrations and incubation times are much higher than for determination of the specific enzyme activity as given in panel E). Radiolabeled probes were subjected to SDS−PAGE and the autoradiography is shown. (B) 1 μg of the indicated GST-GTPases ($\sim$2 μM) were ADP-ribosylated by rWT C3cer (10 nM final, 5 min at 37 °C) in the presence of [^{32}P] NAD. Radiolabeled probes were subjected to SDS−PAGE and the autoradiography is shown. (C) Alignment of partial sequence of RhoA, B, C, and Rac1. Numbers indicate the position of the residues in the whole protein. Residues, which have been identified in RhoA to be critical for ADP-ribosylation, are boxed in Rac1. An arrow marks the acceptor amino acid Rho Asn41 in the C3-catalyzed ADP-ribosylation. (D) K_m for NAD in the ADP-ribosylation of RhoA, B, and C catalyzed by rWT C3cer and specific ADP-ribosyltransferase activities of rWT C3cer either for RhoA, B, or C or RhoC I43V. (E) Specific ADP-ribosyltransferase activities of C3-like exoenzymes for RhoA, B, and C. Recombinant Rho GTPases were ADP-ribosylated as described under Materials and Methods. Specific activities for each Rho protein were calculated from the linear phase of the reaction (2 nM final toxin concentration for RhoA, 5 nM toxin for RhoB and 20 nM toxin for RhoC for 0.5, 1, or 2 min at 37 °C) using Phosphor-imager and are given in mol ADP-ribosylated Rho per mol toxin per minute. (F) Glucosylation of GST-RhoA, B, and C by *C. difficile* active fragment CDB 1−546. Reaction was performed as described under Materials and Methods, and the autoradiography is shown.

of rWT C3cer was 0.29 mol of hydrolyzed [^{32}P] NAD (mol of toxin)$^{-1}$ min^{-1}. On the other hand, C3cer R59K, R97K, R59K/R97K, Y151V, R155E, or E185Q exhibited strongly decreased or no NAD glycohydrolase activity (Table 1). C3cer T178A, T178A/Q183E and Q183E, which also had no ADP-ribosyltransferase activity, exhibited only a 50−70% reduced NAD glycohydrolase activity as compared to recombinant wild type. In contrast, C3cer C19A and Y180T possessed a 2−5-fold increased NAD glycohydrolase activity (Table 1).

Binding of Recombinant C3cer Proteins to RhoA. To examine the ability of rWT C3cer and mutants to bind RhoA, we set out a precipitation assay with these proteins. All tested proteins, except C3cer Y180T, were capable of binding to immobilized RhoA, although they were unable to ADP-ribosylate RhoA (Table 1 and Figure 4B). The amount of bound protein was comparable to that of rWT C3cer, indicating a normal binding affinity for their protein substrate RhoA. Moreover, all tested C3er proteins in this assay were recognized equally well by the antiserum (data not shown).

Cytotoxicity of Recombinant C3er. C3-like ADP-ribosyltransferases exhibited very poor cell accessibility. Due to this fact, we constructed a fusion toxin consisting of the N-terminal part of the C2I toxin from *C. botulinum* and the complete ADP-ribosyltransferase from *B. cereus* and compared it to the original described C2IN−C3lim fusion toxin. In combination with the activated binding component C2II, increasing concentrations of C2IN−C3cer led to rounding up of Vero cells with only small extensions remaining (Figure 5B−D) as it was observed with the control toxin C2IN−C3lim (Figure 5E). Conversely, activated C2II or C2IN−C3cer alone had no effect on Vero cells (Figure 5F,G). To test whether these morphological effects were related to ADP-ribosylation of Rho proteins, toxin-treated cells were lysed and cellular Rho was ADP-ribosylated in the lysate by C2IN−C3cer fusion toxin in the presence of [^{32}P]NAD. A concentration-dependent decrease of radiolabeling of Rho proteins was observed (Figure 5H). Moreover, Western Blot analysis showed that these findings were not due to the degradation of cellular RhoA, because equal amounts of RhoA could be detected in each probe (Figure 5I), indicating that the fusion toxin C2IN−C3cer modified Rho in intact cells.

