Rho-ADP-Ribosylating Exoenzyme from *Bacillus cereus*. Purification, Characterization, and Identification of the NAD-Binding Site†

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ABSTRACT: The ADP-ribosyltransferase produced by a pathogenic strain of *Bacillus cereus* was purified to near homogeneity. The transferase is a 28 000 Da molecular mass enzyme with a p*I* of 10.3. The specific enzyme activity is 7.0 nmol of ADP-ribose min^{-1} mg^{-1} with a K_m for NAD of 0.3 μM. Partial amino acid sequence analysis of the exoenzyme reveals no significant homology to *Clostridium botulinum* C3 nor to *Clostridium limosum* exoenzyme. The novel exoenzyme selectively modifies the small GTP-binding proteins of the Rho family presumably at the same acceptor amino acid (Asn-41) as determined for C3. Besides cellular Rho, recombinant RhoA and -B are substrates for the exoenzyme. However, recombinant Rac1 and CDC42, although belonging to the Rho family, are not modified. *B. cereus* exoenzyme was photolabeled with [*carbonyl*-^{14}C]NAD resulting in inhibition of ADP-ribosyltransferase and NAD-glycohydrolase activity. A glutamic acid residue was identified as part of the NAD-binding site which corresponds to Glu-174 of C3. This glutamic acid is located in a domain which shows high homology with the C-terminal part of C3 exoenzyme, *C. limosum* exoenzyme, and *Staphylococcus aureus* EDIN and which probably represents the catalytic site of the transferases. The data indicate that *B. cereus* exoenzyme is a novel member of the family of C3-like ADP-ribosyltransferases which share the same substrate protein Rho and which have an identical highly conserved catalytic domain.

Various bacterial toxins affect essential cell functions of eukaryotic cells via ADP-ribosylation of regulatory proteins. Besides the well-known diphtheria, cholera, pertussis, and C2 toxins, the family of C3-like transferases has been recently characterized. This family comprises *Clostridium botulinum* C3[1] exoenzyme (Aktories et al., 1987, 1988; Nemoto et al., 1991; Rubin et al., 1988; Segev, 1991), *Clostridium limosum* exoenzyme (Just et al., 1992a), and EDIN (epidermal differentiation inhibitor) produced by *Staphylococcus aureus* E-1 (Sugai et al., 1992). These transferases are basic proteins (p*I* >10) and exhibit a molecular mass of 25 000 to 30 000 Da and a K_m for NAD of the transferase reaction of about 0.3 μM. So far, two isoforms of C3 [C3A from *C. botulinum* type C strain 468 and type D strain 1873 (Popoff et al., 1990, 1991) and C3B from *C. botulinum* type D strain 003-9 (Nemoto et al., 1991)] have been cloned and sequenced and reveal an identity of 64% to each other. These isoforms (C3A: 23 400 Da and C3B: 23 213 Da) consist of 211 and 204 amino acids, respectively, including a signal peptide (40 amino acids) which is removed during the export process. The same features were found for EDIN (23 800 Da) with the exception that EDIN shows only 28% identity to C3A. *C. limosum* exoenzyme is more closely related to the C3 exoenzymes (66% identity to C3A) than to EDIN (27%) (J. Böhmer, I. Just, and K. Aktories, unpublished data). Besides structural similarities, the C3-like transferases have the common substrate protein Rho. Rho proteins A, B, and C are small GTP-binding proteins (22 000 Da) which are involved in the regulation of the microfilament cytoskeleton and the formation of adhesion plaques (Hall, 1992; 1993). The active GTP-bound form induces formation of a dense microfilament network whereas ADP-ribosylation renders Rho inactive, resulting in disaggregation of the actin stress fibers (Chardin et al., 1989; Paterson et al., 1990). ADP-ribosylation of Rho catalyzed by C3 or *C. limosum* exoenzyme was shown to occur at asparagine-41 (Sekine et al., 1989; Just et al., 1992a) which is located in the putative effector domain of the GTP-binding protein (Ueda et al., 1990). The C3-like transferases have been proven valuable tools in the identification and characterization of Rho functions.

Recently, a putative novel member of the C3-like family has been reported (Just et al., 1992b). A strain of *Bacillus cereus* was shown to produce an exoenzyme which selectively ADP-ribosylates 20−25 kDa proteins from platelet membranes. *B. cereus* species are widely distributed in the environment, and the majority are apparently harmless. Some subspecies, however, have been identified to cause food poisoning (Kramer & Gilbert, 1989). A 45 kDa

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[1] Abbreviations: *B. cereus*, *Bacillus cereus*; C3, *Clostridium botulinum* C3 exoenzyme; C3A, C3 exoenzyme from C strain 468 and D strain 1873; C3B, C3 exoenzyme from D strain 003-9; ECL, enhanced chemiluminescence; EDIN, epidermal differentiation inhibitor from *Staphylococcus aureus*; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; PBS, phosphate buffered saline; TLC, thin layer chromatography; SDS−PAGE, SDS−polyacrylamide gel electrophoresis.

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enterotoxin has been incriminated to be the causative agent for the gastrointestinal affection (Shinagawa, 1990). Moreover, in patients immunologically compromised and in intravenous drug users some *Cereus* subspecies can cause severe diseases, e.g., septicemia and endocarditis (Steen et al., 1992). Here we report on the purification, characterization, and identification of the catalytic domain of the ADP-ribosyltransferase, which is produced by a *Bacillus cereus* strain isolated from the blood of a patient with septicemia.