Table 1: Summary of Properties of Recombinant C3cer Wild Type (rWT) and Recombinant C3cer Mutants[a]

toxin	ADP-ribosyl-transferase activity [relative activity in %]	NAD glyco-hydrolase activity [relative activity in %]	RhoA binding
C3cer WT	100	100	+
C3cer C19A	118 ± 0.09	197 ± 10.8	n.d.
C3cer R59K	1.1 ± 0.09	<0.01	+
C3cer R97K	1.9 ± 0.05	<0.01	+
C3cer R59K/R97K	<0.01	<0.01	+
C3cer Y151V	1.6 ± 0.08	4.1 ± 0.03	+
C3cer R155E	0.2 ± 0.001	<0.01	+
C3cer T178A	1.3 ± 0.2	52 ± 1.4	+
C3cer T178A/Q183E	<0.01	29 ± 2.0	n.d.
C3cer Y180T	<0.01	545 ± 10.4	(+)
C3cer Q183E	<0.01	45 ± 3.5	+
C3cer E185Q	<0.01	<0.01	+

[a] The specific ADP-ribosyltransferase and NAD glycohydrolase activities were measured as described under experimental procedures and determined as 22.25 mol (mol of toxin)$^{-1}$ min^{-1} for transferase and 0.29 mol of hydrolyzed [^{32}P] NAD (mol of toxin)$^{-1}$ min^{-1} for glycohydrolase activity, respectively. For calculation of the relative enzyme activities, rWT C3cer activity was set as 100% and SE is given from three independent experiments. For immunoprecipitation experiments, glutathione Sepharose beads-coupled RhoA was incubated with the indicated proteins. Beads were washed, subjected to SDS−PAGE, and blotted onto nitrocellulose. Bound toxin was detected with a specific polyclonal C3cer antibody. +: normal binding to RhoA, (+): reduced binding to RhoA, n.d. not determined. Data are summarized from Figure 4B.

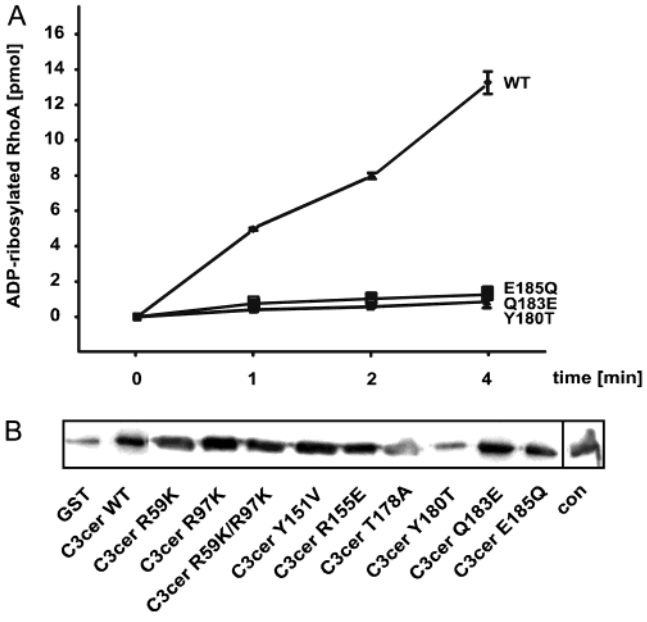

FIGURE 4: (A) Time-dependent ADP-ribosylation of GST-RhoA by rWT C3cer and mutant recombinant C3cer. 1 μg of RhoA (~2 μM) was incubated with the indicated toxins in the presence of [^{32}P]NAD for up to 4 min. Radiolabeled probes were subjected to SDS−PAGE, and the amount of modified RhoA was quantified by phosphorimaging. The concentrations of rWT C3cer were 10 nM and 1 μM for C3cer Y180T, Q183E, and E185Q. (B) Precipitation of rWT C3cer and mutant C3cer with GST-RhoA immobilized to glutathione Sepharose beads. 1 μg of GST or 2 μg of GST-RhoA were incubated with either 300 ng of rWT C3cer and mutant C3cer for 45 min at 4 °C as described under Materials and Methods. Bound toxin was detected with a specific polyclonal antibody against rWT C3cer. Con: recombinant C3cer (200 ng) as a control for the antibody.

DISCUSSION

Here we report on the cloning and characterization of the recombinant ADP-ribosyltransferase C3cer from *B. cereus* strain 2339, which is a member of the C3-like exoenzymes known to modify Rho GTPases. The enzyme was identified previously (*25*) and partially characterized (*26*). These earlier studies resulted in determination of the amino acid sequences of four peptides of C3cer, already suggesting that the transferase from *B. cereus* is rather distantly related to other C3-like transferases. Because the previously identified "peptide 4" (*26*) from native C3cer exhibited significant sequence identity with the C-terminal end of other known C3-like transferases, we used it for further detection of the toxin gene. By construction of a library of *B. cereus* genomic DNA and applying a PCR-based method, we identified the nucleotide sequence and cloned the ADP-ribosyltransferase from genomic DNA. The deduced amino acid sequence of the transferase and additional biochemical studies showed that recombinant C3cer shares structural and biochemical features common to other C3-like transferases, including the high isoelectric point of >9, the typical molecular mass between 23 and 25 kDa and the protein substrate specificity of RhoA, B, and C for the ADP-ribosyltransferase reaction. On the other hand, C3cer exhibits the lowest sequence amino acid identity to all other C3-like exoenzymes. Therefore, C3cer seems to represent a distinct branch in the family of C3-like ADP-ribosyltransferases.

Previously, Just et al. identified the NAD-binding site of C3cer by photolabeling with [carbonyl-^{14}C] NAD (*16*). After digestion of the labeled transferase, they showed that the labeling takes place at a peptide, covering the sequence X-Leu-Leu-Leu-Pro-Arg, with X being an unknown PTH-amino acid derivative most likely caused by photolabeling of Glu. The above-mentioned peptide covers residues Glu^{185} through Arg^{190} in the sequence of C3cer. In fact, the sequence comparison with other ADP-ribosyltransferases, the photolabeling data and the site-directed mutagenesis studies shown in this report indicate that Glu^{185} is the so-called "catalytic glutamic acid", which is conserved in prokaryotic as well as eukaryotic ADP-ribosyltransferases (*31*, *32*). Consistent with this fact, C3cer E185Q exhibited no NAD glycohydrolase and no transferase activity, but normal binding to RhoA. This glutamate is located in turn 2 of the so-called "ARTT motif" that has been described by Han and co-workers (*33*) and is present also in actin-ADP-ribosylating toxins such as the vegetative insecticidal protein VIP (*34*). The motif consists of two short loops, turn 1 (residues 178−181 in C3cer) and turn 2 (residues 182−185 in C3cer). A further functional residue, C3cer Gln^{183}, is located in turn 1 and is conserved in all asparagine-modifying C3-like transferases. Exchange in C3cer (Q183E) completely inhibited the transferase activity but caused only a modest reduction in NAD glycohydrolase activity. Similar results were obtained recently with C3stau2 (*17*).

Besides the mentioned glutamine, a hydrophobic residue in turn 1 (Phe^{169} in C3bot, note the different numbering as compared to the reference) was proposed to be involved in protein substrate recognition. Recently, we confirmed this hypothesis by site-directed mutagenesis studies of C3stau2 (*17*). Exchange of the equivalent residue C3cer Tyr^{180} to threonine reduced either ADP-ribosyltransferase activity and binding to immobilized RhoA. Conversely, the NAD