EXPERIMENTAL PROCEDURES

Materials. *C. botulinum* C3 exoenzyme (Aktories, 1988) and *C. limosum* exoenzyme (Just et al., 1992a) were purified as decribed. [*carbonyl*-^{14}C]NAD (54 mCi/mmol; 50 mCi/mL) was obtained from Amersham (Braunschweig, Germany), and [*adenylate*-^{32}P]NAD (800 Ci/mmol; 1 mCi/mL) was purchased from Dupont NEN (Bad Homburg, Germany). Trypsin (modified; sequencing grade) was obtained from Boehringer (Mannheim, Germany). The recombinant RhoA and RhoB (Paterson et al., 1990; Hall & Self, 1986), Rac1 (Didsbury et al., 1989), and CDC42 (Shinjo et al., 1990) were prepared as described. Human platelet membranes were prepared according to Jakobs et al. (1982). All other reagents were of analytical grade and from commercial sources.

EDIN (epidermal differentiation inhibitor from *S. aureus* E-1) and anti-EDIN antiserum were kindly donated by Dr. M. Sugai, Bethesda, Maryland.

Preparation of B. cereus exoenzyme. Stock cultures of *B. cereus* were inoculated into a medium containing tryptone (1% w/v) and sodium chloride (0.5% w/v), adjusted to pH 7.2, and grown under aerobic conditions for 5 days at 35 °C. The culture (2000 mL) was centrifuged (20 min at 10 000*g*), and the supernatant was precipitated with ammonium sulfate to 90% saturation. The precipitate was collected by centrifugation (20 min at 10 000*g*), and the pellets were extracted with 10 mL of 50 mM triethanolamine hydrochloride (pH 7.5) and passed through membrane filters (0.45 μm pore size). (Although the recovery after ammonium sulfate precipitation was below 20%, this step was necessary for the following purification steps.) After dialysis against the extraction buffer, the extract was subjected to affinity chromatography on Blue-Sepharose CL-4B (1.5 × 8 cm) (Pharmacia, Freiburg, Germany). The column was washed with 50 mL of 50 mM triethanolamine hydrochloride (pH 7.5), and the transferase was eluted with 200 mM NaCl/50 mM triethanolamine hydrochloride (pH 7.5). The fractions containing activity were pooled (5 mL) and concentrated by lyophilization. The lyophilized proteins were dissolved in 1 mL of ddH_2O and loaded on a Superdex-75-HR FPLC column (1 × 20 cm, Pharmacia, Freiburg, Germany) with 200 mM NaCl/50 mM triethanolamine hydrochloride (pH 7.5) as elution buffer and a flow rate of 21 mL/h. The transferase was eluted with an apparent molecular mass of 30 000 Da.

ADP-Ribosylation Assay. The ADP-ribosylation reaction was performed essentially as described (Just et al., 1992a). Human platelet membranes/ cytosolic proteins (30 μg) or recombinant proteins (RhoA/B, Rac1, CDC42) (0.3 μg) were incubated in a medium (100 μL) containing 2 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/50 mM triethanolamine hydrochloride (pH 7.5) and 0.5 μM [α-^{32}P]NAD for 10 min at 37 °C or as indicated. For determination of the specific activity, human platelet membranes were used and the incubation time was 4 min. In preliminary experiments this incubation time was proven to cover the linear phase of the reaction. ADP-ribosylated proteins were analyzed by SDS–PAGE according to Laemmli (1970) with subsequent autoradiography. For quantitative determination of the amount of incorporated ADP-ribose, the reaction (100 μL) was terminated by addition of 1 mL of trichloroacetic acid (20% w/v). The precipitated proteins were collected on nitrocellulose (0.45 μm pore size, Schleicher & Schüll) and washed with 15 mL of 6% trichloroacetic acid, and the remaining radioactivity was measured. For determination of the stoichiometry of the ADP-ribosylation reaction, two different assays were used because the filter assay does not give reliable data about the exact stoichiometry. (i) In the pre-ADP-ribosylation assay, Rho was ADP-ribosylated with C3 in the presence of unlabeled NAD followed by [^{32}P]ADP-ribosylation with *B. cereus* exoenzyme (and vice versa). The absence of incorporated label indicates complete ADP-ribosylation by either exoenzyme. (ii) Autoradiography of 2-D gel electrophoresis of [^{32}P]ADP-ribosylated Rho (formed after different incubation times) revealed only one single spot, indicating that maximally 1 mol of ADP-ribose/mol of Rho was incorporated.

De-ADP-ribosylation. Human platelet membranes (90 μg) were ADP-ribosylated in the presence of [α-^{32}P]NAD with C3 (1 μg/mL) or *B. cereus* exoenzyme (1 μg/mL) for 30 min at 30 °C. Anti-C3 IgG or anti-*B. cereus* exoenzyme antiserum was added, and the samples were incubated for 30 min on ice. Thereafter, membranes were washed to remove nonreacted NAD and the toxin–antibody complex. De-ADP-ribosylation was initiated by addition of 30 mM nicotinamide in 30 mM *N*-morpholinoethanesulfonic acid (MES) buffer (pH 6.0) and, if indicated, in the presence of C3 (1 μg/mL) or *B. cereus* exoenzyme (1 μg/mL).

NAD Glycohydrolase Assay. For determination of the NAD-glycohydrolase activity, *B. cereus* exoenzyme (100 μg/mL) or C3 exoenzyme (100 μg/mL) was incubated in a medium (100 μL) containing 100 mM triethanolamine hydrochloride (pH 7.5)/2 mM $MgCl_2$/10 μM [^{32}P]NAD for 4 h at 37 °C. The time dependent determination of the NAD glycohydrolase revealed that the selected incubation time was in the linear phase of the reaction. A 10 μL aliquot of the reaction mixture was separated by TLC on silica gel plates (silica gel 60, Merck, Darmstadt, Germany) with 2-propanol (66%)/1% ammonium sulfate (33%). The spots corresponding to ADP-ribose and NAD were scraped off, and the radioactivity was measured by liquid scintillation counting. The amount of ADP-ribose formed was calculated as pmol min^{-1} mg^{-1} ± SE.

Immunoblot Analysis. Immunoblotting was performed according to Towbin et al. (1979) with either anti-C3 IgG (1:25 000), anti-*B. cereus* exoenzyme antiserum (1:5000), or anti-EDIN antiserum (1:5000). The second antibody was anti-rabbit IgG coupled to peroxidase, and visualization was performed with the ECL system from Amersham.

Antiserum against *B. cereus* exoenzyme was produced in rabbits; the titer was 1:20 000 at 0.5 μg of exoenzyme.

Isoelectric Focusing. IEF was performed in polyacrylamide gels (7%, 70 × 100 × 0.5 mm) containing Servalytes (Serva, Heidelberg, Germany) (pH range 2–11 and 9–11, each 2.5%). After prefocusing (300 V for 20 min), 1 μg of

each protein was applied per slot and focusing was continued for 180 min at 1700 V. The sheets were fixed with trichloroacetic acid (20% w/v), stained with Coomassie Blue, and destained with methanol/acetic acid/water (40:10:50 v/v).

Protein Concentration. Protein concentration was determined according to Peterson (1977) with bovine serum albumin as standard.

Photolabeling of B. cereus Exoenzyme. B. cereus exoenzyme (3 nmol = 100 μg) was incubated in 25 mM ammonium bicarbonate (pH 7.7)/1 mM $MgCl_2$ without or with [*carbonyl*-^{14}C]labeled NAD (30 nmol) on a microtiter plate for 1h at 4 °C. UV irradiation (254 nm; 3 cm distance; 3000 μW/cm^2) was performed for 3 h at 4 °C. After separation of the photolabeled exoenzyme by reversed-phase HPLC, the amount of labeling was determined by liquid scintillation counting. The ADP-ribosyltransferase activity of the radiolabeled exoenzyme and the exoenzyme irradiated without NAD was determined by [^{32}P]ADP-ribosylation of platelet membranes (as described under ADP-Ribosylation Assay) and subsequent SDS−PAGE analysis of the labeled proteins.

NAD Glycohydrolase Activity of Irradiated B. cereus Exoenzyme. For determination of the NAD-glycohydrolase activity, *B. cereus* exoenzyme (50 μg/mL) was UV-irradiated in the absence or presence of NAD (5 μM) for 3 h. Thereafter, samples were incubated in a medium (100 μL) containing 100 mM triethanolamine hydrochloride (pH 7.5)/2 mM $MgCl_2$/5 μM [^{32}P]NAD for 4 h at 37 °C. Separation and determination of formed ADP-ribose were performed as described above. The amount of ADP-ribose formed was calculated as a percentage of control (UV irradiation without NAD).

Isolation of Peptides. For analysis of the radiolabeled amino acid residues of *B. cereus* exoenzyme, the irradiated exoenzyme was separated from unreacted NAD by HPLC chromatography on a Sephasil RP-C18 5 mm SC 2.1/10 column (Pharmacia). The proteins were eluted with a linear gradient from 0.1% trifluoroacetic acid (TFA) in water to 70% acetonitrile and 0.1% TFA in water. After lyophilization the dried photolabeled exoenzyme was dissolved in 200 μL of a medium containing 100 mM Tris-HCl (pH 8.5)/25 mM methylamine/1 mM $CaCl_2$/1 M urea and incubated with 10 μg of trypsin for 15 h at 37 °C. The digestion was stopped by addition of 0.1% TFA, and the peptides were again loaded on a Sephasil RP-C18 column. They were eluted with a linear gradient of 0−70% acetonitrile in 0.1% TFA and collected in 400 μL fractions. Radioactive peptides were determined by counting 5 μL aliquots of each fraction by liquid scintillation. The radioactive peptides were pooled and rechromatographed on the same column with a 25−45% gradient and, thereafter, lyophilized. The HPLC system was from Pharmacia and consisted of an injector, the gradient pump 2249, and the UV detector 2141.

Two hundred micrograms of unlabeled exoenzyme was also cleaved with trypsin (25 μg for 18 h at 37 °C) and the peptides were separated on a narrow bore HPLC system as described below.