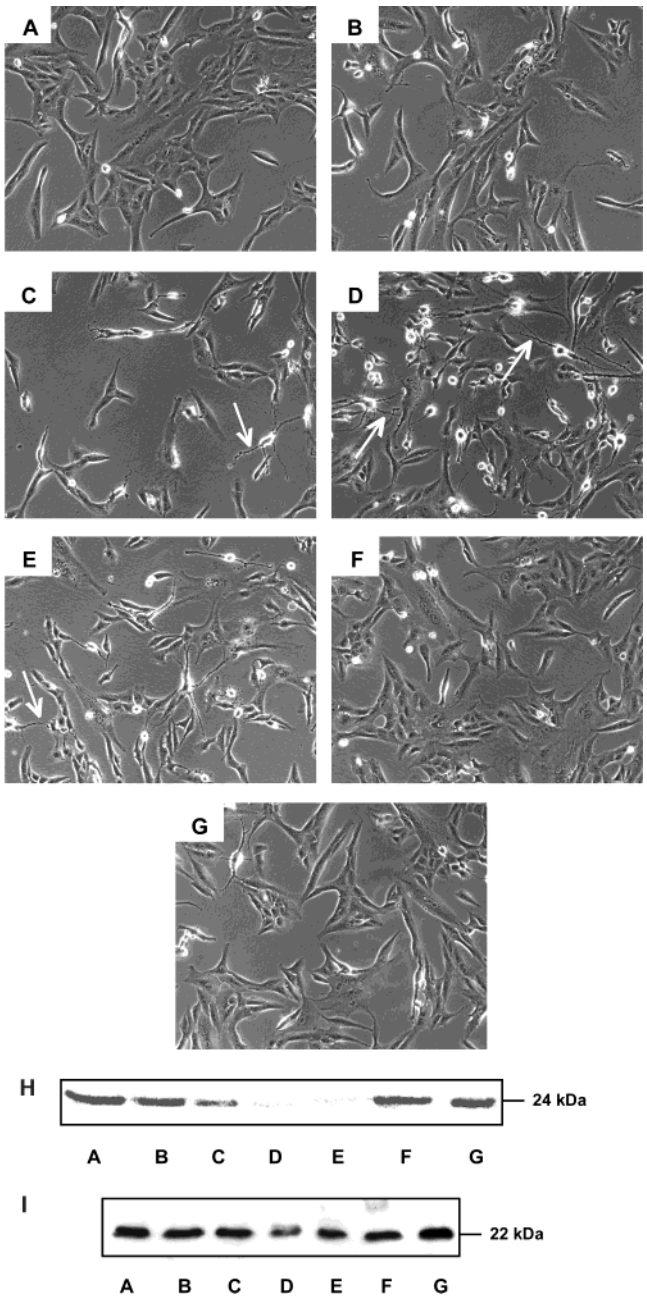

FIGURE 5: (A−G) Cytotoxic effects of C2IN−C3cer and C2IN−C3lim fusion toxin on Vero cells. Cells were grown in DMEM plus 10% fetal calf serum (FCS) to subconfluency. Medium was changed to DMEM without serum, (A) buffer or proteins were added and cells were further incubated at 37 °C. After 3.5 h, cells were photographed and arrows indicate remaining extensions of intoxicated cells. Proteins used are (B) 200 ng/mL C2IIa plus 100 ng/mL C2IN−C3cer, (C) 200 ng/mL C2IIa plus 200 ng/mL C2IN−C3cer, (D) 500 ng/mL C2IIa plus 500 ng/mL C2IN−C3cer, (E) 200 ng/mL C2IIa plus 100 ng/mL C2IN−C3lim, (F) 500 ng/mL C2IN−C3cer alone and (G) 500 ng/mL C2IIa alone. (H) Vero cells, similar treated as described above, were scraped from a 10-cm culture dish, washed, and lysed by sonication. About 30 μg of total cell lysate (Bradford) was [^{32}P]-ADP-ribosylated in the presence of 10 nM C2IN−C3cer for 30 min at 37 °C. Probes were subjected to SDS−PAGE and the autoradiography is shown with numbering the same as in panels A−G. (H) Western Blot analysis with an anti-RhoA antibody of the same probes described under panels A−G.

glycohydrolase activity of C3cer Y180T was increased by about 5-fold, as we observed it with C3stau2. From tryptophan-quenching studies performed with this exoenzyme in the presence of NAD, it was suggested that the affinity for NAD is slightly reduced after mutation, resulting in an increased rate of NAD turnover at the enzyme (*17*). Unfortunately, we were not able to proof this hypothesis with C3cer, because the transferase contains three tryptophan residues (positions 28, 36, and 102). Therefore, no quenching of tryptophan fluorescence could be detected after adding increasing concentrations of NAD to C3cer in solution. Taken together, we propose that within the ARTT-motif, C3cer Gln^{183} is involved the interaction with the acceptor amino acid residue Asn41 of RhoA, thereby defining the specificity of the ADP-ribosylation reaction, while C3cer Tyr^{180} being responsible for the affinity to the protein substrate.

Recently, the structure of C3bot cocrystallized with NAD was reported by Menetrey et al. (*19*). They found that the ARTT motif undergoes a major movement from solvent exposure toward the interior of the NAD binding cleft upon NAD binding thereby covering the nicotinamide ribose by the side chains of Ser^{167}, Phe^{169}, and Gln^{172} (note the different numbering as compared to the reference). C3bot Gln^{172} plays a critical role in this formation of the active site by forming a network of interactions by interacting with C3bot Gln^{142}, which in turn lays along the aromatic ring of Phe^{143} and with a water molecule that links Ser^{167} and Glu^{174}. We compared these structural features with the recently solved crystal structure of ART2.2. This eukaryotic Ecto-ADP-ribosyltransferase shares a glutamine (ART2.2 Gln^{187}) and a glutamate residue (ART2.2 Glu^{189}) at equivalent positions. In addition, a serine (equivalent to Ser^{167} in C3bot and Thr^{178} in C3cer) is involved in the coordination of a water molecule as described for C3bot (*32*). Because this water molecule would be an ideal acceptor for the ADP-ribose in the NAD glycohydrolase reaction, we decided to exchange it to alanine in C3cer. However, C3cer T178A exhibited reduced ADP-ribosyltransferase activity but almost the same NAD glycohydrolase activity as compared to rWT C3cer. These findings suggest that the coordination of this water molecule is not essential for the NAD glycohydrolase activity but rather involved in the ADP-ribosyltransferase reaction of C3cer. Interestingly, the double mutant (T178A/Q183E) exhibited also only 70% reduction of NAD glycohydrolase activity. So far, we are unable to explain our findings in regard to the mentioned network of interactions, which is postulated for these two key residues.

Menetrey and co-workers (*19*) described a novel second structural feature involved in the binding of NAD. It covers residues 137−146 in C3bot (note different numbering). Within this stretch, termed phosphate nicotinamide loop (PN loop), the aromatic side of Phe^{143} stacks against the nicotinamide moiety and Arg^{146} forms hydrogen bonds to one phosphate group of NAD. We were able to confirm the role of the equivalent amino acids in C3cer by mutational analysis, showing that this structural element, which is conserved in all C3-like ADP-ribosyltransferases, has most likely the same function in all C3-like exoenzymes.

Comparing the substrate specificity of C3cer with other C3-like ADP-ribosyltransferases, we found that the toxin modified only RhoA, B, and C. Interestingly, there are major differences in the kinetics of the modification of Rho GTPases, although the amino acid identity between all three is more than 85%. Moreover, we found these differences in the ADP-ribosylation of RhoA not only for C3cer, but also

for C3bot1, C3lim, and C3stau1/2. While RhoA was the preferred substrate, the specific ADP-ribosyltransferase activities for RhoB and C, were 5 and 23 times lower, respectively. Earlier ADP-ribosylation studies on the substrate properties of RhoA and Rac suggested that the RhoA residues Glu^{40} and Val^{43} are essential for efficient ADP-ribosylation by C3bot (*35*). Because in RhoC the latter residue is changed to isoleucine, we tested the substrate properties of Ile43Val RhoC in ADP-ribosylation experiments showing that RhoA Ile^{43} is critical for the difference in the kinetics of the ADP-ribosylation reaction observed with the Rho protein subtypes. Vice versa, the exchange of valine to isoleucine, leads to a decreased ADP-ribosylation by C3cer (data not shown). Individual differences in the stability of the Rho isoforms appear not to be of importance, because all three GTPases were modified by *C. difficile* active fragment CDB 1−546 with similar kinetics.

Due to the absence of a specific receptor binding and translocation domain, the cell accessibility of C3-like ADP-ribosyltransferases is poor (*2*, *3*). Therefore, C3-like ADP-ribosyltransferases are often described as exoenzymes than as toxins. To overcome the shortage of low cell accessibility, protein delivery systems on the basis of bacterial toxins (*29*, *36*) or short peptides have been used (*36*). We studied the cellular effects of rWTC3cer by means of a protein delivery system, which is based on the binary *C. botulinum* C2 toxin, which consists of the enzyme component C2I and the binding/translocation component C2II (*37*−*40*). A fusion protein was constructed consisting of the N-terminal part (amino acids 1−225) of C2I and the complete C3cer. Upon binding to C2II, the fusion toxin was transported into cells. Treatment of cells with the fusion toxin caused redistribution of the cytoskeleton similarly as observed with other C3 transferases, indicating the inactivation of Rho GTPases. Accordingly, subsequent C3cer-catalyzed ADP-ribosylation of RhoA in the cell lysate was largely reduced after pretreatment of intact cells with the toxin.