Amino Acid Sequence Analysis. ^{14}C-Labeled peptides and peptides from the tryptic digestion of unlabeled exoenzyme were chromatographed on a narrow bore HPLC system [Applied Biosystems solvent delivery system 140A, UV detector 759A, and Vydac C18-column (2.1 mm i.d. × 250 mm)] . The peptides were eluted with a linear gradient starting from 0.1% TFA in water to 80% of acetonitrile in 0.1% TFA. Sequence analysis was carried out with an Applied Biosystems 477A protein sequencer with an on-line 120A PTH-amino acid analyzer. The instrument was run with the standard programs according to the manufacturer's instructions. During the sequencing procedure a constant aliquot (40%) of every formed PTH-amino acid was directed to the fraction collector for further radioactivity counting. For determination of the N-terminal sequence, the protein was purified onto Sephasil RP-C18 as described above.

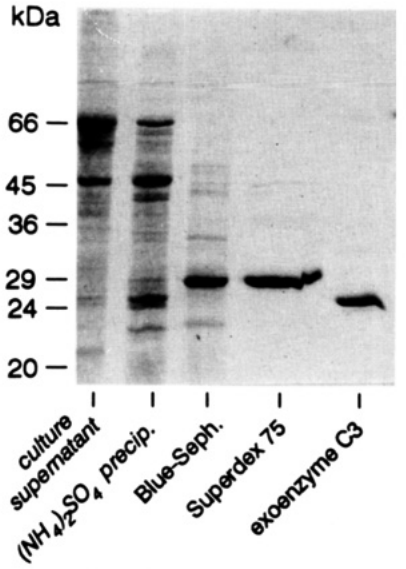

FIGURE 1: SDS−PAGE of various steps of the purification of *B. cereus* exoenzyme. The exoenzyme was purified from the culture supernatant by ammonium sulfate precipitation to 90% saturation (25 μg). The collected pellet was extracted with 50 mM triethanolamine hydrochloride (pH 7.5) (25 μg) and loaded onto Blue-Sepharose. The transferase was eluted with 200 mM NaCl (3 μg) and subsequently resolved by gel permeation chromatography (Superdex 75) (3 μg). The right lane shows the C3 exoenzyme (2 μg).

RESULTS

A human pathogenic strain of *B. cereus* was reported to produce an exoenzyme which selectively ADP-ribosylated 22 kDa GTP-binding proteins (Just et al., 1992b). To further characterize this novel exoenzyme, we purified the ADP-ribosyltransferase from the culture supernatant (Figure 1). The ammonium sulfate precipitate of the culture supernatant was extracted with 50 mM triethanolamine hydrochloride (pH 7.5) and dialyzed against the same buffer. The dialyzed extract was then loaded onto a Blue-Sepharose affinity chromatography column, and the transferase activity was eluted with 200 mM NaCl. The eluate was concentrated by lyophilization and applied to gel permeation chromatography (Superdex 75 column). The transferase was eluted with an apparent molecular mass of 30 000 Da. On SDS−PAGE the novel transferase showed a molecular mass of 28 000 Da whereas *C. limosum* exoenzyme and C3 exhibited a slightly lower molecular mass of 25 000 Da. The isoelectric point was 10.3. The steps of the purification procedure are summarized in Table 1.

Immunological Characterization. Next we studied whether *B. cereus* exoenzyme is immunologically related to the family of Rho-ADP-ribosylating exoenzymes. *C. botulinum* C3 and *C. limosum* exoenzyme show immunological cross-reactivity

Table 1: Purification of *B. cereus* Exoenzyme from the Culture Supernatant

step	volume (mL)	protein (mg/mL)	total protein (mg)	specific activity (nmol min^{-1} mg^{-1})	total activity (nmol/min)	recovery (%)
culture supernatant	2500	0.092	230	0.226	52.9	100
ammonium sulfate precipitation	20	1.08	21.6	0.41	8.85	17
Blue-Sepharose	12	0.117	1.4	1.624	2.27	4.5
Superdex 75	1.8	0.06	0.11	6.96	0.77	1.5

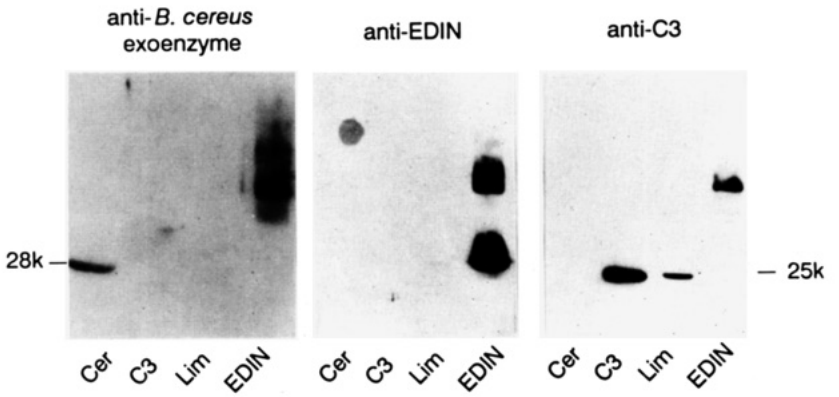

FIGURE 2: Immunoblot analysis of *B. cereus* exoenzyme. *B. cereus* exoenzyme (Cer), C3 exoenzyme (C3), *C. limosum* exoenzyme (Lim), and EDIN (each 0.5 μg) were electrophoresed and subsequently electroblotted to nitrocellulose. The immunoblot was performed as described under Experimental Procedures. Visualization was carried out with the ECL detection system. Antibody dilutions: anti-*B. cereus* exoenzyme antiserum (1:5000), anti-C3 IgG (1:25 000), and anti-EDIN antiserum (1:5000).