Because of the poor cell entry ability, the biological functions of C3 exoenzymes as virulence factors and their pathophysiological role are still largely enigmatic. It is possible that a specific transporter for C3-like transferases exists, which has not been identified so far. At least two other possibilities have to be discussed. First, it was found that C3stau-producing *S. aureus* are invasive bacteria (*41*, *42*) and are most likely able to release C3 directly into the cytosol (*43*). Second, it was shown recently that the pore-forming toxin streptolysin O from *Streptococcus pyogenes* specifically facilitates the cell entry of the bacterial exoenzyme NAD glycohydrolase, which by itself is not able to enter eukaryotic target cells (*44*). A similar mechanism may exist for some C3 exoenzymes including C3cer. In this respect, it is notable that *B. cereus* produces several pore-forming toxins (*20*). Thus, whether one of these possibilities applies for the cellular entry of C3cer has to be studied.

ACKNOWLEDGMENT

We thank Motoyuki Sugai from Hiroshima University, Japan, for providing the EDIN A (C3stau1) gene.

REFERENCES

1. Aktories, K., Mohr, C., and Koch, G. (1992) *Curr. Top. Microbiol. Immunol. 175*, 115−131.
2. Aktories, K., Barth, H., and Just, I. (2000) in *Handbook of Experimental Pharmacology*, pp 207−233, Springer-Verlag, Berlin.
3. Wilde, C., and Aktories, K. (2001) *Toxicon 39*, 1647−1660.
4. Aktories, K., Weller, U., and Chhatwal, G. S. (1987) *FEBS Lett. 212*, 109−113.
5. Rubin, E. J., Gill, D. M., Boquet, P., and Popoff, M. R. (1988) *Mol. Cell. Biol. 8*, 418−426.
6. Rösener, S., Chhatwal, G. S., and Aktories, K. (1987) *FEBS Lett. 224*, 38−42.
7. Just, I., Mohr, C., Schallehn, G., Menard, L., Didsbury, J. R., Vandekerckhove, J., van Damme, J., and Aktories, K. (1992) *J. Biol. Chem. 267*, 10274−10280.
8. Inoue, S., Sugai, M., Murooka, Y., Paik, S.-Y., Hong, Y.-M., Ohgai, H., and Suginaka, H. (1991) *Biochem. Biophys. Res. Commun. 174*, 459−464.
9. Wilde, C., Chhatwal, G. S., Schmalzing, G., Aktories, K., and Just, I. (2001) *J. Biol. Chem. 276*, 9537−9542.
10. Yamaguchi, T., Hayashi, T., Takami, H., Ohnishi, M., Murata, T., Nakayama, K., Asakawa, K., Ohara, M., Komatsuzawa, H., and Sugai, M. (2001) *Infect. Immun. 69*, 7760−7771.
11. Sekine, A., Fujiwara, M., and Narumiya, S. (1989) *J. Biol. Chem. 264*, 8602−8605.
12. Braun, U., Habermann, B., Just, I., Aktories, K., and Vandekerckhove, J. (1989) *FEBS Lett. 243*, 70−76.
13. Aktories, K., Braun, U., Rösener, S., Just, I., and Hall, A. (1989) *Biochem. Biophys. Res. Commun. 158*, 209−213.
14. Chardin, P., Boquet, P., Madaule, P., Popoff, M. R., Rubin, E. J., and Gill, D. M. (1989) *EMBO J. 8*, 1087−1092.
15. Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990) *J. Cell Biol. 111*, 1001−1007.
16. Böhmer, J., Jung, M., Sehr, P., Fritz, G., Popoff, M., Just, I., and Aktories, K. (1996) *Biochemistry 35*, 282−289.
17. Wilde, C., Just, I., and Aktories, K. (2002) *Biochemistry 41*, 1539−1544.
18. Han, S., Arvai, A. S., Clancy, S. B., and Tainer, J. A. (2001) *J. Mol. Biol. 305*, 95−107.
19. Ménétrey, J., Flatau, G., Stura, E. A., Charbonnier, J.-B., Gas, F., Teulon, J.-M., Le Du, M.-H., Boquet, P., and Ménez, A. (2002) *J. Biol. Chem. 277*, 30950−30957.
20. Drobniewski, F. A. (1993) *Clin. Microbiol. Rev. 6*, 324−338.
21. Kramer, J. M., and Gilbert, R. J. (1989) in *Foodborne Bacterial Pathogens* (Doyle, M. P., Ed.) pp 22−70, Dekker, M., Inc., New York, Basel.
22. Ombui, J. N., Schmieger, H., Kagiko, M. M., and Arimi, S. M. (1997) *FEMS Microbiol. Lett. 149*, 245−248.
23. Lund, T., De Buyser, M., and Granum, P. E. (2000) *Mol. Microbiol. 38*, 254−261.
24. Beecher, D. J., and Wong, A. C. L. (2000) *Microbiology 146*, 3033−3039.
25. Just, I., Schallehn, G., and Aktories, K. (1992) *Biochem. Biophys. Res. Commun. 183*, 931−936.
26. Just, I., Selzer, J., Jung, M., van Damme, J., Vandekerckhove, J., and Aktories, K. (1995) *Biochemistry 34*, 334−340.
27. Laemmli, U. K. (1970) *Nature 227*, 680−685.
28. Hofmann, F., Busch, C., Prepens, U., Just, I., and Aktories, K. (1997) *J. Biol. Chem. 272*, 11074−11078.
29. Barth, H., Hofmann, F., Olenik, C., Just, I., and Aktories, K. (1998) *Infect. Immun. 66*, 1364−1369.
30. Han, S., Craig, J. A., Putnam, C. D., Carozzi, N. B., and Tainer, J. A. (1999) *Nat. Struct. Biol. 6*, 932−936.
31. Domenighini, M., Magagnoli, C., Pizza, M., and Rappuoli, R. (1994) *Mol. Microbiol. 14*, 41−50.
32. Mueller-Dieckmann, C., Ritter, H., Haag, F., Koch-Nolte, F., and Schulz, G. E. (2002) *J. Mol. Biol. 322*, 687−696.
33. Wilde, C., Just, I., and Aktories, K. (2002) *Biochemistry 41*, 1539−1544.
34. Han, S., Craig, J. A., Putnam, C. D., Carozzi, N. B., and Tainer, J. A. (1999) *Nat. Struct. Biol. 6*, 932−936.
35. Wilde, C., Genth, H., Aktories, K., and Just, I. (2000) *J. Biol. Chem. 275*, 16478−16483.
36. Aullo, P., Giry, M., Olsnes, S., Popoff, M. R., Kocks, C., and Boquet, P. (1993) *EMBO J. 12*, 921−931.
37. Aktories, K., and Just, I. (1990) in *ADP-Ribosylating Toxins and G-Proteins* (Moss, J., and Vaughan, M., Eds.) pp 79−95, American Society for Microbiology, Washington, DC.
38. Aktories, K., Sehr, P., and Just, I. (1997) in *Bacterial Toxins* (Aktories, K., Ed.) pp 93−101, Chapman & Hall, Weinheim.

39. Ohishi, I., Iwasaki, M., and Sakaguchi, G. (1980) *Infect. Immun. 30*, 668−673.
40. Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K. H., and Habermann, E. (1986) *Nature 322*, 390−392.
41. Sinha, B., Francois, P. P., Nüsse, O., Foti, M., Hartford, O. M., Vaudaux, P., Foster, T. J., Lew, D. P., Herrmann, M., and Krause, K.-H. (1999) *Cell. Microbiol. 1*, 101−117.
42. Lowy, F. D. (2000) *Trends Microbiol. 8*, 341−344.
43. Wilde, C., Chhatwal, G. S., and Aktories, K. (2001) *Trends Microbiol. 10*, 5−7.
44. Madden, J. C., Ruiz, N., and Caparon, M. (2001) *Cell 104*, 143−152.

BI034583B