Table 2: Comparison of the Enzyme Kinetic Characteristics

	K_m (μM NAD)	specific activity (nmol min^{-1} mg^{-1})
B. cereus exoenzyme	0.3 ± 0.03	7.0 ± 0.6
C. limosum exoenzyme	0.3 ± 0.04	3.1 ± 0.4
C. botulinum C3A	0.4 ± 0.04	6.4 ± 0.6

(Just et al., 1992a), and therefore, only C3 was used. As shown in Figure 2, polyclonal anti-*B. cereus* exoenzyme antiserum did not recognize C3 (25 kDa) or EDIN (28 kDa). Conversely, neither anti-EDIN nor anti-C3 recognized the novel exoenzyme. Furthermore, neither EDIN nor C3 showed immunological cross-reactivity. However, all exoenzyme antibodies cross-reacted with an unknown 44 kDa protein from the EDIN preparation which was not present in the *B. cereus* and C3 exoenzyme preparations. The anti-C3 antibody, which was reported to inhibit C3- and also *C. limosum* exoenzyme-catalyzed ADP-ribosylation (Just et al., 1992a), did not affect ADP-ribosylation by *B. cereus* exoenzyme, not even in the presence of high concentration of the antibody (data not shown). Consistent with this finding, anti-*B. cereus* exoenzyme antibody was unable to block ADP-ribosylation by C3. Thus, the novel ADP-ribosyltransferase is immunologically distinct from C3, *C. limosum* exoenzyme, and *S. aureus* EDIN.

Enzyme Characteristics. The K_m value of the ADP-ribosylation reaction for NAD was determined as 0.30 ± 0.03 (±SE, $n = 3$) μM NAD for *B. cereus* exoenzyme, which is identical with the K_m of C3 and of the *C. limosum* exoenzyme, respectively. The three exoenzymes revealed almost the same specific enzyme activity which was 7.0 ± 0.6 (±SE, $n = 3$) nmol min^{-1} mg^{-1} for *B. cereus* exoenzyme (Table 2 summarizes the comparative studies). Determination of the stoichiometry of the ADP-ribosylation reaction revealed that each of the three exoenzymes catalyzed incorporation of maximally 1 mol of ADP-ribose/mol of Rho. Further similarity concerning transferase activity was the ability of all exoenzymes to catalyze the ADP-ribosylation reaction at 0 °C (not shown). The specific enzyme activity of the NAD glycohydrolase was 178 ± 12 (±SE, $n = 3$) pmol min^{-1} mg^{-1} for *B. cereus* exoenzyme and 93 ± 5.6 (±SE, $n = 4$) pmol min^{-1} mg^{-1} for C3 exoenzyme, respectively.

Substrate Specificity. ADP-ribosylation of platelet membranes by C3 in the presence of unlabeled NAD prevented subsequent ADP-ribosylation by *B. cereus* exoenzyme with [^{32}P]NAD (Just et al., 1992b). The same was true when the first ADP-ribosylation was performed with *B. cereus* exoenzyme. These findings indicate that C3 and *B. cereus* exoenzyme share the same substrate proteins. To compare the substrate specificity in more detail, we studied various recombinant GTP-binding proteins (Figure 3). Platelet cytosol which exclusively contained the RhoA isoform (Nemoto et al., 1992) was modified by all three transferases. The incorporation of ADP-ribose was stimulated by 0.01% sodium dodecyl sulfate as recently reported for C3 and *C. limosum* exoenzyme (Just et al., 1993). Recombinant RhoA which lacks the posttranslational polyisoprenylation showed the same property as endogenous RhoA. ADP-ribosylation of recombinant RhoB was catalyzed by all three exoenzymes, but in contrast to RhoA, sodium dodecyl sulfate (0.01%) decreased incorporation of ADP-ribose. Recombinant Rac1, which belongs to the Rho family (Didsbury et al., 1989), was only modified by C3. However, the maximal ADP-ribosylation was about 10% in the presence of 0.01% SDS. Cdc42, a further member of the Rho family (Hart et al., 1991), was not a substrate for any of the three transferases, not even in the presence of SDS (not shown).

Acceptor Amino Acid for the ADP-Ribose Moiety. The Rho protein is ADP-ribosylated by C3 in asparagine-41 (Sekine et al., 1989). To test whether *B. cereus* exoenzyme modifies its substrate Rho at the same acceptor amino acid,

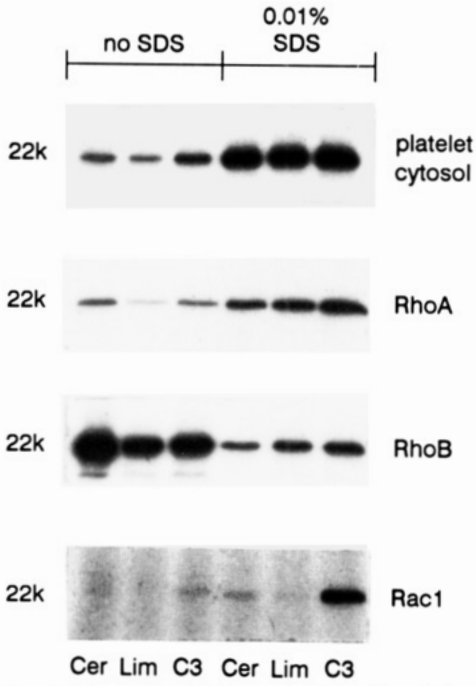

FIGURE 3: Substrate specificity of ADP-ribosylation by the exoenzymes from *B. cereus* (Cer), *C. limosum* (Lim), and *C. botulinum* C3 (C3). Human platelet cytosol (30 μg), recombinant RhoA (0.3 μg), RhoB (0.3 μg), and Rac1 (0.3 μg) were ADP-ribosylated by the exoenzymes without or with 0.01% SDS as described under Experimental Procedures. The proteins were analyzed by SDS−PAGE followed by autoradiography. Note the different exposure times: RhoA and RhoB (4 h); platelet cytosol and Rac1 (14 h).

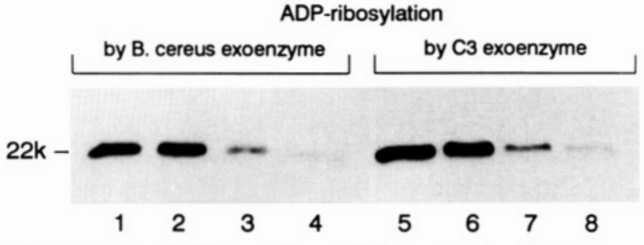

FIGURE 4: De-ADP-ribosylation of Rho protein in platelet membranes. Human platelet membranes (90 μg) were [^{32}P]ADP-ribosylated by *B. cereus* exoenzyme (lanes 1−4) or by C3 exoenzyme (lanes 5−8) for 30 min at 30 °C. The exoenzymes were removed by washing the membranes after addition of the corresponding anti-exoenzyme antibody. De-ADP-ribosylation was started by addition of nicotinamide (30 mM) and MES buffer (30 mM, pH 6.0). Rho ADP-ribosylated by *B. cereus* exoenzyme (lanes 1−4): control (1); de-ADP-ribosylation after removal of *B. cereus* exonzyme (2); de-ADP-ribosylation after removal of *B. cereus* exonzyme and addition of C3 (3); de-ADP-ribosylation by *B. cereus* exonzyme (4). Rho ADP-ribosylated by C3 exoenzyme (lanes 5−8): control (5); de-ADP-ribosylation after removal of C3 (6); de-ADP-ribosylation after removal of C3 and addition of *B. cereus* exonzyme (7); de-ADP-ribosylation by C3 (8).

we applied the method of de-ADP-ribosylation. Human platelet membrane Rho, which had been [^{32}P]ADP-ribosylated by C3 exoenzyme, was subjected to the reversal reaction. In the absence of NAD and the presence of high concentration of nicotinamide and low pH (6.0), C3 exoenzyme cleaved the ADP-ribose from the Rho protein and formed NAD. When C3 was removed by antibody treatment, the reversal reaction was inhibited. However, after addition of *B. cereus* exoenzyme, de-ADP-ribosylation of Rho was detected (Figure 4). De-ADP-ribosylation of Rho was also observed when the first ADP-ribosylation was performed with *B. cereus* exoenzyme and the reversal reaction was carried out with C3. The findings with the mutual de-ADP-ribosylation indicate that the *B. cereus* exoenzyme modifies Rho most likely at amino acid asparagine-41.

Table 3: Partial Amino Acid Analysis of Tryptic Peptides of *B. cereus* Exoenzyme and Comparison with Sequences of *C. botulinum* C3, *C. limosum* Exoenzyme, and *S. aureus* EDIN[a]

A.	
N-terminus	
CER	GNIPSKPKDXNNVDKYKLXTNKEEADAT
LIM	PYADSFKEFTNIDEARAWGDKQFAKYKLSS
C3A	AYSNTYQEFTNIDQAKAWGNAQYKKYGLSK
C3B	SYADTFTEFTNVEEAKKWGNAQYKKYGLSK
EDIN	ADVKNFTDLDEATKWGNKLIKQAKYSS
B.	↓
CER peptide 4	VGSGTHGAYMNSDDLTAYPGQY*E*LLLPR
LIM	VLDGSKAGYIE PISTFKGQL*E*VLLPRSSTY
C3A	VAKGSKAGYID PISAFAGQL*E*MLLPRHSTY
C3B	VTNGSKGGYID PISYFPGQL*E*VLLPRNNSY
EDIN	LPKGTKAAYLNSKDLTAYYGQQ*E*VLLPRGTEY
C.	
CER peptide 1	TRPVMTEFK
CER peptide 2	IYIAIDNNTQK
CER peptide 3	GDDAWIFGK

[a] X indicates a position where due to injection failue during analysis no residue could be assigned.

Partial Amino Acid Sequence. The structural relationship of *B. cereus* exoenzyme compared to the C3-like transferases was further confirmed by partial amino acid sequence analyses. These were done on four peptides (numbered 1, 2, 3, and 4). Furthermore, the N-terminal end of the undigested protein was directly sequenced. Comparison of one large and three small peptides and the N-terminal sequence covering together 83 residues (about one-third of the molecule) reveals an identity of only 11% with C3 (Table 3). Peptide 4 includes a stretch of nine amino acids which shows high homology (identity of seven of nine amino acids) to a C-terminal stretch found in all C3-like transferases.

Catalytic Site. Next we studied the NAD-binding site of the novel transferase. Therefore, *B. cereus* exoenzyme was photolabeled with [*carbonyl*-^{14}C]NAD by UV irradiation and subsequently digested with trypsin. Radioactive peptides formed were separated on a Sephasil RP-C18 column (Figure 5A). The peak, which contained major radioactivity (12 000 cpm), was selected for rechromatography, which resulted in two peptide peaks. Only the second peak contained radioactivity (8000 cpm) and was used for sequence analysis (Figure 5B). The resulting sequence X-Leu-Leu-Leu-Pro-Arg overlapped with that of peptide 4 (Table 3) except for X, which elutes as an unknown PTH-amino acid derivative (eluting between dimethylphenylthiourea and PTH-Ala in the conventional seperation system; results not shown). The corresponding amino acid in the labeled enzyme is Glu. It is therefore assumed that X represents the NAD-modified target residue. Amino acid sequence comparison with the DNA-derived sequence of *C. botulinum* C3 revealed that the identified NAD-binding amino acid correponds to Glu-174 of C3A. The amino acids adjacent to this glutamic acid, Tyr-Pro-Gly-Gln-Tyr-**Glu**-Leu-Leu-Leu-Pro, show high ho-

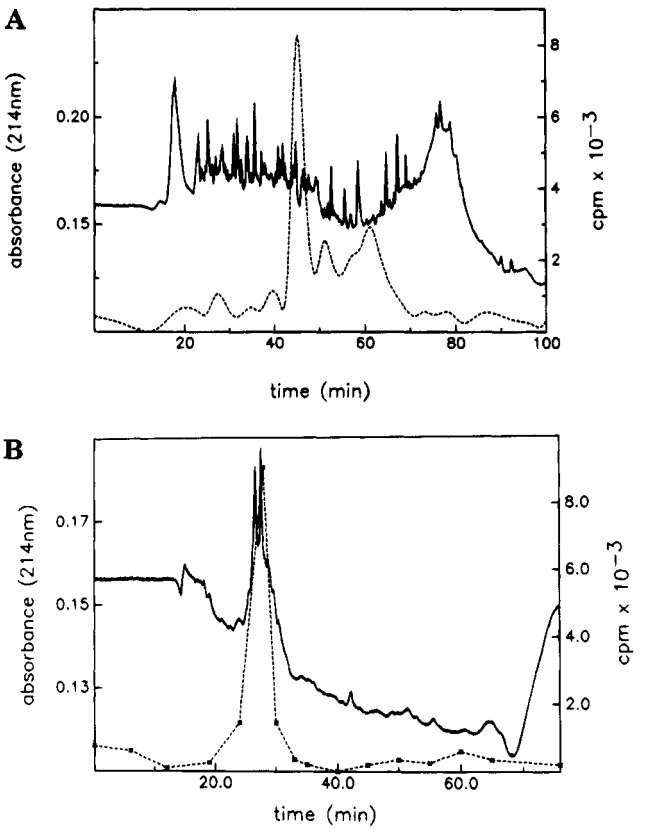

FIGURE 5: Elution profile of the tryptic peptides from *B. cereus* exoenzyme photolabeled with [*carbonyl*-^{14}C]NAD. (A) *B. cereus* exoenzyme (3 nmol) was treated by UV irradiation in the presence of [*carbonyl*-^{14}C]NAD (30 nmol) for 3 h at 4 °C. After removal of nonreacted NAD by reversed-phased HPLC, the labeled exoenzyme was digested with 10 μg of trypsin for 15 h at 37 °C. The peptides formed were separated by HPLC-chromatography on a Sephasil RP-C18 column. (B) The fractions corresponding to the large radioactive peak were pooled and rechromatographed with a linear 25−45% acetonitrile gradient in 0.1% TFA. Elution profiles of proteolytic fragments (—) and of radioactivity (- - -) are shown.

mology to the corresponding C-terminal domains of *C. limosum* exoenzyme, EDIN, and the two isoforms of C3 (Table 3). If NAD is cross-linked to the pivotal amino acid of the catalytic center, enzyme activity should be altered or blocked. Therefore, we compared enzyme activity of the exoenzyme which was irradiated in the presence or absence of NAD. The ADP-ribosyltransferase and NAD-glycohydrolase activity was inhibited after irradiation in the presence of NAD, whereas control irradiation in the absence of NAD did not alter the enzyme activities (data not shown).

DISCUSSION

Here we report on the purification and characterization of an ADP-ribosyltransferase which is produced by a human pathogenic strain of *B. cereus*. This novel exoenzyme exhibits high similarity to C3 and *C. limosum* exoenzyme with respect to the K_m for NAD, the specific enzyme activity, and substrate specificity. In contrast, *B. cereus* exoenzyme shows low amino acid sequence homology (11%) to C3, *C. limosum* exoenzyme, and EDIN, respectively, except for its putative catalytic site, which is highly conserved in all Rho-ADP-ribosylating transferases.

Detailed analysis of the substrate specificity reveals that *B. cereus* exoenzyme ADP-ribosylates Rho proteins in cell extracts as well as recombinant RhoA. Recombinant Rho differs from cellular Rho in C-terminal polyisoprenylation and carboxymethylation. This posttranslational modification is important for the functional activity of Rho but is not essential for ADP-ribosylation. Sodium dodecyl sulfate (0.01%) has the same effects on *B. cereus* exoenzyme-catalyzed ADP-ribosylation of Rho as reported for C3 (Just et al., 1993); i.e., ADP-ribosylation of RhoA and recombinant RhoA is stimulated, whereas modification of RhoB is decreased. Further increase in the SDS concentration caused abrupt inhibition of ADP-ribosylation due to denaturation of the protein. Stimulation of Rho-ADP-ribosylation in cytosolic fractions by SDS (0.01%), is most likely caused by cleavage of the Rho complex with the guanine nucleotide dissociation inhibitor (Bourmeyster et al., 1992) and release of free Rho which is then substrate. In addition to Rho (A, B, C), the Rac and Cdc42 proteins belong to the Rho family. Rac and Cdc42 show about 65% homology to the Rho proteins and contain an asparagine at position 41, which is located inside a highly conserved region (amino acids 34−42) known as the putative effector domain. However, Rac and Cdc42 are not substrates for *B. cereus* nor for *C. limosum* exoenzyme. Only C3 is able to modify Rac in the presence of SDS to a minor extent (<10%). High homology of the ADP-ribose acceptor region is requisite but not sufficient for small GTP-binding proteins to serve as substrate for C3-like ADP-ribosyltransferases. It seems that additional structural requirements thus far unknown are essential. Under physiological conditions, the Rho isoforms A, B, and C are the exclusive substrates for *B. cereus* transferase.

To assess whether *B. cereus* exoenzyme modified Rho at the same acceptor amino acid as C3, we studied the de-ADP-ribosylation reaction. ADP-ribose incorporated by *B. cereus* exoenzyme was cleaved by C3 and vice versa. Because this reaction is highly stereospecific, the mutual de-ADP-ribosylation provides evidence that the novel exoenzyme also modifies Rho at asparagine-41 (Sekine et al., 1989). The acceptor amino acid is located in the putative effector domain of Rho, and attachment of the bulky ADP-ribose moiety could therefore prevent the association with thus so far unknown effector protein of Rho to cause disaggregation of the actin cytoskeleton (Aktories et al., 1993).

We used photolabeling of *B. cereus* exoenzyme with NAD to assess the catalytic site of the transferase. Glutamic acid was identified as acceptor amino acid for the radioactive label. According to the proposed reaction scheme (Carroll et al., 1985), only the nicotinamide moiety of NAD is convalently linked to glutamic acid. This glutamic acid is probably involved in the catalytic activity, because NAD cross-linking results in inhibition of ADP-ribosyltransferase and NAD-glycohydrolase activity, whereas control irradiation does not alter the enzyme activities. A glutamic acid residue as NAD-binding amino acid has been also identified in *C. limosum* exoenzyme (Jung et al., 1993). Furthermore, comparison of the amino acid sequences of the NAD-binding peptide from *C. limosum* exoenzyme revealed high homology of the following motif: Tyr/Phe-X-Gly-Gln-X-**Glu**-A-Leu-Leu-Pro-Arg (X, any amino acid; A, aliphatic amino acid). Further comparison with the DNA-derived amino acid sequence of two isoforms of C3 (A and B) and of EDIN revealed that this motif is found in all Rho-ADP-ribosylating transferases. The amino acids adjacent to the NAD-binding amino acid Glu are highly conserved and located at the C-terminal part of the enzyme. A glutamic acid residue as

NAD-binding amino acid has also been identified in other ADP-ribosyltransferases: Glu-148 in diphtheria toxin (Carroll et al., 1985; Carroll & Collier, 1984), Glu-553 in *Pseudomonas* exotoxin A (Carroll & Collier, 1987), and Glu-129 in pertussis toxin (Barbieri et al., 1989). These glutamic acids are essential for coordinating the NAD in the transferase and are pivotal for enzyme activity. It is likely that the identified NAD-binding glutamic acid of the C3-like exoenzymes are functionally equivalent to these glutamic acids.

Although *B. cereus* exoenzyme exhibits the same enzyme kinetic characteristics (K_m, specific enzyme activity) and substrate specificity as the other C3-like transferases, important structural differences are observed: (i) *B. cereus* exoenzyme has a higher molecular mass (28 000 Da) than C3 and *C. limosum* exoenzyme (23 500 Da). (ii) Although *B. cereus* shows the same basic isoelectric point of about 10, the novel transferase behaves quite differently with respect to the purification procedure used for C3 and *C. limosum* exoenzyme. (iii) The novel transferase shows no immunological relationship to any member of the family of Rho-ADP-ribosylating exoenzymes. Deduced from immunoblot analysis, the family of Rho-ADP-ribosylating exoenzymes comprises three immunological distinct subfamilies: C3/*C. limosum* exoenzyme, EDIN, and *B. cereus* exoenzyme. The NAD-binding site is highly conserved in all C3-like exoenzymes, but the absence of cross-reactivity of the polyclonal anti-exoenzyme antibodies suggests low antigenicity of the catalytic site. Partial amino acid sequencing reveals only a homology of about 10% to C3 and corroborates the immunological findings. All these findings confirm the structural differences between the novel transferase, C3, and *C. limosum* exoenzyme, respectively. Similar large differences were found between diphtheria toxin and *Pseudomanas* exotoxin A which both ADP-ribosylate elongation factor EF-2 at a diphthamide but show no obvious amino acid homology to each other (Gray et al., 1984).

In summary, we identified a further member of the family of C3-like transferases which selectively ADP-ribosylates the small GTP-binding protein Rho. Comparison of the amino acid sequences and biochemical properties reveals great structural differences. However, all the members share a highly conserved motif which is apparently involved in the transferase activity.

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